Coll. Antropol. **35** (2011) 4: 1243–1249
Original scientific paper

The Distribution of HLA Alleles among Children with Atopic Asthma in Croatia

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A B S T R A C T

*Allergic asthma is a multifactorial disease involving well known environmental factors and less identified genetic components. In several studies the HLA genes have been implicated in the development of asthma and atopy, but the importance of these associations remains unclear. The aim of the present study was to analyse the distribution of specificities at HLA class I loci (-A and -B) and HLA class II locus (-DRB1) in a group of 143 Croatian children with atopic asthma, regarding total serum IgE and specific IgE against common inhalant allergens, as well as their connection with different asthmatic phenotypes and to identify HLA genotype which increases the risk for atopy or asthma or which has a protective effect. As controls we used a group of 163 healthy unrelated individuals. HLA class I antigens were determined by serology, while DRB1 specificities were detected by polymerase-chain reaction amplification and hybridisation with sequence specific oligonucleotide probes method (PCR-SSOP). We found no significant correlation between any of the HLA-A antigens and asthma, atopy or associated atopic phenotypes. At HLA-B locus, HLA-B8 antigen was significantly increased among asthmatic patients ($p=0.002$), patients with high total serum IgE ($p=0.002$), as well as among patients sensitizated to Dermatophagoides pteronyssinus (Der p) ($p=0.014$) and among patients sensitizated to Der p + Dactylis glomerata (Dact g) or Ambrosia elatior (Amb a) ($p=0.004$). Among HLA-DRB1 specificities, HLA-DRB1*01 showed positive correlation with asthma and atopy ($p=0.034$), while HLA-DRB1*03 specificity was observed with significantly higher frequency among patients with total serum IgE ≥ 400 KU/L ($p=0.048$). HLA-DRB1*16 specificity was observed with significantly lower frequency among patients with asthma only in comparison to healthy controls ($p=0.027$) and to patients with asthma and allergic rhinitis ($p=0.005$). In conclusion, our data suggest that HLA specificities play a relevant role in predisposition to asthma, as well as in different clinical forms of atopic diseases. HLA-B8, HLA-DRB1*01 and HLA-DRB1*03 genotype increases the risk for atopic asthma and high serum IgE.*

Key words: *atopy, asthma, children, HLA class I and class II*

Introduction

Allergic asthma is a multifactorial disease involving well-identified environmental factors and less-known genetic components. It is the most common chronic childhood disease, the prevalence of which is on the rise in Croatia, as well as in most of the other countries worldwide. Products of mayor histocompatibility complex (MHC), located within 6p21.3 region, play a fundamental role in regulating immune responses since they encode the molecules that represent the linkage elements between environmental allergens and the immune system. MHC genes and human leucocyte antigen (HLA) system have been implicated in the development of asthma and atopy, but the importance of associations between HLA genes and asthma remains unclear. Different HLA genes may represent factors conferring risk or protection for the development of allergic diseases. It is assumed that HLA genes as genetic markers have influence on the atopy and asthma as well as on sensitization against specific inhalant allergens. Their identification in Croatian children suffering from atopic asthma, as well as their connection with different atopic phenotypes, could help to identify individuals with increased risk for the disease. This could enable both the primary prevention and timely introduction of the appropriate treatment.

In general, the availability of data regarding the influence of HLA genes on atopy is much lower for children

Received for publication October 1, 2009

than for grownups, with results for children population being rather inconsistent. The antigens which are most often linked with atopy and asthma in children are HLA-B8, -B17, -Bw44, HLA-DRB1*03, -DRB1*01 and -DRB1*013, although results differ for diverse population groups[1–5]. Based on our review of the available current literature, only two studies have so far examined the relationship between HLA types and asthma severity[4,6]. At the same time, studies of this kind have not been performed for our population. For that reason and in order to elucidate the relationship between asthma and related phenotypes and HLA genes in the Croatian children with atopic asthma, we have analysed HLA-A and HLA-B *loci* within the class I region, as well as the most polymorphic of HLA class II loci: HLA-DRB1 locus, which has demonstrated a connection to asthma and atopy in most studies on other populations[3–5,7–9]. In this study we assess the potential influence of specific HLA genes on the severity of childhood asthma Objectives of our study were to (I) determine frequencies of HLA-A, HLA-B antigens and HLA-DRB1 specificities, (II) analyse the association between HLA-A, HLA-B antigens and HLA-DRB1 specificities and atopy – with regard to total serum IgE levels and specific IgE against three common aeroallergens (*Der p, Amb e* and *Dact g),* (III) analyse the association between HLA-A, HLA-B antigens and HLA-DRB1 specificities and different atopic phenotypes (coexistence of allergic rhinitis – AR, clinical severity of the disease and response to treatment) and (IV) identify HLA specificities which increase the risk for atopy and/or asthma or have a protective effect.

Material and Methods

Study design and subjects

The study was a case-control study. The subjects for this study were a stratified random sample of the children treated for atopic asthma in our outpatient clinic. We obtained data on family history of asthma or atopy from medical records of the subjects. The samples were collected during one year period, from April 2004 to April 2005.

The study protocol was approved by the institutional review board and written informed consent was obtained from at least one parent of the study participants.

Controls

A total of 163 unrelated healthy individuals, (older than 18 years, 64% males and 36% females) without any signs of allergic diseases or sensitization to common inhalant allergens were chosen as controls for HLA class I and HLA class II polymorphisms. They are citizens of Zagreb but they originate from different regions of Croatia. They form a representative sample of the Croatian population and have been published in the literature[10,11]. As HLA frequencies do not vary with age in the general population, this control group is considered to be matched with patients of every age.

Ascertainment of asthma case

Patients were considered to have asthma if a physician had made a diagnosis of asthma upon the following criteria: a history of recurrent episodes of cough, wheezing, shortness of breath and chest tightness with favourable clinical response to bronchodilator. In children older than 6 years, airflow reversibility was documented by positive response to bronchodilator (rise of FEV_1 ≥15% measured 10 min after β_2-agonist inhalation).

Atopy was defined as positive skin prick-test (presence of a wheal ≥3 mm) to at least one of the common aeroallergens, elevated total serum IgE antibodies and elevated specific IgE against the same allergens.

Total and specific IgE in serum were determined using monoclonal-based immunoassay (Uni-Cap-system, Pharmacia-Upjohn Diagnostics, Uppsala, Sweden).

Patients having any other chronic condition affecting the respiratory system, except asthma, were excluded from the study.

Severity of asthma

There is no gold standard for diagnosing asthma, or for rating the severity of the condition, especially in children. However, some recent studies concluded that the Global Initiative for Asthma (GINA) guidelines, the most widely used recommendations for asthma management; seem to offer a valid basis for classifying asthma severity[12–14]. We based our primary definition of asthma severity on GINA guidelines which classify patients at initial assessment into four categories: intermittent, mild persistent, moderate persistent and severe persistent asthma[12]. We determined the severity of asthma at the time when a patient met the criteria for asthma. Levels of asthma control were assessed according to the same guidelines, referring to the control of the manifestations of the disease[5]. Diagnosis of AR was made according to ARIA guidelines[15]. All patients had nasal smears for eosinophils and ENT (ear-nose-throat) examination.

HLA typing

HLA-A and HLA-B typing was performed on T+B lymphocyte suspension according to standard microcytotoxicity test[16]. The typing sera were obtained from our laboratory and from commercial sources. The detection of 14 different HLA-A antigens and 25 HLA-B antigens was possible with those sera.

Class II HLA-DRB1 alleles were typed using the PCR--SSOP method. Generic amplification of DRB1 genes was carried out according to the previously reported procedure[16]. PCR products were blotted on nylon membranes and hybridized with biotinylated oligonucleotide probes as specified by the XIIth International Histocompatibility Workshop[17]. The conditions of hybridization and washing were as previously reported[18]. Thirteen main DRB1 specificities could be determined with this set of probes.

The results were compared to those of 163 (for HLA class I antigens) and of 141 (for HLA-DRB1 specificities) healthy unrelated controls from the same population.

Satistics

Statistical analysis was made using the »Sta-Win« software package. HLA antigen frequencies observed in the patients were compared to those determined in local ethnically matched controls.

Frequencies were compared using chi-squared test with Yates continuitiy correction. When the conditions were unsuitable for chi-squared test, we used Fisher's exact test. P values were corrected by multiplying them with the number of antigens tested (P_{corr}). A P value less than 0.05 was considered to indicate a statistically significant difference between groups.

The odds ratio (OR) for each HLA specificity was calculated from the 2x2 tables.

Results

Study cohort

One hundred and forty-three children with atopic asthma (88 boys and 55 girls), aged 2.8 to 18.8 years (median 10.9 years), underwent HLA typing. Patient characteristics are listed in Table 1. AR was present in 59.4% of patients, which is less than expected, probably due to the lower age bracket share of patients (less than 4 years of age), where AR is rare. The majority of patients had mild asthma (48.3%), while only 3.4% patients had severe persistent asthma. Moderate persistent asthma and intermittent asthma were equally represented (27.3% and 21.0% respectively). All patients were sensitized to house-dust mite (*Der p*); among them 29 patients (20.3%) were also sensitized to both ragweed (*Amb e*) and mugwort (*Dact g*), while 33 patients (23.1%) were – in addition to house-dust mite – also sensitized to either ragweed or mugwort. Atopic diseases (asthma, allergic rhinitis and/or atopic eczema) in family members were present in 84 patients (58.7 %). Levels of serum total IgE were above the upper referent value in all patients.

Since in some healthy individuals the total serum IgE can be elevated even up to double normal value for age without any clinical significance, we have decided to set the cut-off point above the highest total IgE level in the non-atopic group. Therefore, patients with total IgE levels ≥400 kU/L were considered as high IgE responders (65.7%). Considering the response to treatment, assessment of asthma control after six months of therapy revealed that 60 patients (42.0%) had their asthma under control, 72 patients (50.3%) were partly controlled, while in 11 patients (7.7%) asthma remained uncontrolled in spite of therapy.

Class I HLA antigens

Thirteen different HLA-A antigens were identified in our patients, the most common being HLA-A2 (49.7%), HLA-A9 (29.4%) and HLA-A1 (26.6%). The same antigens were the most common in controls as well (Table 2).

TABLE 1
PATIENT CHARACTERISTICS

	n	%
Number of patients	143	
Females	55	38.5
Males	88	61.5
Atopic phenotype		
Asthma	58	40.6
Asthma + allergic rhinitis	85	59.4
Severity of asthma		
Intermittent	30	21.0
Mild persistant	69	48.3
Moderate persistent	39	27.3
Severe persistent	5	3.4
Sensitization		
Der p	81	56.6
Der p + Dact g or Amb e	33	23.1
Der p + Dect g + Amb e	29	20.3
Atopy in the family		
Yes	84	58.7
No	59	41.3
Total serum IgE		
≥ 400 kU/L	94	65.7
< 400 kU/L	49	34.3
Asthma control after therapy		
Controlled	60	42.0
Partly controlled	72	50.3
Uncontrolled	11	7.7

Legend: Der p – Dermatophagoides pteronyssinus; Dact g – Dactylis glomerata; Amb e – Ambrosia elatior

On HLA-B locus, 19 different antigens were identified among our patients, the most common were HLA-B35 (21.7%), HLA-B8 (21.0%) and HLA-B5 (20.3%).

HLA-B8 antigen was significantly increased in asthmatic patients (χ^2=9.62, p=0.002, OR=3.06, p_{corr}=0.038) in comparison to controls.

HLA-B8 antigen was also significantly increased in patients with high total serum IgE (χ^2=9.50, p=0.002, OR=3.37, p_{corr}=0.042), and also showing its positive correlation with atopy, as well as with allergic rhinitis (χ^2= 7.74, p=0.005, OR=3.09, p_{corr}=0.108) (Table 3). When we analysed HLA class I antigens with regard to the sensitization to inhalant allergens, HLA-B8 antigen was significantly more frequent in patients sensitized only to *Der p* (χ^2=6.09, p=0.014, OR=2.84, p_{corr} =0.272) and in patients sensitized to *Der p* and either *Amb e* or *Dact g* (χ^2=8.41, p=0.004, OR=4.33, p_{corr}=0.074), although these correlations showed no significance after correction. At the same time HLA-B8 antigen showed a negative cor-

TABLE 2
DISTRIBUTION OF THE MOST FREQUENT HLA-A, -B AND –DRB1 SPECIFICITIES AMONG THE CROATIAN PATIENTS WITH ASTHMA AND CONTROLS

HLA-	Patients with atopic asthma (N=143)		Controls*	
	n	%	n	%
A1	38	0.133	30	0.092
A2	71	0.248	83	0.255
A3	22	0.077	35	0.107
A9	42	0.147	53	0.163
A11	18	0.063	17	0.052
A25	6	0.021	12	0.037
A26	13	0.046	15	0.046
A28	10	0.035	14	0.043
B5	29	0.101	42	0.129
B7	22	0.077	28	0.086
B8	30	0.105 [1]	13	0.039
B12	23	0.080	27	0.083
B13	16	0.056	8	0.025
B14	15	0.052	7	0.022
B15	12	0.042	22	0.067
B18	21	0.073	37	0.114
B27	23	0.080	19	0.058
B35	31	0.108	36	0.110
DRB1*01	35	0.129	25	0.089
DRB1*03	31	0.115[2]	19	0.067
DRB1*04	29	0.107	29	0.103
DRB1*07	25	0.093	27	0.096
DRB1*11	47	0.174	44	0.156
DRB1*13	34	0.126	36	0.128
DRB1*15	23	0.085	30	0.106
DRB1*16	21	0.0778[2]	33	0.117

relation with response to treatment and was significantly more often found in patients who remained on the same level according to the severity of asthma in comparison to patients whose asthma was completely under control (χ^2=8.10, p=0.004, OR=3.30, p_{corr} =0.088).

HLA-B14 antigen showed significant positive correlation with sensitization to all three tested inhalant allergens, although the correlation lost significance after correction (χ^2=5.01, p=0.025, OR=4.45, p_{corr}=0.512).

HLA-DRB1 specificities

Among patients with asthma, 13 different specificities of HLA-DRB1 locus were identified. The most common specificities were HLA-DRB1*11 (17.4% of patients), HLA--DRB1*01 (13.0%) and HLA-DRB1*13 (12.6%). There were no significant differences in frequencies of HLA--DRB1 specificities between asthmatics and controls. Higher frequencies of HLA-DRB1*01 and -DRB1*03 specificities observed among patients were marginally significant, as well as a lower frequency of HLA-DRB1*16 specificity (Table 2).

In patients suffering from asthma without AR, HLA--DRB1*01 specificity was significantly increased (χ^2=4.52, p=0.034, OR=2.10, p_{corr}=0.442), while HLA-DRB1*16 specificity was decreased in comparison to patients suffering from both asthma and AR, as well as to control subjects (χ^2=5.31, p=0.027, OR=0.28, p_{corr}=0.350) (Table 3).

HLA-DRB1*03 specificity was significantly increased compared to controls in patients with high serum IgE levels (≥400 kU/L) (χ^2=3.89, p=0.048, OR=2.0, p_{corr}=0.623).

We found no significant correlation between HLA--DRB1 specificities and specific sensitization against three tested allergens, or the correlation with different asthmatic phenotypes regarding the response to treatment.

TABLE 3
THE LIST OF HLA SPECIFICITIES POSITIVELY AND NEGATIVELY ASSOCIATED WITH DIFFERENT FEATURES OF ATOPY AND ASTHMATIC PHENOTYPE

Patients	HLA-	Patients %	Controls %	P	OR
Asthma only	B8	0.210	0.039	0.002	3.06
	DRB1*01	0.170	0.089	0.034	2.10
	DRB1*16	0.036	0.111	0.027	0.28
Asthma + allergic rhinitis	B8	0.212	0.039	0.005	3.09
Total serum IgE ≥ 400 kU/L	B8	0.223	0.039	0.002	3.37
	DRB1*03	0.126	0.067	0.048	2.00
Sensitization to inhalant allergens					
Der p	B8	0.198	0.039	0.014	2.84
Der p + Dact g or Amb e	B8	0.273	0.039	0.004	4.33
Der p + Dact g + Amb e	B14	0.172	0.043	0.025	4.45
Response to treatment			Controlled		
Partly controlled	B8	0.222	0.039	0.004	3.30

Legend: *N=163 for HLA-B, while N=141 for HLA-DRB1

Discussion and Conclusion

Results of the previously conducted investigations point to the linkage of HLA antigens with atopy and asthma, showing that different HLA genes can represent an increased risk or protection in the development of allergic diseases[19–21]. Immunogenetic studies on the linkage between hereditary factors and asthma in children's populations also demonstrate differences between various ethnic groups[19,7,9,22–24].

In the present study we analysed a group of 143 patients with asthma. The importance of this study stems from the fact that it is the first genetic study of atopic asthma in the Croatian population. The distribution of HLA-A antigens in our population is similar to those reported in other studies for children[22,23,7]. We found no significant correlation between any of the HLA-A antigens and asthma, atopy or associated atopic phenotypes. These results coincide with data from literature, so it seems that the HLA-A locus does not have an influence on either atopy or atopic phenotypes[7,9,19–24]. On the other hand, according to our results, HLA-B8 antigen represents a risk factor for asthma in Croatian children's population. The connection between HLA-B8 antigen and asthma has been established in other populations as well, e.g. in adults and children with allergic asthma in English, Greek and Egyptian populations[7,22,23,25]. Data presented in this paper point out that HLA-B8 antigen is also a risk factor for atopy.

Among the genetic factors with an influence on atopy, HLA antigens have a role in the allergen-specific IgE-mediated response. A positive linkage has been established between the hypersensitivity to *Der p I* and HLA-DRB1*04 and HLA-DRB1*03 specificities[26] but a weak linkage or a complete lack of association has been reported in studies that have analyzed the response to complex antigens, *i.e.* mixtures of unpurified allergens, or the response to allergens with large molecular mass[24,27–29]. According to the published results, it seems that individual antigens do not have a significant influence on the intensity of specific IgE immunological response. It is more probable that environmental factors or other *loci* (e.g. genes for T-cell receptor or TNF-α) are important in determining the individual sensitization to a specific allergen[30]. In our patients, HLA-B8 antigen has shown influence on the production of specific IgE antibodies against inhalant allergens. This antigen is significantly more frequent in patients sensitized to house-dust mite only, and in patients which are also sensitized to one of the other tested allergens (*Der p+Amb e* or *Dact g*). In patients who are sensitized to all three of the tested allergens, HLA-B14 antigen is the most frequent one. It is difficult to compare results of our study with the literature, because there is no previous epidemiologic study that assessed the association between HLA class I antigens and specific IgE response. In order to reach any definite conclusion, a larger group of patients should be analysed, and it would be helpful to include data for other populations worldwide as well.

At the same time, HLA-B8 antigen showed a negative correlation with the response to treatment and was found significantly more often in patients who remained on the same level according to the severity of asthma in comparison with the patients who achieved complete control after treatment. To the best of our knowledge, there are no published results on connection between HLA class I antigens and asthma severity.

For the purpose of analyzing the linkage between the HLA-class II antigens and asthma and atopy in our patients, we have chosen the HLA-DRB1. In the largest number of immunogenetic studies, this gene is covered by the most consistent data concerning its association with atopy and asthma. In patients suffering from asthma without AR, HLA-DRB1*01 specificity was significantly increased, which is in concordance with published data for other populations. Namely, in most of the reported studies, an association between the HLA-DRB1*01 specificity and atopy has been established[8,19,31–35]. This particular specificity appears significantly more often in asthmatics with high concentrations of total serum IgE (above 200–400 IU/mL), both in adults and in children[19,21,31,32,8]. In our group of asthmatic children with high concentration of IgE we have found that the HLA--DRB1*03 specificity was significantly increased in comparison to controls, and therefore is proved to be a risk factor for atopy in our patients. It is probably a result of well-known linkage disequilibrium between HLA-B8 and DRB1*03, to be precise, HLA-B8 antigen showed higher value of OR (OR=3.37) and p value in comparison to HLA-DRB1*03 (OR=2.00; p=0.0480). The association of this specificity with asthma and with high total serum IgE has been established by other authors as well[3,4,21,29,2]. It is well documented that the 8.1 ancestral haplotype (8.1 AH) encompassing the alleles HLA-A1, -B8 and DR3 is one of the most frequent Caucasian haplotypes. Aside from the HLA markers which could be responsible for this association, the non-HLA constituens of 8.1AH haplotype should also be considered. For example, 8.1 AH include the fourth component of human complement (C4) which is an essential factor of the innate immunity. Furthermore, alleles TNF2 (–308*A), HSP70-2 1267*G, LTA 252*G and C4A*Q0 which are located in the HLA class III region are also a part of this haplotype.

It is also important to point out that certain HLA antigens are probably in linkage disequilibrium with another – as yet unidentified – gene that controls the non-specific IgE synthesis. HLA antigens act together with other *loci* involved in the synthesis of IgE (e.g. *loci* on chromosomes 5q and 11q).

It is interesting to note that HLA-DRB1*16 specificity showed significantly lower presence in the group of the Croatian children suffering from asthma without AR in comparison to patients having both asthma and AR (3.57% *vs*. 10.76%, p=0.05; data not shown) which is in concordance with results from Woszczek et al.[8].

We found no significant correlation between HLA--DRB1 specificities and specific sensitization against tested allergens, which is again compatible with most of the

published results[27–29]. No correlation was found with different asthmatic phenotypes regarding the response to treatment, either. There are limitations to the ability for making definitive conclusions concerning the genetic influences on atopy and asthma in Croatian children, because of the relatively small sample size and the absence of a simultaneous analysis of other candidate genes in combination with HLA markers.

In conclusion, this study represents an attempt to assess the genetic susceptibility to childhood asthma and atopy in the Croatian population. Importantly, it is a part of the effort to define the genes responsible for asthma and atopy in different population groups. Our data suggest that HLA-B8, HLA-DRB1*01 and HLA-DRB1*03 specificities present a risk factor for atopic asthma and high serum IgE. This information may help clinicians and researchers to better characterize asthma for the purpose of improved patient care *e.g*. in early identification and treatment. These research findings deserve further investigations involving more patients with different forms of the disease, and including fine gene mapping.

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HLA ANTIGENI I ATOPIJSKA ASTMA U DJECE U HRVATSKOJ

S A Ž E T A K

Alergijska astma je multifaktorski uzrokovana bolest u čiju su patogenezu uključeni dobro poznati čimbenici iz okoliša, ali i manje poznati čimbenici nasljeđa. Brojne studije ukazuju na utjecaj gena HLA na razvoj astme i atopije. Cilj ovog istraživanja bio je analizirati raspodjelu antigena HLA razreda I (-A i -B) i specifičnosti HLA-DRB1 u skupini od 143 hrvatske djece s atopijskom astmom, s osvrtom na ukupni IgE u serumu, specifični IgE na uobičajene inhalacijske alergene, povezanost HLA antigena s različitim astmatskim fenotipovima te identificirati HLA genotip koji povećava rizik za atopiju/astmu ili ima protektivni učinak. Kontrolnu grupu činile su 163 zdrave nesrodne osobe. Antigeni HLA razreda I određivani su testom mikrolimfocitotoksičnosti (MLCT), dok su specifičnosti lokusa DRB1 određene metodom PCR-SSOP (engl. Polymerase Chain Reaction – Sequence Specific Oligonucleotide Probes*).* Istraživanjem nije utvrđena statistički značajna povezanost između antigena HLA-A i astme, atopije ili asociranih atopijskih fenotipova. Na lokusu HLA-B, antigen HLA-B8 je značajno češći u bolesnika u usporedbi s kontrolnom skupinoim (p=0,002). Isti antigen također je značajno češći u bolesnika s visokim ukupnim IgE u serumu (p=0,002), te u bolesnika senzibiliziranih na *Dermatophagoides pteronyssinus* (*Der p)* (p=0,014) i bolesnika senzibiliziranih na *Der p* i *Dactylis glomerata (Dact g)* ili *Der p* i *Ambrosia elatior* (*Amb e*) (p=0,004). Među specifičnostima lokusa HLA-DRB1, za HLA-DRB1*01 utvrđena je pozitivna povezansot s astmom i atopijom (p=0,034), dok je HLA-DRB1*03 značajno češće prisutan u bolesnika s ukupnim serumskim IgE ≥ 400 KU/L (p=0,048). Specifičnost HLA-DRB1*16 značajno je smanjen među bolesnicima koji boluju jedino od astme u usporedbi s kontrolom (p=0,027) kao i u usporedbi s bolesnicima koji boluju i od alergijskog rinitisa (p=0,005). U zaključku, naši rezultati ukazuju da regija HLA ima značajnu ulogu u predispoziciji za astmu, kao i u različitim kliničkim oblicima alergijskih bolesti. HLA-B8, HLA-DRB1*01 i HLA-DRB1*03 specifičnosti značajno povećavaju rizik za atopijsku astmu i visoki serumski IgE.