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# The investigation of HLA microsatellites influence in predisposition to sarcoidosis among Croatians

*Z. Grubić[1], T. Peroš-Golubičić[2], K. Štingl[1], R. Žunec[1]*

[1] Tissue Typing Centre, University Hospital Centre Zagreb; [2] University Hospital for Lung Disease "Jordanovac", Zagreb, Croatia

**Abstract.** The aim of the present study was to analyze the distribution of HLA alleles (A, B, DRB1, DQB1) and HLA microsatellite alleles (TNFa, TNFb, TNFd, D6S273, D6S1014) in the Croatian patients with acute (N=93), as well as chronic sarcoidosis (N=40), in comparison to healthy controls (N=177), and investigate whether the polymorphism within the HLA region could be associated with different forms of sarcoidosis. Genomic DNA was isolated from peripheral blood. Patients were analyzed for HLA class I loci (A, B) by serology, while PCR-SSP method was used for HLA class II loci (DRB1, DQB1). Five HLA microsatellites were analyzed by PCR and electrophoresis in an automated sequencer. No significant deviation in the distribution of frequencies at HLA class I alleles was observed between the two patients' subgroups and controls. Regarding the HLA class II alleles, a statistically significant increase in frequency of HLA-DRB1*03 and DQB1*0201 allele was found among patients with acute sarcoidosis in comparison to controls as well as in comparison to patients with chronic sarcoidosis. The same finding was observed for HLA-DRB1*03/DQB1*0201 haplotype (Pcorr=0.0168; OR=2.83). In the group of patients with chronic sarcoidosis DRB1*11 (P=0.0219; OR=2.44), DRB1*15 (P=0.0414; OR=2.47) demonstrated statistically significant difference in comparison to controls only, while a lower frequency of DRB1*13 (P=0.0156; OR=0.24) in this group was statistically significant when compared to both patients with acute sarcoidosis and controls. None of the alleles at TNFa microsatellite showed significant difference in distribution among both subgroups of patients and controls. Significant difference between patients with acute form of disease and controls was found for the following alleles: TNFd-2 (Pcorr=0.00007; OR=4.89), D6S273-7 (Pcorr=0.0213; OR=2.96), and D6S1014-7 (Pcorr=0.0028; OR=3.97). On the other hand, patients with chronic sarcoidosis differed from control subjects for D6S1014-8 (Pcorr=0.0296; OR=8.35) allele. This study suggests the existence of an association of non-HLA markers with sarcoidosis and the involvement of the region between HLA-DQB1 and D6S273 loci in its pathophysiology. *(Sarcoidosis Vasc Diffuse Lung Dis 2011; 28: 18-26)*

**Key words:** sarcoidosis, HLA, TNF, D6S273, D6S1014, Croatians

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Correspondence: Zorana Grubić
Tissue Typing Centre
University Hospital Centre Zagreb
Kispaticeva 12, 10000 Zagreb
Croatia
Tel. + 385 1 23 67 287
Fax: + 385 1 23 67 337
E-mail: zgrubic@kbc-zagreb.hr

## Introduction

Sarcoidosis is a multisystem granulomatous disease of unknown etiology characterized by an accumulation of activated, proliferating T lymphocytes and mononuclear phagocytes that predominantly affects the lungs (1). Disease occurs among men and women of all races and ages. A few hypotheses attempted to explain the cause of sarcoidosis, but the

most widely accepted theory is that a T-helper cell/macrophage alveolitis in pulmonary sarcoidosis is caused by an unknown stimulus which induces the alveolar macrophages and lymphocytes to release mediators such as tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2) (2).

Most studies on the association between sarcoidosis and genetic factors have focused on the HLA genes (3-6). The strongest association has been found with HLA-A and DRB1 alleles (7-9). We have also reported about the association between HLA-B8, -DRB1*0301 and -DQB1*0201 alleles and different onset of sarcoidosis in the Croatian population (7). A role of this haplotype has been implicated in many autoimmune diseases. However, approximately 40% patients with the acute onset of the disease have a predisposing HLA-DRB1*0301/DQB1*0201 haplotype, while the remaining patients do not carry this combination. Other loci might play a relevant role in the linkage of the HLA region with sarcoidosis, such as Tumor Necrosis Factor (TNF) genes.

Numerous investigations of the TNF cluster have indicated that these locus/loci might contribute to disease susceptibility (10-13). A large number of biallelic polymorphisms have been identified within the TNF gene cluster, as well as 6 microsatellites (TNFa-TNFf). With the exception of TNFb microsatellite which is composed out of mononucleotide tandem repeats $(G/A)_n$, TNF microsatellites are based on a dinucleotide repeat motif: TNFa – $(GT)_n$, TNFc – $(GA)_n$, TNFd – $(GA)_n$, TNFe – $(GA)_n$ and TNFf – $(CA)_n$ (13, 14). Data which support the inclusion of this region in the investigation of sarcoidosis was provided by various studies (15-18).

At the same time, it is well documented that the microsatellites in the region between TNF gene cluster and DRB loci also play a role in the predisposition to some autoimmune diseases such as rheumatoid arthritis (RA), type 1 diabetes, Addison's disease as well as in psoriatic arthritis and scleroderma (19-22). An example of such a dinucleotide microsatellite locus is D6S273, which was a subject of various studies that reported about its role as an additional genetic marker (23-25).

The D6S1014 microsatellite is another such example of association with RA, multiple sclerosis, schizophrenia, etc. (26-29).

In the context of the possible role of TNF gene cluster in the etiology of sarcoidosis, and with respect to the possible association of microsatellites located between TNF and DRB1, the aim of the present study was to determine, by analyzing well-characterized subgroups (acute and chronic) of Croatian sarcoidosis patients, whether an association between sarcoidosis and HLA class I (A, B) and HLA class II (DRB1, DQB1) loci as well as with HLA microsatellites (TNFa, TNFb, TNFd, D6S273, and D6S1014) exists.

## Materials

### *Patients and controls*

All the patients (N=133) included in this study were treated at the University Hospital for Lung Diseases "Jordanovac", Zagreb. All patients were followed up for 2-18, average 4.5 years in the period 1990-2009. Patients were recruited from Zagreb area and were all of Croatian origin. The University Hospital for Lung Diseases "Jordanovac", Zagreb ethics committee approved the study. All patients signed their informed consent. The diagnosis of sarcoidosis was based on consistent clinical features, together with biopsy-proven noncaesating epitheloid cell granulomas according to the international guidelines (16). A summary of patient's characteristics is listed in Table 1.

The control group (N=177) was comprised out of ethnically matched, healthy volunteer citizens of Zagreb, the capital city of Croatia.

The entire investigated sample was previously typed for HLA specificities (7). Typing for HLA class I (A and B) was done by serology, while the DNA typing method as described in the published data was used for the determination of HLA class II (DRB1 and DQB1) by PCS-SSP (Polymerase Chain Reaction – Sequence Specific Primers) (30).

## Methods

### *Microsatellite typing*

DNA was extracted from EDTA anti-coagulated blood using a commercial kit (Nucleospin Blood, Macherey-Nagel, Duren, Germany).

**Table 1.** Patients' characteristics

| | n (%) |
|---|---|
| Number of patients | 133 |
| Females | 76 (57.1%) |
| Males | 57 (42.9%) |
| Biopsy confirmation | 100% |
| Mean age at disease onset | 42 yrs (23-65) |
| Form of disease | |
| Acute (AcS) | 93 (69.9%) |
| With Erythema nodosum | 43/93 |
| With Erythema nodosum and Löfgren syndrome | 19/93 |
| Chronic (ChrS) | 40 (30.1%) |
| Pulmonary sarcoidosis | 78 (48.6%) |
| Extra-thoracic sarcoidosis | 55 (41.4%) |
| Chest radiograph | |
| Stage 0 | 66 (49.6% ) |
| Acute/Chronic | 34/32 |
| Stage I | 15 (11.3%) |
| Acute/Chronic | 4/11 |
| Stage II | 29 (21.8%) |
| Acute/Chronic | 9/20 |
| Stage III | 22 (16.5%) |
| Acute/Chronic | 2/20 |
| Stage IV | 1 (0.8%) |
| Acute/Chronic | 0/1 |
| Lung function tests | |
| FVC < 80% | 32 (24.1%9 |
| $FEV_1$ < 80% | 36 (27.1%) |
| $D_{LCO}$ < 80% | 65 (48.9%) |

FVC – forced vital capacity; $FEV_1$ – forced expiratory volume in one second; $D_{LCO}$ – diffusing capacity for carbon monoxide

Samples were amplified for 5 microsatellites (TNFa, TNFb, TNFd, D6S273, D6S1014). The amplification for each microsatellite was carried out in a separate reaction with 10 μl as a total reaction volume per sample. The forward primers were labeled with Cy5 fluorescent dye which enabled the laser detection of the amplified fragments. Primer sequences and conditions of amplification were as reported in a previously published study (31). Following the polymerase chain reaction (PCR) amplification, the PCR products were mixed with loading buffer and an internal standard, denatured and immediately placed on ice. Fragments were separated on a 6% polyacrylamide gel using an automated sequencer (ALFexpress; Pharmacia Biotech, Uppsala, Sweden). Allele identification was performed using fragment analysis software (AlleleLocator, Pharmacia Biotech). B cell lines from the 11th International Histocompatibility Workshop (IHW) with known microsatellite alleles were included on each gel as additional internal standards: VAVY - TNFa2, TNFb3, TNFd1, D6S1014-143pb; TUBO-TNFa3/TNFa13, TNFb1/b4, TNFc1/c2, TNFd4/d5; OMW – TNFa7, TNFb1, TNFd2; IBW9-TNFa4, TNFb7, TNFc2, TNFd5; BTB – D6S1014-137pb; TAB089 - D6S273-134pb; BTB - D6S273-134pb.

*Statistics*

Phenotype frequencies of the HLA specificities as well as the HLA microsatellite alleles were calculated for the patient's groups and control group. The chi-squared test was used to compare the expected value of genotype with its observed value in order to confirm whether it satisfies the Hardy-Weinberg law. The significance of associations between loci was estimated from 2X2 tables by chi-squared test with Yate's correction or by Fisher's exact test when the number of cases was less than five. The multiple comparisons were performed using Bonferroni correction for the number of comparisons made (P value was multiplied by 13 for A, 18 for B, 13 for DRB1, 14 for DQB1, 13 for TNFa, 5 for TNFb, 4 for TNFd, 7 for D6S273 and 7 for D6S1014). The odds ratio (OR) of disease risk for a given marker was also calculated from the 2X2 contingency table and a value of Pcorr<0.05 was considered as statistically significant.

## Results

*HLA class I analysis*

Among 14 HLA-A class I antigens observed in both subgroups (acute sarcoidosis – AcS and chronic sarcoidosis – ChrS) and twenty-one different antigens at HLA-B locus there were no statistically significant differences in comparison to control group (data not shown).

*HLA class II analysis*

The phenotype frequencies of HLA-DRB1 and DQB1 alleles are listed in Table 2. DRB1*03 specificity was significantly more present among the patients with the acute form of the disease in comparison to control subjects (31/93-33.3% vs. 19/141-13.5%; P=0.0005; Pcorr=0.0069) while in comparison to patients with chronic sarcoidosis the difference was not significant after correction (31/93-

**Table 2. T**he distribution of DRB1 and DQB1 alleles among patients with sarcoidosis and controls

| HLA- | Acute sarcoidosis | | Chronic sarcoidosis | | Controls | |
|---|---|---|---|---|---|---|
| | n | % PF | n | % PF | n | % PF |
| **DRB1*** | **N=93** | | **N=40** | | **N=141** | |
| 01 | 15 | 16.13 | 3 | 7.50 | 24 | 17.02 |
| 15[1] | 22 | 23.65 | 13 | 32.50 | 23 | 16.31 |
| 16 | 20 | 21.21 | 5 | 12.50 | 30 | 23.40 |
| 03[2] | 31 | 33.33 | 6 | 15.00 | 19 | 13.48 |
| 04 | 11 | 11.83 | 6 | 15.00 | 29 | 20.56 |
| 11[3] | 31 | 33.33 | 21 | 52.50 | 44 | 31.20 |
| 12 | 4 | 4.30 | 2 | 5.00 | 5 | 3.54 |
| 13[4] | 24 | 25.81 | 3 | 7.50 | 36 | 25.53 |
| 14 | 6 | 6.45 | 7 | 17.50 | 12 | 8.52 |
| 07 | 10 | 10.75 | 5 | 12.50 | 27 | 19.14 |
| 08 | 5 | 5.38 | 2 | 5.00 | 9 | 6.38 |
| 09 | 0 | 0.0 | 1 | 2.50 | 1 | 0.72 |
| 10 | 2 | 2.15 | 1 | 2.50 | 5 | 3.54 |
| **DQB1*** | **N=93** | | **N=40** | | **N=119** | |
| 0501 | 17 | 18.28 | 5 | 12.50 | 29 | 24.37 |
| 0502 | 21 | 22.58 | 7 | 17.50 | 26 | 21.85 |
| 0503 | 6 | 6.45 | 7 | 17.50 | 12 | 10.08 |
| 0601 | 1 | 1.08 | 0 | 0.0 | 3 | 2.52 |
| 0602 | 18 | 19.35 | 9 | 22.50 | 21 | 17.65 |
| 0603 | 11 | 11.83 | 2 | 5.00 | 16 | 13.44 |
| 0604 | 14 | 15.05 | 2 | 5.00 | 9 | 7.56 |
| 0201 | 31 | 33.33 | 6 | 15.00 | 16 | 13.44 |
| 0202 | 8 | 8.60 | 5 | 12.50 | 13 | 10.92 |
| 0301 | 40 | 43.01 | 24 | 60.00 | 56 | 47.08 |
| 0302 | 7 | 7.53 | 4 | 10.00 | 9 | 7.56 |
| 0303 | 2 | 2.15 | 1 | 2.50 | 3 | 2.52 |
| 0401 | 0 | 0,0 | 0 | 0.0 | 2 | 1.68 |
| 0402 | 5 | 5.38 | 2 | 5.00 | 2 | 1.68 |

Legend: PF-phenotype frequency;
[1] Patients with acute sarcoidosis vs. controls P=0.0414, Pcorr>0.05;
[2] Patients with acute sarcoidosis vs. controls P=0.0005; Pcorr=0.0069;
[2] Patients with acute sarcoidosis vs. patients with chronic sarcoidosis P=0.0354, Pcorr>0.05;
[3] Patients with chronic sarcoidosis vs. controls P=0.0219; Pcorr>0.05;
[4] Patients with chronic sarcoidosis vs. controls P=0.0156, Pcorr>0.05;
[4] Patients with chronic sarcoidosis vs. patients with acute sarcoidosis P=0.0181, Pcorr>0.05

33.3% vs. 6/40-15.0%; P=0.0354; Pcorr>0.05). The increase of the DRB1*11 frequency among patients with chronic sarcoidosis was observed in comparison to controls (21/40-52.5% vs. 44/141-31.2%; P=0.0219; Pcorr>0.05; OR=2.44) but not in comparison to patients with acute sarcoidosis (21/40-52.5% vs. 31/93-33.3%; P>0.05). The situation was the same with the frequency of DRB1*15 specificity, which was also more present among ChrS patients in comparison to the control subjects (13/40-32.5% vs. 23/141-16.3%, P=0.0414), but not in comparison to the patients with AcS. At the same time, the lower frequency of DRB1*13 specificity among ChrS group revealed a difference in comparison to AcS (P=0.0181; Pcorr>0.05) as well as to controls (P=0.0156; Pcorr>0.05; OR=0.24). None of the other DRB1 specificities has reached a statistically significant difference in the frequency when the tested groups were compared. At DQB1 locus, only DQB1*0201 allele showed a significantly higher frequency among AcS patients in comparison to controls (21/93-33.3% vs. 16/119-13.4%; P=0.0009; Pcorr=0.0117) but not to ChrS patients (21/93-33.3% vs. 6/40-15.0%; P=0.0354; Pcorr>0.05).

Analysis of the presence of the HLA-DRB1*03/DQB1*0201 haplotype among patients with sarcoidosis showed that this haplotype is more present in cases of the acute form of the disease than in subgroup of patients with the chronic form of sarcoidosis (P=0.0354; Pcorr>0.05; OR=2.83). This difference was significant in comparison to controls (P=0.0001; Pcorr=0.0168; OR=3.22).

*HLA microsatellite analysis*

The distribution of TNFa microsatellite alleles was also determined in the same groups of patients and controls. No significant differences were found between the two subgroups of patients. A tendency to an increased frequency was found for TNFa-4 allele among patients with acute sarcoidosis (19/93-20.4%) in contrast to the group of patients with the chronic form of the disease (3/40-7.5%), whereas TNFa-11 allele was decreased (13/93-14.0% vs. 9/40-22.5%; P>0.05).

The comparison of the AcS patients group versus ChrS patients group did not reveal significant differences for any of the TNFb alleles. Compared with the controls, a slightly increased frequency of TNFb-3 allele in the group of patients with AcS was found (34/91-37.4% vs. 37/155-23.9%; P=0.0350; Pcorr>0.05).

At the TNFd locus the comparison of patient and control groups showed a statistically significant increase of the TNFd-2 allele frequency among patients with AcS (37/93-39.8% vs. 20/168-11.9%; P<0.00001; Pcorr =0.00007), while the increase of this allele's frequency was not statistically significant among patients with ChrS (12/40-30.0% vs. 20/168-11.9%; P =0.0091; Pcorr >0.05). The distribution of all other alleles at TNFd locus did not differ from the distribution found among controls.

The distribution of D6S273 allele frequencies showed that D6S273-7 allele is significantly more present among patients with AcS in comparison to control subjects (22/88-25.0% vs. 17/168-10.1%; P=0.0003; Pcorr=0.0021), while the difference found for patients with ChrS was not statistically significant (7/33-21.2% vs. 17/168-10.1%; P>0.05). The low frequency of D6S273-3 allele among patients with ChrS did not reach a significant P value in comparison of the frequency of this allele among controls (3/33-9.1% vs. 54/168-32.1%; P=0.0100; Pcorr>0.05), as well as in comparison to patients with AcS. The difference in the frequency of D6S273-3 allele among patients with AcS and controls was also observed but without statistical significance after correction (17/88-19.3% vs. 54/168-32.1%; P=0.0424; Pcorr>0.05).

The last analyzed microsatellite locus was D6S1014. Four out of eight alleles known so far (D6S1014-1, D6S1014-4, D6S1014-7, and D6S1014-8) demonstrated a difference in frequencies between patients' groups and controls. Only the frequency of D6S1014-8 allele varied between two subgroups of patients (AcS: 6/93-6.5% vs. ChrS: 6/40-15.0%) but without a significant P value. At the same time, this allele was observed with a significantly higher frequency among ChrS patients in comparison to the controls (6/40-15.0% vs. 3/145-2.1%; P=0.0037; Pcorr=0.0296). The D6S1014-4 allele was less present among AcS patients in comparison to controls (45/93-48.4% vs. 93/145-64.1%; P=0.0219; Pcorr>0.05), but also among patients with ChrS in comparison to healthy individuals (17/40-42.5% vs. 93/145-64.1%; P=0.0223; Pcorr>0.05). Contrary to this allele, D6S1014-7 allele was increased among patients with AcS (23/93-24.7% vs. 12/145-8.3%; P=0.0004; Pcorr=0.0028) and patients with ChrS (9/40-22.5% vs. 12/145-8.3%; P=0.0151; Pcorr>0.05). The presence of D6S1014-1 allele was slightly lower among both subgroups of patients in comparison to the control group.

*Extended HLA haplotype analysis*

The final aim of the study was to investigate the extended haplotypes among the tested groups. This analysis showed that there were no significant differences between patient groups as well as in comparison of patients with the control group. With respect to the HLA-DRB1 specificities that have shown significant difference between patient's groups or one patient group and controls, the following haplotypic associations were observed: HLA-DRB1*03/DQB1*0201/TNFa3/TNFb3/TNFd2/D6S273-7/D6S1014-7 (AcS: 25/93-26.8% vs. ChrS: 4/40-10.0%; P=0.0387; Pcorr>0.05 and AcS: 25/93-26.8% vs. controls: 15/141-10.6%; P=0.0019; Pcorr>0.05), HLA-DRB1*11/DQB1*0301/TNFa 10/TNFb4/TNFd4/D6S273-4/D6S1014-6 (ChrS: 12/40-30.0% vs. AcS: 12/93-12.9%; P=0.0353; Pcorr>0.05), and HLA-DRB1*13/DQB1*06/TNFa10/TNFb1/TNFd5/D6S273-4/D6S1014-4 (AcS: 14/93-15.1% vs. ChrS: 1/40-2.5%; P=0.0384; Pcorr>0.05).

There were no statistically significant differences between patient's groups or one patient group and controls when we included HLA class I specificities in extended haplotypic association analysis. This was the case for all previously mentioned hap-

**Table 3.** The distribution of TNFa, TNFb, TNFd, D6S273 and D6S1014 alleles among patients with sarcoidosis and controls

| Microsatellite | Acute sarcoidosis | | Chronic sarcoidosis | | Controls | |
|---|---|---|---|---|---|---|
| | n | % PF | n | % PF | n | % PF |
| **TNFa (bp)** | **N=93** | | **N=40** | | **N=177** | |
| 1 (97) | 5 | 5.38 | 3 | 7.50 | 3 | 1.69 |
| 2 (99) | 31 | 33.33 | 18 | 45.00 | 60 | 33.90 |
| 3 (101) | 8 | 8.60 | 1 | 2.50 | 6 | 3.39 |
| 4 (103) | 19 | 20.43 | 3 | 7.50 | 21 | 11.86 |
| 5 (105) | 12 | 12.90 | 3 | 7.50 | 25 | 14.12 |
| 6 (107) | 16 | 17.20 | 5 | 12.50 | 42 | 23.73 |
| 7 (109) | 17 | 18.28 | 8 | 20.00 | 32 | 18.04 |
| 8 (111) | 2 | 2.15 | 2 | 5.00 | 0 | 0.00 |
| 9 (113) | 8 | 8.60 | 7 | 17.50 | 14 | 7.91 |
| 10 (115) | 29 | 31.18 | 16 | 40.00 | 80 | 45.20 |
| 11 (117) | 13 | 13.98 | 9 | 22.50 | 41 | 23.16 |
| 12 (119) | 6 | 6.45 | 1 | 2.50 | 0 | 0.00 |
| 13 (121) | 4 | 4.30 | 1 | 2.50 | 21 | 11.86 |
| 14 (123) | 0 | 0.00 | 0 | 0.00 | 1 | 0.56 |
| **TNFb (bp)** | **N=91** | | **N=39** | | **N=155** | |
| 1 (124) | 16 | 17.58 | 9 | 23.08 | 42 | 27.10 |
| 2 (125) | 0 | 0.00 | 0 | 0.00 | 9 | 5.81 |
| 3[1](126) | 34 | 37.36 | 9 | 23.08 | 37 | 23.87 |
| 4 (127) | 39 | 42.86 | 23 | 58.97 | 102 | 65.91 |
| 5 (128) | 38 | 41.76 | 13 | 33.33 | 54 | 35.48 |
| 6 (129) | 1 | 1.10 | 0 | 0.00 | 2 | 1.29 |
| 7 (130) | 0 | 0.00 | 0 | 0.00 | 5 | 3.23 |
| **TNFd (bp)** | **N=93** | | **N=40** | | **N=168** | |
| 1 (124) | 1 | 1.07 | 2 | 5.00 | 11 | 6.55 |
| 2[2] (126) | 37 | 39.78 | 12 | 30.00 | 20 | 11.90 |
| 3 (128) | 0 | 0.00 | 0 | 0.00 | 8 | 4.76 |
| 4 (130) | 64 | 68.82 | 30 | 75.00 | 144 | 83.72 |
| 5 (132) | 31 | 33.33 | 14 | 35.00 | 70 | 41.66 |
| 6 (134) | 14 | 15.05 | 5 | 12.50 | 27 | 16.08 |
| 7 (136) | 4 | 4.30 | 0 | 0.00 | 5 | 2.98 |
| **D6S273 (bp)** | **N=88** | | **N=33** | | **N=168** | |
| 1 (126) | 4 | 4.55 | 3 | 9.09 | 11 | 6.55 |
| 2 (128) | 15 | 17.05 | 5 | 15.15 | 12 | 7.14 |
| 3[3] (130) | 17 | 19.32 | 3 | 9.09 | 54 | 32.14 |
| 4 (132) | 53 | 60.23 | 19 | 57.58 | 102 | 60.71 |
| 5 (134) | 35 | 39.77 | 19 | 57.58 | 82 | 48.81 |
| 6 (136) | 14 | 15.91 | 5 | 15.15 | 14 | 8.33 |
| 7[4] (138) | 22 | 25.00 | 7 | 21.21 | 17 | 10.12 |
| **D6S1014 (bp)** | **N=93** | | **N=40** | | **N=145** | |
| 1 (125) | 13 | 13.98 | 4 | 10.00 | 33 | 22.76 |
| 2 (128) | 5 | 5.38 | 2 | 5.00 | 0 | 0.00 |
| 3 (131) | 0 | 0.00 | 0 | 0.00 | 3 | 2.06 |
| 4[5] (134) | 45 | 48.39 | 17 | 42.50 | 93 | 64.14 |
| 5 (137) | 37 | 39.78 | 15 | 37.50 | 56 | 39.62 |
| 6 (140) | 25 | 26.88 | 15 | 37.50 | 44 | 30.34 |
| 7[6] (143) | 23 | 24.73 | 9 | 22.50 | 12 | 8.28 |
| 8[7] (146) | 6 | 6.45 | 6 | 15.00 | 3 | 2.06 |

Legend: PF-phenotype frequency;
[1]Patients with acute sarcoidosis vs. controls P=0.00350, Pcorr>0.05;
[2]Patients with acute sarcoidosis vs. controls P=0.00001; Pcorr=0.00007;
[2]Patients with chronic sarcoidosis vs. controls P=0.0091, Pcorr>0.05;
[3]Patients with acute sarcoidosis vs. controls P=0.0424, Pcorr>0.05;
[3]Patients with chronic sarcoidosis vs. controls P=0.0100, Pcorr>0.05;
[4]Patients with acute sarcoidosis vs. controls P=0.0003; Pcorr>0.0021;
[5]Patients with acute sarcoidosis vs. controls P=0.0219, Pcorr>0.05;
[5]Patients with chronic sarcoidosis vs. controls P=0.0223, Pcorr>0.05;
[6]Patients with acute sarcoidosis vs. controls P=0.0004, Pcorr=0.0028;
[6]Patients with chronic sarcoidosis vs. controls P=0.0151, Pcorr>0.05;
[7]Patients with chronic sarcoidosis vs. controls P=0.0037, Pcorr>0.0296;

lotypic associations which included only HLA class II region. Even the increase in frequency of HLA-A1/B8/DRB1*03/DQB1*0201/TNFa3/TNFb3/TNFd2/D6S273-7/D6S1014-7 among patients with acute form of disease (17/93-18.3%) did not reach statistically significant P value in comparison to patients with chronic form of sarcoidosis (4/40-10.%) nor controls (10/141-7.1%). The difference was even less pronounced for haplotypic associations: HLA-2/B18/DRB1*11/DQB1*0301/TNFa10/TNFb4/TNFd4/D6S273-4/D6S1014-6 (AcS: 5/93-5.4%; ChrS: 3/40-7.5%; controls: 12/141-8.5%) HLA-A2/B44/DRB1*13/DQB1*06/TNFa10/TNFb1/TNFd5/D6S273-4/D6S1014-4 (AcS: 4/93-4.3%; ChrS: 0/40-0%; controls: 3/141-2.1%).

## Discussion

The research on the association between sarcoidosis and HLA region has been carried out for a long period of time, but many questions are still open. A consensus about which HLA locus is directly involved in the pathogenesis of sarcoidosis has not been established to date. In our previous case–control study in the Croatian population we reported about the association between HLA-B8/DRB1*0301/DQB1*0201 haplotype with acute onset of disease, radiological stage I, erythema nodosum, Löfgren syndrome, no-medical therapy, and pulmonary sarcoidosis.

The present study is an extension of the previous study with the aim of determining which genetic markers within the HLA region are associated with acute or chronic form of disease. Consistent with previous report, we have demonstrated the association of HLA-DRB1*03/DQB1*0201 haplotype with the acute form of the disease. This confirms the findings from United Kingdom, Poland, Czech Republic, Italy, and Germany which reported about the HLA-DRB1*0301 association with a good prognosis and the acute form of the disease (32-35).

At the same time, we did not find a correlation between HLA genes or haplotypes with the chronic form of sarcoidosis. Namely, the higher frequency of HLA-DRB1*11 specificity in this subgroup of sarcoidosis patients was significant only before correction. This is similar to the findings from Poland, India and USA who have also reported about an association between DRB1*11 specificity and the chronic form of sarcoidosis (36, 37). Simultaneously, various groups of authors reported about a higher frequency of HLA-DRB1*1501 and DRB1*14 among patients with chronic sarcoidosis (38-40). HLA-DRB1*15 specificity was also increased in our sample of patients with chronic form of sarcoidosis but only before correction of the P value, while the higher frequency of DRB1*14 specificity did not reach a statistically significant P value even before correction. A lower frequency of DRB1*13 specificity among patients with the chronic sarcoidosis but only before correction, was also observed in the Croatian population. All these differences could be explained by the fact that the association of HLA-DRB1 specificities with sarcoidosis activation and disease outcome is ethnically dependent; on the other hand, this could also be explained by a smaller number of patients in our study which affected the statistical power of analysis.

The results obtained after analysis of HLA-DQB1 among patients and controls are in good concordance with previous reports with respect to DQB1*0201 which is in association with DRB1*0301. On the other hand, no such significant association was found for DQB1*0602 and persistent disease as was reported by other authors (41). This correlates well with our finding for DRB1*15 which is in linkage disequilibrium with this DQB1 allele and can also be explained by a lower statistical power of analysis due to relatively small group of patients.

This study is not the first attempt to discover other genetic markers which play a role in the immunopathogenesis of sarcoidosis. A few studies have reported about the association between TNF polymorphisms and sarcoidosis (11, 14-16). More precisely, a higher frequency of the TNF-307A allele was found among patients with Löfgren's syndrome, while a study from the Czech Republic reported about an association between two other single nucleotide polymorphisms (SNPs) and Löfgren's syndrome (TNF-308*G/A and LTA*252*A/G). Additional support for the investigation of the polymorphisms within TNF gene cluster was given by a Japanese study in which the authors reported about the association between TNFa2 allele and cardiac sarcoidosis (17, 18). The clinical course of sarcoidosis has already been associated with patient's individ-

ual capacity of spontaneous TNF-α production by alveolar macrophages (42). The protein level of this cytokine has been shown to correlate with the polymorphisms located within the regulatory region of its gene, especially with the TNF-308 polymorphism (43). According to our knowledge, this is the first time that the polymorphisms of TNF microsatellites were analyzed among patients with sarcoidosis. Our data did not reveal any association between TNFa and TNFb microsatellite alleles and sarcoidosis. On the other hand, the only statistically significant difference concerning the TNFd locus, even after the correction of P value, was observed for TNFd-2 allele. The increase of the TNFd-2 allele frequency was found for both subgroups of patients. While among patients with AcS this result could be interpreted with respect to the fact that this allele is also a part of 8.1 AH (ancestral haplotype), the fact that HLA-B*08 specificity, as well as DRB1*0301 allele frequencies were not increased among patients with ChrS suggests that this TNFd allele might be considered as an independant risk factor for chronic sarcoidosis.

Regarding the increased frequency of D6S273-7 allele among patients with AcS in comparison to the controls, after the stratification analysis was performed for this allele, we can conclude that this increase is a result of linkage disequilibrium of this allele with DRB1*03 specificity. At the same time, on the base of our results we can suggest that D6S273-3 allele is protective for the chronic form of sarcoidosis (OR=0.21).

Considering that the HLA-DRB1 locus is in linkage disequilibrium with D6S1014 locus, we have also performed a stratification analysis taking into account polymorphisms at these loci. Results demonstrated that the higher frequency of D6S1014-8 allele and odds ratio (OR=8.35) are not a result of LD with DRB1*11 or DRB1*15 which have also shown a positive correlation with ChS (OR=2.44 and OR=2.47, respectively). Our data suggest that D6S1014-8 allele independently contributes to the susceptibility to chronic sarcoidosis. On the base of our results we can hypothesize that gene(s) associated with sarcoidosis is located between D6S273 and D6S1014 microsatellites in the HLA class III region (G6D gene) (43). This gene is a member of the leukocyte antigen 6 superfamily, which is important in leukocyte maturation and for that reason it might be very helpful to screen sarcoidosis patients for their polymorphisms.

Haplotypic association analysis which included markers located in HLA class II region demonstrated positive association HLA-DRB1*03/DQB1*0201/TNFa3/TNFb3/TNFd2/D6S273-7/D6S1014-7 and HLA-DRB1*13/DQB1*06/TNFa10/TNFb1/TNFd5/D6S273-4/D6S1014-4 combinations with acute sarcoidosis, while for patients with chronic form of sarcoidosis a positive association was found for the HLA-DRB1*11/DQB1*0301/TNFa10/TNFb4/TNFd4/D6S273-4/D6S1014-6 combination. No statistically significant association was found for any of the haplotypic combinations which included the HLA class I region which supports the hypothesis that genetic markers in HLA class II region are more involved in predisposition to this disease than those located in the HLA class I region.

In conclusion, the present study identified non-HLA markers associated with sarcoidosis giving a new point of view on the genetic background for this disease and suggesting the involvement of the region between HLA-DQB1 and D6S273 loci in its pathophysiology.

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