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Association of Cytokine Gene Polymorphisms with Bronchial Asthma in Macedonians

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ABSTRACT

Bronchial asthma is a multifactorial disease whereby both environmental and genetic factors contribute to its aetiology and/or clinical severity. The aim of this study was to examine the association of 22 cytokine gene polymorphism in the Macedonian population with bronchial asthma (BA).

The sample of the population comprised of 301 normal unrelated individuals and 74 patients with BA. Cytokine genotyping was performed by PCR.

Susceptible cytokine polymorphisms for BA for ten genotypes (IL-4 -1098/T:T, TNF-α -238/A:G, IL-4 -590/C:C, IL-2 +166/T:T, IL-2 -330/T:T, IL-10 -1082/G:G, IFNγ utr5644/T:T, IL-10 -1082/A:A, IL-1β +3962/T:T, IL-6 -174/G:G), six diplotypes, four haplotypes, and two alleles were found. Protective cytokine polymorphisms for BA for seven cytokine genotypes (IL-4 -1098/G:T, TNF- α -238/G:G, IL-2 -330/G:T, IL-4 -590/C:T, IFNγ utr5644/A:T, IL-1β +3962/C:T, IL-10 -1082/A:G), six cytokine diplotypes, four cytokine haplotypes, and four cytokine alleles were found.

We concluded that several cytokine polymorphisms are protective, or susceptible associated with BA in population of Macedonians.

Key words: Bronchial asthma; Cytokine polymorphism; Macedonians

INTRODUCTION

Bronchial asthma (BA) remains a significant problem worldwide, because it affects more than 300 million of people all over the world.[1] Although in recent years there has been advances in pathophysiology of bronchial asthma, its cause is still unknown. Bronchial asthma is a multifactorial disease whereby both environmental and genetic factors contribute to its aetiology and/or clinical severity.[2-5] Genetics of bronchial asthma is complex, involving multiple genes, and Mendelian patterns of inheritance do not follow.[6-9] More likely is that the pathophysiology of bronchial asthma involves the interaction of multiple sets of genes.[10,11] Bronchial asthma is a multi-complex chronic disease

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characterized with inflammation of airway mucosa.[12] Because of that, factors that regulate the inflammation response when direct contact with airborne agents exists, plays an important role in the pathogenesis of bronchial asthma. This inflammation is regulated by a number of different cytokines originated from inflammatory and structural tissue cells. It has been postulated that clinical symptoms in bronchial asthma may reflect an imbalance in pro- and anti-inflammatory cytokine content.

Several cytokine gene polymorphisms in the cytokine gene regulatory regions correlate with cytokine secretion,[13,14] and one individual may have a cytokine expression pattern quite different from an other.[15] Studies of disease association have been done in order to understand the correlation with immune activation, which may determine the risk or protection from disease expression.[15-18] Analysis and interpretation of allelic and haplotype distributions are taken in the context of a normal population and these distributions may vary by ethnic group.

Many data have been published about the relationship between cytokine polymorphism and bronchial asthma. While some authors show positive association of certain cytokine polymorphisms with bronchial asthma, others report quite opposite results.[19-28]

We have published data about cytokine polymorphism in healthy ethnic Macedonians,[29] however there are no data about the associations of cytokine polymorphisms and various diseases in the Republic of Macedonia. The aim of this study was to examine the association of cytokine gene polymorphisms in the healthy Macedonian population and in patients with bronchial asthma in order to add knowledge about the genetic background of this disease, and to provide data for Meta- analysis.

Our results have shown several susceptible and protective cytokine polymorphisms for bronchial asthma in Macedonian population, and for the first time it was shown an association between *IL-2* polymorphisms and bronchial asthma.

PATIENTS AND METHODS

Groups

The total studied sample consisted of 375 examinees, divided into two different groups as follows: normal individuals, and patients with bronchial asthma.

Normal individuals. There were 301 unrelated individuals, born in different parts of Macedonia. They were age and sex non-matched normal individuals who attended the Institute of Immunobiology and Human Genetics for DNA donation between May 1, 2001 and April 25, 2002 and agreed to take part in this study as a control group. Individuals with family history of bronchial asthma were excluded from the investigation.

Bronchial asthma. There were 74 patients with bronchial asthma fulfilling the criteria of National Institutes of Health (Bethesda, MD).[30] They were 37-59 years old patients who attended the Clinic for Pulmoallergology, University School of Medicine for outpatient treatment between May 5, 2003 and April 25, 2004.

All individuals were of Macedonian origin and nationality, Christian Orthodox religion, and residents of different regions of the Republic of Macedonia. Each individual was interviewed on a one-to-one basis, his/her genealogy was recorded for the past three generations, and a signed consent was obtained. Admixture, if any, was recorded for each individual. Individuals with only one Macedonian parent were excluded from the study.

All of the patients and normal individuals included in this study signed a written consent to participate in the study which was approved by the Committee of the Ministry of Education and Science from Republic of Macedonia (No 087405), and Ethical Committee of the Medical Faculty in Skopje.

Genomic DNA Isolation and Storage

DNA was isolated from peripheral blood leukocytes by phenol-chlorophorm extraction method or with BioRobot EZ1 workstation (QIAGEN).[31] The quality and quantity of DNA were analyzed by GeneQuant (Pharmacia Biotech, Uppsala, Sweden). Isolated DNA samples were stored in the Macedonian Human DNA Bank.[32]

Typing Methods

Thirteen cytokine genes were identified: gamma-interferon (*IFNγ*); interleukin (IL) 1 alpha (*IL-1a*); IL-1 beta (*IL-1β*); IL-1 receptor (*IL-1R*); IL-1R antagonist (*IL-1RA*); *IL-2; IL-4;* IL-4 receptor alpha (*IL-4R a); IL-6; IL-10; IL-12;* TGF beta 1 (*TGF-β1*); and TNF alpha (*TNF-a*). Cytokine genotyping was performed by polymerase chain reaction with sequence-specific

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priming (PCR-SSP) (Heidelberg kit, from the Institute of Immunology, Department of Transplantation Immunology, University of Heidelberg, Heidelberg, Germany). Briefly, PCR-SSP typing by the Heidelberg kit consisted of 48 PCR primers mixes aliquotted in 96 well PCR trays (two typing per tray). Master mix, which was supplied along with the reagents and consisted of $MgCL_2$, buffer, dNTP's, and glycerol was mixed with 1.2-3.0 µg DNA and 20 U Taq polymerase and dispensed in the 48 wells. Agarose gel electrophoresis on a 2% agarose gel revealed either a positive or a negative specific amplification for each well.[33] Subsequently, the results were entered in the Cytokine-SCORE software[34] and analyzed automatically. Manual interpretation was also possible according to the interpretation scheme provided along with the kit.

Statistical Analysis

The population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop,[35-37] was used for analysis of the cytokine data for this report. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each single nucleotide polymorphism (SNP) were determined.[38] The exact test for genotype frequency deviation from HWP was calculated using the Arlequin implementation accessed via PyPop.[39] Those SNPs that did not fit HWP were evaluated to determine whether there was an excess of homozygotes or heterozygotes, or if any particular genotypes significantly differed from the expected frequencies by the chi square test. Comparisons of frequencies for two groups were tested by the χ^2 test. Crude odds ratios (OR) (as estimates of the relative risk) were calculated with 95% confidence interval (CI).

RESULTS

Cytokine Alleles

Cytokine allele frequency, Fisher exact p-value, Odds ratio and Wald's 95% confidence interval in bronchial asthma patients and normal Macedonian population are shown (Table 1). The biggest positive (susceptible) odds ratio was found for *TNFα -238/A* (p<0.001) (OR=4.752, Wald's 95% CI between 2.692-8.389), meaning that people with *TNFα -238/A* allele have 4.75 times higher risk to develop bronchial asthma in comparison to others with *TNFα -238/G* allele. Positive (susceptible) odds ratio was also found for *IL-4 -590/C* (p<0.001), odds ratio 3.403 (2.038-5.682). Negative (protective) association for bronchial asthma was found for the following alleles: *IL-4 -1098/G* (p<0.001), odds ratio 0.223 (0.123-0.406); *IL-2 +166/G* (p<0.001), odds ratio 0.516 (0.354-0.754); *IL-2 -330/G* (p=0.003), odds ratio 0.519 (0.335-0.803); *IL-6 -174/C* (p=0.025), odds ratio 0.611 (0.397-0.942) (Table 1).

Cytokine Genotypes

Cytokine genotype frequency, Fisher exact p-value, Odds ratio and Wald's 95% confidence interval in bronchial asthma patients and normal Macedonian population are shown (Table 2). We found positive (susceptible) association between patients with bronchial asthma and following genotypes (according to the level of susceptibility): *IL4 -1098/T:T* (p<0.001), odds ratio 7.155 (Wald's 95% CI between 3.751-13.65); *TNF-α -238/A:G* (p<0.001), odds ratio 6.944 (3.675-13.12); *IL-4 -590/C:C* (p<0.001), odds ratio 5.608 (3.143-10.01); *IL-2 +166/T:T* (p<0.001), odds ratio 4.430 (2.352-8.344); *IL-2 -330/T:T* (p<0.001), odds ratio 3.353 (2.008-6.217); *IL-10 -1082/G:G* (p=0.007), odds ratio 2.896 (1.294-6.486); *IFNγ utr5644/T:T* (p=0.001), odds ratio 2.365 (1.384-4.042); *IL-10 -1082/A:A* (p=0.001), odds ratio 2.359 (1.383-4.022); *IL-1β +3962/T:T* (p=0.031), odds ratio 2.031 (1.056-3.905); and *IL-6 -174/G:G* (p=0.027), odds ratio 1.791 (1.063-3.017) (Table 2).

Negative (protective) association between patients with bronchial asthma and following genotypes (according to the protectively level) was found for: *IL-4 -1098/G:T* (p<0.001), odds ratio 0.142 (Wald's 95% CI between 0.074-0.271); *TNF-α -238/G:G* (p<0.001), odds ratio 0.158 (0.084-0.295); *IL-2 -330/G:T* (p<0.001), odds ratio 0.174 (0.086-0.352); *IL-4 -590/C:T* (p<0.001), odds ratio 0.190 (0.107-0.338); *IFNγ utr5644/A:T* (p<0.001), odds ratio 0.201 (0.098-0.409); *IL-1β +3962/C:T* (p<0.001), odds ratio 0.241 (0.107-0.546); *IL-10 -1082/A:G* (p<0.001), odds ratio 0.313 (0.185-0.528), and *IL-2 +166/G: T* (p=0.014), odds ratio 0.458 (0.243-0.861). Genotypes *TNF-α -238/A:A, IL-4 -1098/G:G* and *IL-4-590/T:T* were present only in normal Macedonian population, while only patients with bronchial asthma had *TGF-β1 cdn25/C:C* genotype (Table 2).

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Table 1. Cytokine allele frequency, Fisher exact p-value, Odds ratio and Wald's 95% confidence interval in Bronchial Asthma patients and normal Macedonian population.

Cytokine Polymorphism	Allele	Bronchial Asthma (n=74)		Control (n=301)		Fisher exact p-value	Odds ratio	Wald's 95% CI
		N	F	N	F			
IL-1α -889	C	129	0.872	482	0.814	0.100	1.550	0.917-2.617
	T	19	0.128	110	0.186			
IL-1β -511	C	100	0.676	404	0.671	0.915	1.021	0.696-1.499
	T	48	0.324	198	0.329			
IL-1β +3962	C	109	0.736	439	0.729	0.859	1.038	0.690-1.560
	T	39	0.264	163	0.270			
IL-1R psti1970	C	91	0.615	399	0.662	0.272	0.812	0.560-1.178
	T	57	0.385	203	0.337			
IL-1RA mspa11100	T	105	0.709	420	0.698	0.779	1.058	0.713-1.570
	C	43	0.291	182	0.302			
IL-4Rα +1902	A	115	0.777	502	0.834	0.105	0.694	0.446-1.081
	G	33	0.223	100	0.166			
IL-12 -1188	A	120	0.811	433	0.744	0.090	1.475	0.939-2.316
	C	28	0.189	149	0.256			
IFNγ utr5644	T	62	0.431	259	0.520	0.058	0.698	0.480-1.014
	A	82	0.568	239	0.480			
TGF-β1 cdn10	T	67	0.453	282	0.502	0.288	0.821	0.571-1.181
	C	81	0.547	280	0.498			
TGF-β1 cdn25	G	135	0.912	532	0.947	0.118	0.586	0.297-1.153
	C	13	0.088	30	0.053			
TNF-α -308	A	11	0.074	74	0.123	0.095	0.573	0.296-1.109
	G	137	0.926	528	0.877			
TNF-α -238	A	27	0.182	27	0.045	<0.001*	4.752	2.692-8.389
	G	121	0.818	575	0.955			
IL-2 -330	G	30	0.205	191	0.332	0.003*	0.519	0.335-0.803
	T	116	0.795	383	0.667			
IL-2 +166	G	86	0.589	422	0.735	<0.001*	0.516	0.354-0.754
	T	60	0.411	152	0.264			
IL-4 -1098	G	13	0.090	176	0.308	<0.001*	0.223	0.123-0.406
	T	131	0.910	396	0.692			
IL-4 -590	C	125	0.868	377	0.659	<0.001*	3.403	2.038-5.682
	T	19	0.132	195	0.341			
IL-4 -33	C	127	0.882	479	0.837	0.185	1.450	0.835-2.521
	T	17	0.118	93	0.163			
IL-6 -174	C	31	0.209	182	0.302	0.025*	0.611	0.397-0.942
	G	117	0.791	420	0.698			
IL-6 nt565	A	31	0.209	173	0.287	0.056	0.657	0.426-1.014
	G	117	0.791	429	0.713			
IL-10 -1082	A	94	0.635	352	0.589	0.302	1.217	0.839-1.765
	G	54	0.365	246	0.411			
IL-10 -819	C	102	0.689	435	0.727	0.354	0.831	0.562-1.230
	T	46	0.311	163	0.272			
IL-10 -592	A	45	0.304	173	0.289	0.724	1.073	0.725-1.589
	C	103	0.696	425	0.710			

N= absolute number; F=frequency; CI=Confidence Interval; * statistically significant

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Table 2. Cytokine genotype frequency, Fisher exact p-value, Odds ratio and Wald's 95% confidence interval in Bronchial Asthma patients and normal Macedonian population.

Polymorphism	Geno-type	Bronchial Asthma (n=74)		Controls (n=301)		Fisher exact p-value	Odds ratio	Wald's 95% CI
		N	F	N	F			
	C:C	57	0.770	204	0.689	0.171	1.512	0.834-2.741
IL-1α -889	C:T	15	0.203	74	0.250	0.395	0.763	0.408-1.425
	T:T	2	0.027	18	0.061	0.250	0.429	0.097-1.892
	C:C	35	0.423	143	0.475	0.974	0.992	0.596-1.650
IL-1β -511	C:T	30	0.405	118	0.392	0.833	1.057	0.630-1.776
	T:T	9	0.122	40	0.133	0.797	0.904	0.417-1.956
	C:C	51	0.689	174	0.578	0.080	1.618	0.941-2.785
IL-1β +3962	C:T	7	0.095	91	0.302	<0.001*	0.241	0.107-0.546
	T:T	16	0.216	36	0.120	0.031*	2.031	1.056-3.905
	C:C	26	0.351	133	0.442	0.158	0.684	0.403-1.161
IL-1R psti1970	C:T	39	0.527	133	0.442	0.188	1.408	0.845-2.344
	T:T	9	0.122	35	0.116	0.898	1.052	0.482-2.298
	C:C	5	0.088	30	0.100	0.395	0.655	0.245-1.749
IL-1RA mspa11100	C:T	33	0.446	122	0.405	0.525	1.181	0.707-1.972
	T:T	36	0.486	149	0.495	0.895	0.966	0.581-1.608
	A:A	44	0.595	212	0.704	0.069	0.616	0.364-1.042
IL-4Rα +1902	A:G	27	0.365	78	0.259	0.070	1.642	0.958-2.816
	G:G	3	0.040	11	0.037	0.871	1.114	0.303-4.099
	A:A	48	0.649	160	0.550	0.125	1.512	0.890-2.569
IL-12 -1188	A:C	24	0.324	113	0.388	0.310	0.756	0.440-1.299
	C:C	2	0.027	18	0.062	0.240	0.421	0.096-1.858
	A:A	26	0.361	64	0.257	0.051	1.748	0.995-3.072
IFNγ utr5644	A:T	10	0.139	111	0.446	<0.001*	0.201	0.098-0.409
	T:T	36	0.500	74	0.297	0.001*	2.365	1.384-4.042
	C:C	14	0.189	65	0.231	0.438	0.775	0.407-1.477
TGF-β1 cdn10	C:T	39	0.526	150	0.534	0.917	0.973	0.583-1.626
	T:T	21	0.365	66	0.235	0.384	1.291	0.726-2.296
	C:G	7	0.095	30	0.107	0.761	0.874	0.368-2.078
TGF-β1 cdn25	G:G	64	0.865	251	0.893	0.492	0.765	0.355-1.646
	C:C	3	0.040	0	/	&	&	&
	A:G	9	0.122	66	0.219	0.060	0.493	0.233-1.042
TNF-α -308	G:G	64	0.865	231	0.768	0.067	1.939	0.946-3.977
	A:A	1	0.013	4	0.013	0.988	1.018	0.112-9.237
	A:G	27	0.365	23	0.076	<0.001*	6.944	3.675-13.12
TNF-α -238	G:G	47	0.635	276	0.917	<0.001*	0.158	0.084-0.295
	A:A	0	/	2	0.007	&	&	&
	G:G	10	0.137	27	0.094	0.281	1.529	0.704-3.321
IL-2 -330	G:T	10	0.137	137	0.477	<0.001*	0.174	0.086-0.352
	T:T	53	0.726	123	0.429	<0.001*	3.533	2.008-6.217
	G:G	36	0.493	162	0.565	0.274	0.751	0.449-1.256
IL-2 +166	G:T	14	0.192	98	0.341	0.014*	0.458	0.243-0.861
	T:T	23	0.315	27	0.094	<0.001*	4.430	2.352-8.344
	G:T	13	0.181	174	0.608	<0.001*	0.142	0.074-0.271
IL-4 -1098	T:T	59	0.819	111	0.388	<0.001*	7.155	3.751-13.65
	G:G	0	/	1	0.004	&	&	&

Continued.

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Table 2. Continued.

Polymorphism	Geno-type	Bronchial Asthma (n=74)		Controls (n=301)		Fisher exact p-value	Odds ratio	Wald's 95% CI
		N	F	N	F			
IL-4 -590	C:C	53	0.736	95	0.332	<0.001*	5.608	3.143-10.01
	C:T	19	0.264	187	0.654	<0.001*	0.190	0.107-0.338
	T:T	0	/	4	0.014	&	&	&
IL-4 -33	C:C	57	0.792	209	0.731	0.291	1.400	0.749-2.618
	C:T	13	0.180	61	0.213	0.540	0.813	0.419-1.579
	T:T	2	0.028	16	0.056	0.328	0.482	0.108-2.146
IL-6 -174	C:C	3	0.040	25	0.083	0.213	0.467	0.137-1.589
	C:G	25	0.339	132	0.439	0.116	0.653	0.383-1.113
	G:G	46	0.621	144	0.478	0.027	1.791	1.063-3.017
IL-6 nt565	A:A	3	0.040	25	0.083	0.213	0.467	0.137-1.589
	A:G	25	0.339	123	0.409	0.264	0.738	0.433-1.259
	G:G	46	0.622	153	0.508	0.080	1.589	0.944-2.677
IL-10 -1082	A:A	31	0.425	70	0.234	0.001*	2.359	1.383-4.022
	A:G	32	0.425	212	0.709	<0.001*	0.313	0.185-0.528
	G:G	11	0.150	17	0.057	0.007*	2.896	1.294-6.486
IL-10 -819	C:C	35	0.473	155	0.518	0.484	0.834	0.501-1.388
	C:T	32	0.432	125	0.418	0.823	1.061	0.634-1.774
	T:T	7	0.095	19	0.064	0.348	1.540	0.622-3.812
IL-10 -592	A:A	6	0.081	28	0.094	0.737	0.854	0.340-2.145
	A:C	33	0.446	117	0.391	0.391	1.252	0.749-2.093
	C:C	35	0.473	154	0.515	0.517	0.845	0.508-1.407

N= absolute number; F=frequency; CI=Confidence Interval; &, cannot be calculated because expected <5, $\chi 2$ test; * statistically significant

Cytokine Haplotypes

Cytokine haplotypes frequency, Fisher exact p-value, crude odds ratio and Wald's 95% confidence interval in the patients with bronchial asthma and normal Macedonian population are presented in Table 3. With the Heidelberg kit it was possible to analyse haplotypes for *TGF-β1, TNF-α, IL-2, IL-4, IL-6* and *IL-10*.

Positive (susceptible) association between the patients with bronchial asthma and following haplotypes was found (according the level of susceptibility): *IL-4/TCC* (p<0.001), odds ratio 5.926 (3.890-9.029); *TNF-α/GA* (p<0.001), odds ratio 4.943 (2.787-8.769); *IL-2/TT* (p<0.001), odds ratio 2.204 (1.506-3.227); and *IL-6/GG* (p=0.025), odds ratio 1.636 (1.061-2.520). Negative (protective) association was found between the patients with bronchial asthma and haplotypes for: *IL-4/TTC* (p<0.001), odds ratio 0.120 (0.044-0.331); *IL-4/GCC* (p<0.001), odds ratio 0.249 (0.137-0.453); *TNF-α/GG* (p=0.006), odds ratio 0.557 (0.364-0.851); and *IL-2/GG* (p=0.013), odds ratio 0.575 (0.371-0.892). Haplotypes *IL-2/GT, IL-4/GCT, IL-4/GTC, IL-4/GTT, IL-6/CG, IL-6/GA, IL-10/ACA* and *IL-10/ATC* were present only in normal Macedonian population, while only patients with bronchial asthma had *IL-10/GTC* haplotype (Table 3).

Cytokine Diplotypes (Haplotype Zygosity)

Cytokine diplotypes (haplotype zygosity), Fisher exact p-value, crude odds ratio and Wald's 95% confidence interval for each SNP in the patients with bronchial asthma and normal Macedonian population are shown (Table 4). Positive (susceptible) association between the patients with bronchial asthma and following diplotypes was found (according to the level of susceptibility): *TNF-α/GA:GG* (p<0.001), odds ratio 6.252 (Wald's 95% CI between 3.317-11.78); *IL-4/TCC:TCC* (p<0.001), odds ratio 5.343 (3.085-9.255); *IL-2/TT:TT* (p<0.001), odds ratio 4.821 (2.537-9.161); *IL-10/ACC:ATA* (p<0.001), odds ratio 4.255 (2.130-8.500); *IL-10/GCC:GCC* (p=0.002), odds ratio 3.211 (1.459-7.064); and *IL-6/GG:GG* (p=0.027), odds ratio 1.791 (1.063-3.017) (Table 4).

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Table 3. Haplotype frequency of cytokine polymorphism, Fisher exact p-value, Odds ratio and Wald's 95% confidence interval in Bronchial Asthma patients and normal Macedonian population.

Polymorphism	Haplotype	Bronchial Asthma (n=74)		Control (n=301)		Fisher exact p-value	Odds ratio	Wald's 95% CI
		N	F	N	F			
TGF-β1	CC	13	0.088	30	0.053	0.118	1.708	0.867-3.363
	CG	54	0.365	250	0.445	0.080	0.717	0.493-1.042
	TG	81	0.547	282	0.502	0.324	1.200	0.835-1.727
TNF-α	AG	12	0.081	74	0.123	0.152	0.630	0.333-1.192
	GA	27	0.182	26	0.043	<0.001*	4.943	2.787-8.769
	GG	109	0.737	502	0.834	0.006*	0.557	0.364-0.851
IL-2	GG	30	0.205	178	0.310	0.013*	0.575	0.371-0.892
	GT	0	/	14	0.024	&	&	&
	TG	56	0.384	244	0.425	0.364	0.842	0.580-1.221
	TT	60	0.411	138	0.240	<0.001*	2.204	1.506-3.227
IL-4	GCC	13	0.090	163	0.285	<0.001*	0.249	0.137-0.453
	GCT	0	/	8	0.014	&	&	&
	GTC	0	/	4	0.007	&	&	&
	GTT	0	/	1	0.002	&	&	&
	TCC	110	0.764	202	0.353	<0.001*	5.926	3.890-9.029
	TCT	2	0.014	4	0.007	0.417	2.000	0.363-11.03
	TTC	4	0.028	110	0.192	<0.001*	0.120	0.044-0.331
	TTT	15	0.104	80	0.140	0.259	0.715	0.399-1.283
IL-6	CA	31	0.209	172	0.286	0.061	0.662	0.429-1.022
	CG	0	/	9	0.150	&	&	&
	GG	117	0.791	420	0.698	0.025*	1.636	1.061-2.520
	GA	0	/	1	0.002	&	&	&
IL-10	ACA	0	/	12	0.020	&	&	&
	ACC	48	0.324	177	0.296	0.501	1.142	0.776-1.680
	ATA	45	0.304	161	0.269	0.396	1.186	0.800-1.759
	ATC	0	/	2	0.003	&	&	&
	GCC	54	0.365	246	0.411	0.302	0.822	0.567-1.193
	GTC	1	0.007	0	/	&	&	&

N= absolute number; F=frequency; CI=Confidence Interval; &, cannot be calculated because expected <5, $\chi 2$ test; *, statistically significant.

Negative (protective) association between patients with bronchial asthma and following diplotypes (according to the protective level) was found for: *IL-4/GCC:TTC* (p<0.001), odds ratio 0.051 (Wald's 95% CI between 0.012-0.211); *IL-2/GG:TG* (p<0.001), odds ratio 0.252 (0.111-0.572); *IL-2/GG:TT* (p=0.028), odds ratio 0.281 (0.084-0.937); *IL-10/ACC:GCC* (p=0.008), odds ratio 0.448 (0.246-0.816); *TNF-a/GG:GG* (p=0.003), odds ratio 0.461 (0.275-0.773); and *IL-10/ATA:GCC* (p=0.038), odds ratio 0.517 (0.275-0.972). Diplotypes *TGF-β1 CC:CG, TNF-α GA:GA, IL-2 GT:TG, IL-2 GT:GG, IL-2 GT/TT, IL-4 GCC:GCC, IL-4 TTT:TTT, IL-4 GCT:TTT, IL-4 GTC:TTC, IL-4 GTT:TTC, IL-6 CG:GG, IL-6 GA:GG, IL-10 ACA:GCC, IL-10 ACA:ATA* and *IL-10 ATC-GCC* were present only in normal Macedonian population, while diplotypes *TGF-β1 CC:CC, TNF-α GA:AG,* and *IL-10 GTC:ATA* had only patients with bronchial asthma (Table 4).

Summary of all susceptible and protective cytokine polymorphisms for bronchial asthma in Macedonian population are presented (Table 5). If the odds ratio showed a significant value above 1.000 we indicate that positive or susceptible association exists, and if the odds ratio showed a significant value below 1.000 then negative or protective association exists.

From the Table 5 we can see that the highest number of cytokine genotypes (10 of them) are susceptible for bronchial asthma with biggest odds ratio of 7.155 for *IL-4 -1098/T:T*, and more than three times bigger risk (p<0.001) for *TNF-a -238/A:G* (6.944), *IL-4 -590/C:C* (5.608), *IL-2 +166/T:T* (4.430), and *IL-2 -330/T:T* (3.353). Six cytokine diplotypes, four cytokine haplotypes, and two cytokine alleles were found to be positively (susceptible) associated with bronchial asthma (Table 5).

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Table 4. Cytokine diplotypes (haplotype zygotes), Fisher exact p-value, Odds ratio and Wald's 95% confidence interval in Bronchial Asthma patients and normal Macedonian population.

Polymorphism	Genotype	Bronchial Asthma (n=74)		Control (n=301)		Fisher exact p-value	Odds ratio	Wald's 95% CI
		N	F	N	F			
TGF- β1	CC:CG	0	/	16	0.057	&	&	&
	CC:TG	7	0.094	14	0.050	0.146	1.993	0.774-5.132
	CG:CG	11	0.149	49	0.174	0.599	0.827	0.406-1.683
	CG:TG	32	0.432	136	0.484	0.429	0.812	0.485-1.361
	TG:TG	21	0.284	66	0.235	0.384	1.291	0.726-2.296
	CC:CC	3	0.041	0	/	&	&	&
TNF- α	AG:GG	9	0.121	66	0.219	0.060	0.493	0.233-1.042
	GA:GG	26	0.351	24	0.080	<0.001*	6.252	3.317-11.78
	GG:GG	37	0.500	206	0.684	0.003*	0.461	0.275-0.773
	AG:AG	1	0.014	4	0.013	0.988	1.017	0.112-9.237
	GA:AG	1	0.014	0	/	&	&	&
	GA:GA	0	/	1	0.004	&	&	&
IL-2	GG:GG	10	0.137	27	0.094	0.281	1.529	0.704-3.321
	GG:TG	7	0.096	85	0.296	<0.001*	0.252	0.111-0.572
	GG:TT	3	0.041	38	0.133	0.028*	0.281	0.084-0.937
	GT:TG	0	/	11	0.058	&	&	&
	TG:TG	19	0.260	50	0.174	0.095	1.688	0.911-3.055
	TG:TT	11	0.151	48	0.168	0.733	0.883	0.433-1.801
	TT:TT	23	0.315	25	0.087	<0.001*	4.821	2.537-9.161
	GT:GG	0	/	1	0.003	&	&	&
	GT:TT	0	/	2	0.007	&	&	&
IL-4	GCC:GCC	0	/	1	0.003	&	&	&
	GCC:TCC	8	0.111	26	0.091	0.601	1.250	0.541-2.891
	GCC:TTC	2	0.028	103	0.360	<0.001*	0.051	0.012-0.211
	GCC:TTT	3	0.041	32	0.112	0.073	0.345	0.103-1.161
	TCC:TCC	45	0.625	68	0.238	<0.001*	5.343	3.085-9.255
	TCC:TTC	2	0.028	7	0.025	0.873	1.139	0.232-5.602
	TCC:TTT	10	0.139	28	0.098	0.313	1.486	0.686-3.221
	TTT:TTT	0	/	4	0.014	&	&	&
	GCT:TTT	0	/	8	0.028	&	&	&
	GTC:TTC	0	/	4	0.014	&	&	&
	TCT:TTT	2	0.028	4	0.014	0.415	2.014	0.362-11.22
	GTT:TTC	0	/	1	0.003	&	&	&
IL-6	CA:CA	3	0.040	25	0.083	0.213	0.467	0.137-1.589
	CA:GG	25	0.338	122	0.405	0.287	0.749	0.439-1.277
	CG:GG	0	/	9	0.030	&	&	&
	GG:GG	46	0.622	144	0.479	0.027*	1.791	1.063-3.017
	GA:GG	0	/	1	0.003	&	&	&
IL-10	ACC:ACC	7	0.095	21	0.070	0.476	1.383	0.565-3.388
	ACC:ATA	18	0.243	21	0.070	<0.001*	4.255	2.130-8.500
	ACC:GCC	16	0.216	114	0.381	0.008*	0.448	0.246-0.816
	ATA:ATA	6	0.081	19	0.064	0.589	1.300	0.500-3.380
	ATA:GCC	14	0.189	93	0.311	0.038*	0.517	0.275-0.972
	GCC:GCC	12	0.162	17	0.057	0.002*	3.211	1.459-7.064
	ACA :GCC	0	/	3	0.010	&	&	&
	ACA :ATA	0	/	9	0.030	&	&	&
	ATC :GCC	0	/	2	0.007	&	&	&
	GTC:ATA	1	0.014	0	0	&	&	&

N= absolute number; F=frequency; CI=Confidence Interval; &, cannot be calculated because expected <5, $\chi 2$ test; *, statistically significant.

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Table 5. Summary of all susceptible and protective cytokine polymorphisms for Bronchial Asthma in Macedonian population.

Cytokine	Susceptible			Protective		
	Polymorphism	p	Odds ratio	Polymorphism	p	Odds ratio
Cytokine Alleles	TNF-α -238/A	p<0.001	4.752	IL-4 -1098/G	p<0.001	0.223
	IL-4 -590/C	p<0.001	3.403	IL-2 +166/G	p<0.001	0.516
				IL-2 -330/G	p=0.003	0.519
				IL-6 -174/C	p=0.025	0.611
Cytokine Genotypes	IL-4 -1098/T:T	p<0.001	7.155	IL-4 -1098/G:T	p<0.001	0.142
	TNF-α -238/A:G	p<0.001	6.944	TNF-α -238/G:G	p<0.001	0.158
	IL-4 -590/C:C	p<0.001	5.608	IL-2 -330/G:T	p<0.001	0.174
	IL-2 +166/T:T	p<0.001	4.430	IL-4 -590/C:T	p<0.001	0.190
	IL-2 -330/T:T	p<0.001	3.353	IFNγutr5644/A:T	p<0.001	0.201
	IL-10 -1082/G:G	p=0.007	2.896	IL-1β+3962/C:T	p<0.001	0.241
	IFNγutr5644/T:T	p=0.001	2.365	IL-10 -1082/A:G	p<0.001	0.313
	IL-10 -1082/A:A	p=0.001	2.359			
	IL-1β+3962/T:T	p=0.031	2.031			
	IL-6 -174/G:G	p=0.027	1.791			
Cytokine Haplotypes	IL-4/TCC	p<0.001	5.926	IL-4/TTC	p<0.001	0.120
	TNF-α/GA	p<0.001	4.943	IL-4/GCC	p<0.001	0.249
	IL-2/TT	p<0.001	2.204	TNF-α/GG	p=0.006	0.557
	IL-6/GG	p=0.025	1.636	IL-2/GG	p=0.013	0.575
Cytokine Diplotypes (Haplotype Zygosity)	TNF-α/GA:GG	p<0.001	6.252	IL-4/GCC:TTC	p<0.001	0.051
	IL-4/TCC:TCC	p<0.001	5.343	IL-2/GG:TG	p<0.001	0.252
	IL-2/TT:TT	p<0.001	4.821	IL-2/GG:TT	p=0.028	0.281
	IL-10/ACC:ATA	p<0.001	4.255	IL-10/ACC:GCC	p=0.008	0.448
	IL-10/GCC:GCC	p=0.002	3.211	TNF-α/GG:GG	p=0.003	0.461
	IL-6/GG:GG	p=0.027	1.791	IL-10/ATA:GCC	p=0.038	0.517

At the same time protective cytokine polymorphisms regarding bronchial asthma for seven cytokine genotypes, six cytokine diplotypes, four cytokine haplotypes, and four cytokine alleles were found. Most of the negative (protective) associations with bronchial asthma were at very high protective levels (p<0.001) (Table 5).

DISCUSSION

Our results of 22 cytokine polymorphisms in patients with BA and in normal Macedonian population are presented in this paper. We did not find any significant association between bronchial asthma and *IL-1a -889* frequencies of alleles and genotypes.

It was found that patients with bronchial asthma showed an increase level of *IL-1β* in BAL fluid compared with normal controls and patients with asymptomatic bronchial asthma.[40-42]

In this study we could not demonstrate any associations between *IL-1β* alleles and bronchial asthma. When we analyzed the genotypes, we found that *IL-1β +3962/T:T* genotype was positively (susceptible) associated with BA, while *IL-1β +3962/C:T* genotype showed negative (protective) association.

IL-1β mediates its functions via its receptor *IL-1R*. IL-1*β* physiological antagonist is IL-1 receptor antagonist *IL-1RA*.[43,44] Unlike to the previously reported results[25] our investigation seems to refute any association between BA and *IL-1R psti1970* and *IL-1RA mspa1110* polymorphisms (alleles or genotypes).

Interleukin-12 (IL-12) acts synergistically with IL-2 to induce differentiation of cytotoxic T lymphocytes and stimulates the proliferation of activated T and NK cells. It also enhances T and NK cell mediated cytolytic activity and secretion of IFNγ.[45] Our data showed no associations between BA and *IL-12 -1188*

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polymorphisms at allele and genotype level contrary to other publications.[46, 47]

Asthmatic patients have reduced production of IFNγ by T cells, which correlates with the severity of BA.[48, 49] Number of eosinophils in BAL fluid of bronchial asthmatic patients is reduced by IFNγ.[50] We did not find any association between BA and *IFNγ* alleles. However, analysis of genotypes showed significant negative (protective) association of *IFNγ utr5644/A:T* genotype, and positive (susceptible) association with *IFNγ utr5644/T:T* genotype. Our results do not conform with others, who did not find association between *IFNγ* gene polymorphisms and BA.[51]

TGF-β1 regulate growth and differentiation of cells, production of extra cellular matrix and repair of tissues,[52, 53] and promotes formation of elastin, which promotes lung damage repair in risk patients.[54, 55] Several studies have analyzed the association between polymorphisms of transforming growth factor-*β*1 (*TGF-β1*) gene and BA.[20, 27, 56, 57] Contrary to the results of several authors,[27, 56, 57] we did not find any significant association in *TGF-β1* codon 10 and codon 25 frequencies of alleles, genotypes, haplotypes, or diplotypes. Similar findings for *TGF-β1* have been published by other authors.[20]

TNF- *a* takes part in the air-way remodelling and alters smooth muscle function in patients with BA.[58] TNF- *a* has an important amplifying effect on asthmatic inflammation by activating the secretion of cytokines from a variety of cells in the airway.[59, 60] Many authors investigated the role of TNF-*a* in the pathogenesis of BA. Some of them found that *TNF-a* polymorphisms is a risk factor for BA[21, 23, 24, 61-65] and the others did not find such results.[22,66] Our results demonstrated susceptible effect of *TNFa -238/A* allele, as well as *TNF- a -238/A:G* genotype. Positive association was also found for *TNF- a /GA* haplotype and *TNF-a /GA:GG* diplotype. However, *TNF-a -238/G:G* genotype, *TNF- a /GG* haplotype and *TNF-a /GG:GG* diplotype showed protective association with BA indicating the protective effect of the G allele in the *TNF-a -238* polymorphism.

Levels of IL-2 are increased in bronchoalveolar lavage fluid of patients with BA.[43] To our knowledge, this is the first study analyzing the association between IL-2 polymorphisms and BA. In our study, we demonstrated that there was protective association between the BA and *IL-2 -330/G* allele, *IL-2 +166/G allele, IL-2 -330/G:T* genotype, *IL-2 +166/G:T* genotype, *IL-2/GG* haplotype, and *IL-2/GG:TG* and *IL-2/GG:TT* diplotypes. We also found positive (susceptible) association between BA and: *IL-2 -330/T:T* genotype, *IL-2 +166/T:T* genotype, *IL-2/TT* haplotype, and *IL-2/TT:TT* diplotype. We could say that patients homozygous for T allele are more susceptible for BA than those homozygous and/or heterozygous for G allele.

Interleukin-4 (IL-4) is a multifunctional T-helper type 2 cytokine that induces production of mucus and hyperplasia of goblet cells in bronchial sub mucosa.[67] Its gene together with the gene of the *IL-13* is located on the chromosome 5q31, only 20 kilo base apart, in the region associated with airway hyper-responsiveness.[68] These two genes have been directly involved in the pathogenesis of bronchial asthma.[69, 70] In this study, we investigated alleles and genotypes of three polymorphisms of *IL-4* (at the positions -1098, -590, and *-33*), as well as haplotypes and diplotypes of investigated polymorphisms. The results showed protective association of BA with *IL-4 -1098/G* allele. We found protective association between BA and two heterozygous genotype polymorphisms of *IL-4 (IL-4 -1098/G:T* and *IL-4 -590/C:T*). *IL-4 -590/C* allele and homozygous genotypes of the same *IL-4* polymorphisms were susceptible for BA (*IL-4-1098/T:T* and *IL-4 -590/CC*). We also found protective association between bronchial asthma and *IL-4/TTC*, and *IL-4/GCC* haplotypes, as well as susceptible association with *IL-4/TCC* haplotype. From the diplotypes analysis, we can see that combination of *IL-4* haplotypes *IL-4/TCC:TCC* has the susceptible association with BA, while *IL-4* dilpotype *GCC:TTC* was negatively associated with BA. Several authors showed no associations between *IL-4* allele and genotype frequency and BA.[71, 72] Our results of *IL-4* haplotypes conform with others, who also found associations between *IL-4* polymorphisms and bronchial asthma.[19, 73-75]

Our results showed no significant differences of *IL-4Ra +1902* frequency at allele and genotype level. These results are in agreement with others.[72, 76, 77]

Although it has been found that alveolar macrophages and epithelial cells in asthmatic patients produce high amounts of IL-6, its role in the pathophysiology of bronchial asthma is still unclear.[43,78,79] We investigated the association for two polymorphisms of *IL-6, -174 C/G* and *nt565 A/G*. Our data showed that *IL-6 -174/C* allele was protective for

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BA, but association analysis showed that patients homozygous for G allele (*IL-6 -174/G:G* genotype, *IL-6/GG* haplotype and *IL-6/GG:GG* diplotypes) were more susceptible for BA.

Interleukin-10 (IL-10) is anti-inflammatory cytokine that reduces production of pro-inflammatory cytokines during inflammatory responses.[80,81] Alveolar macrophages in patients with bronchial asthma produce significantly less IL-10.[82] We did not find any association between the *IL-10* alleles (at the positions -1082, -819, and -592) and BA. Analysis of genotypes showed significant negative (protective) association between BA and *IL-10 -1082/A:G* haplotype, and positive (susceptible) association with *IL-10 -1082/G:G* and *IL-10 -1082/A:A* genotypes. *IL-10* genotypes at the locations -819, and -592 were not significantly association with BA. Neither *IL-10* haplotypes showed associated with BA. Two haplotype combinations of *IL-10* (diplotype or haplotype zygozity) were negatively associated with BA (*IL-10/ ACC:GCC* and *IL-10/ATA:GCC*), and two haplotype combinations were positively associated with BA (*IL-10/GCC:GCC*, and *IL-10/ATA:ACC*). Some data suggested that there is no association between *IL-10* polymorphisms and BA,[71] while other data claimed the opposite.[26]

Inconsistent results obtained from various authors highlight the genetic role among different ethnic groups. Because the complex-trait diseases, like BA, are influenced not only by genetic factor but by gene-environment interaction as well, it is possible that different ethnic groups will show association with different cytokine polymorphisms. Meta analysis of all cytokine polymorphisms and association with BA is needed in order to shed light on the pathogenesis of disease.

In conclusion, we confirmed that several cytokine polymorphisms are positively and several cytokine polymorphism are negatively associated with bronchial asthma in population of ethnic Macedonians, and for the first time we found association between *IL-2* polymorphisms and bronchial asthma.

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