Association between 22 cytokine gene polymorphisms and dilated cardiomyopathy in Macedonian patients

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Abstract

Background: Inflammation is an important component in the pathogenesis of many cardiovascular diseases and one of the commonest mechanisms in cardiomyopathy. There have been several studies on the cytokine polymorphism and dilated cardiomyopathy (DCM), but the results obtained were contradictory.

Aim: To examine a possible role of 22 cytokine gene polymorphisms in host susceptibility to or protection against DCM in Macedonians.

Methods: In this study 301 healthy unrelated individuals and 52 patients with DCM were studied. Cytokine genotyping was performed by PCR with sequence-specific priming (PCR-SSP) (Heidelberg kit).

Results: After the Bonferroni adjustment, the *IL-4 -1098/T, IL-4 -1098/T:T, IL-4/TCC*, and *IL-4/TCC:TTC* cytokine genes were positively associated with DCM, while a negative association was identified for *IL-4 -1098/G*, *IL-4 -1098/G:T, IL-1B +3962/C:C, IL-4/GCC*, and *IL-4/GCC:TTC*.

Conclusions: These results suggest that some cytokine gene polymorphisms are significantly associated and affect host susceptibility/resistance to DCM in Macedonians.

Key words: dilated cardiomyopathy, cytokine polymorphism, Republic of Macedonia

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Introduction

Dilated cardiomyopathy (DCM), a disorder characterised by cardiac dilation and reduced systolic function, represents an outcome of a heterogeneous group of inherited and acquired disorders. Myocarditis, coronary artery disease, systemic diseases and myocardial toxins have been identified as causative factors. Idiopathic DCM, in which these causes are excluded, represents approximately a half of all DCM cases [1]. This condition was diagnosed in 1426 children younger than 18 years from the United States and Canada [2]. Unlike other parts of the world, in which cardiomyopathy is rare, DCM is a major cause of heart failure in Africa [3].

Among cases of idiopathic DCM, familial form accounts for 20-25% of cases, with an exception of rare cases resulting from mutations in dystrophin (e.g. OMIM 300377.0021) [4]. Familial DCM is characterised by an autosomal dominant pattern of inheritance with age-related penetrance. Ventricular dilatation and systolic dysfunction usually develop in the second or third decade of life. Mutations in many other genes have been found to cause various forms of DCM (http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=115200) [4].

Inflammation is an important component in the pathogenesis of many cardiovascular diseases and one of the commonest mechanisms in cardiomyopathy. In most cases, the role of inflammation is a natural response to injury, and an important mechanism for healing and tissue repair. However, the inflammatory response can be either inadequate or exagerated, leading to direct injury or a severe host disease. Accumulating data have revealed an important inflammatory component in the pathogenesis of DCM, and there is growing evidence that myocarditis and DCM are closely related. The term 'cardiomyopathy' is no longer reserved for the idiopathic forms but can be used interchangeably

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with the term 'heart muscle diseases' including specific secondary forms [5].

Several studies on the cytokine polymorphism and DCM have been conducted. The association between *TNF-α* and/or *IL-10* gene polymorphism and DCM was studied [6–9]. However, the results were contradictory. Studies performed in patients with different ethnic origins yielded contrasting results [8, 9].

We have previously published data for the cytokine polymorphisms in a healthy Macedonian population [10--12]. The aim of this study was to investigate the existence of possible associations between 22 cytokine gene polymorphisms and DCM in Macedonians.

Methods

Patients

The total studied sample consisted of 353 subjects, divided into two groups: healthy individuals and patients with DCM.

Healthy individuals

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

Dilated cardiomyopathy

Fifty two patients with a diagnosis of DCM of different aetiologies were included in this study. They were 20-59--year-old consecutive patients who attended the Clinic of Cardiology, Faculty of Medicine, Ss. Cyril and Methodius University, Skopje, Republic of Macedonia for treatment between 18 September 2000 and 21 July 2002. The diagnosis of ischemic (n = 25), valvular (n = 17), and idiopathic cardiomyopathy (n = 10) was confirmed according to the report of the 1995 World Health Organization [13]. All patients underwent transthoracic echocardiography (Hewlett Packard, Sonos 5500), had left ventricular (LV) dilatation (end diastolic diameter ≥ 5.5 cm), and showed an impaired LV function (LV ejection fraction < 40%, or fractional shortening < 25%).

All individuals were of Macedonian origin and nationality, 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 last three generations, and his/her signed consent was obtained. Admixture, if any, was recorded for each individual. Subjects with only one Macedonian parent were excluded from the study.

All of the patients and healthy individuals included in this study signed written consent to participate in the study which was approved by the Committee of the Macedonian Ministry of Education and Science (No.13--874/3-05) and the Ethical Committee of the Medical Faculty in Skopje.

Genomic DNA isolation and storage

The DNA was isolated from peripheral blood leukocytes using the phenol-chlorophorm extraction method or BioRobot EZ1 workstation (QIAGEN) [14]. The quality and quantity of DNA were analysed by GeneQuant (Pharmacia Biotech, Uppsala, Sweden). Isolated DNA samples were stored in the Macedonian Human DNA Bank [15].

Typing methods

Cytokine genotyping was performed by PCR-SSP (Heidelberg kit). Fourteen cytokine genes with 22 single nucleotide polymorphisms (SNP) were typed: *IL-1α -889, IL-1β -511, IL-1β +3962, IL-1R psti1970, IL-1RA mspa11100, IL-4Rα +1902, IL-12 -1188, IFNγ utr5644, TGF-β1 cdn10, TGF-β1 cdn25, TNF-α -308, TNF-α -238, IL-2 -330, IL-2 +166, IL-4 -1098, IL-4 -590, IL-4 -33, IL-6 -174, IL-6 565, IL-10 -1082, IL-10 -819,* and *IL-10 -592*. Briefly, PCR-SSP typing Heidelberg kit consists of 48 PCR primer mixes aliquoted in 96-well PCR trays (two typings 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 48 wells [16]. Agarose gel electrophoresis on a 2% gel revealed a positive or negative signal for specific amplification in each well. Subsequently, the results were analysed according to the interpretation scheme provided with the kit.

Statistical analysis

The population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop [17--19], was used for analysis of the cytokine data in this study. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each SNP were determined [20]. The exact test for genotype frequency deviation from HWP was calculated using the Arlequin implementation accessed via PyPop [21]. Those SNPs that did not fit HWP were evaluated to determine whether there was an excess of homozygotes or heterozygotes, or if any particular genotype frequencies were significantly different from the expected frequencies. Comparisons of different genotypes for two groups were tested by the χ^2 test with Bonferroni corrected p-value [22]. Crude odds ratios (OR), as estimates of the relative risk, were calculated within 95% CI.

Results

Cytokine alleles

Table I shows frequencies of polymorphic cytokine alleles, Fisher exact p-value, odds ratio (OR) and Wald's 95% CI in patients with DCM and healthy Macedonians.

Table I. Cytokine allele frequency, Fisher exact p-value, odds ratio and Wald's 95% confidence interval in patients with dilated cardiomyopathy and healthy Macedonian population

Cytokine polymorphism	Allele	DCM (n = 52)		Control (n = 301)		Fisher exact p-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
IL-1α -889	*C*	85	0.817	482	0.814	0.940	1.021	0.596-1.750
	T	19	0.183	110	0.186	0.940	0.980	0.572-1.679
IL-1β -511	*C*	66	0.635	404	0.671	0.466	0.851	0.552-1.314
	T	38	0.365	198	0.329	0.466	1.175	0.761-1.813
IL-1β +3962	*C*	68	0.654	439	0.729	0.115	0.701	0.451-1.091
	T	36	0.346	163	0.270	0.115	1.426	0.916-2.219
IL-1R psti1970	*C*	74	0.712	399	0.662	0.329	1.255	0.795-1.981
	T	30	0.288	203	0.337	0.329	0.797	0.505-1.258
IL-1RA mspa11100	*T*	67	0.644	420	0.698	0.277	0.785	0.507-1.215
	C	37	0.356	182	0.302	0.277	1.274	0.823-1.974
IL-4Rα +1902	*A*	89	0.856	502	0.834	0.577	1.182	0.657-2.127
	G	15	0.144	100	0.166	0.577	0.846	0.470-1.523
IL-12 -1188	*A*	73	0.702	433	0.744	0.369	0.810	0.512-1.283
	C	31	0.298	149	0.256	0.369	1.234	0.779-1.954
IFNγ utr5644	*T*	52	0.500	259	0.520	0.709	0.923	0.605-1.408
	A	52	0.500	239	0.480	0.709	1.084	0.710-1.654
TGF-β1 cdn10	*T*	59	0.567	282	0.502	0.219	1.302	0.854-1.985
	C	45	0.433	280	0.498	0.219	0.768	0.504-1.171
TGF-β1 cdn25	*G*	101	0.971	532	0.947	0.290	1.899	0.569-6.340
	C	3	0.029	30	0.053	0.290	0.527	0.158-1.759
TNF-α -308	*A*	8	0.078	74	0.123	0.195	0.607	0.284-1.301
	G	94	0.922	528	0.877	0.195	1.647	0.769-3.527
TNF-α -238	*A*	3	0.029	27	0.045	0.475	0.645	0.192-2.168
	G	99	0.971	575	0.955	0.475	1.550	0.461-5.206
IL-2 -330	*G*	33	0.317	191	0.332	0.758	0.932	0.596-1.459
	T	71	0.683	383	0.667	0.758	1.073	0.686-1.679
IL-2 +166	*G*	66	0.635	422	0.735	0.036	0.626	0.403-0.971
	T	38	0.365	152	0.264	0.036	1.599	1.029-2.482
IL-4 -1098	*G*	15	0.144	176	0.308	< 0.001*	0.379	0.213-0.674
	T	89	0.856	396	0.692	< 0.001*	2.637	1.484-4.687
IL-4 -590	*C*	74	0.712	377	0.659	0.296	1.276	0.807-2.017
	T	30	0.288	195	0.341	0.296	0.784	0.496-1.239
IL-4 -33	*C*	85	0.817	479	0.837	0.612	0.869	0.504-1.498
	T	19	0.183	93	0.163	0.612	1.151	0.668-1.985
IL-6 -174	*C*	36	0.346	182	0.302	0.372	1.222	0.787-1.897
	G	68	0.654	420	0.698	0.371	0.819	0.527-1.271
IL-6 nt565	*A*	36	0.346	173	0.287	0.225	1.313	0.845-2.040
	G	68	0.654	429	0.713	0.225	0.762	0.490-1.184
IL-10 -1082	*A*	60	0.577	352	0.589	0.823	0.953	0.625-1.453
	G	44	0.423	246	0.411	0.823	1.049	0.688-1.600
IL-10 -819	*C*	79	0.760	435	0.727	0.494	1.184	0.729-1.922
	T	25	0.240	163	0.272	0.494	0.845	0.520-1.371
IL-10 -592	*A*	25	0.240	173	0.289	0.306	0.777	0.480-1.261
	C	79	0.760	425	0.710	0.306	1.286	0.793-2.086

Abbreviations: N – absolute number, F – frequency, CI – confidence Interval, DCM – dilated cardiomyopathy

** statistically significant after Bonferroni adjustment (p-value × number of alleles) < 0.05*

We found a negative association for *IL-4 -1998/G* allele (OR = 0.379), and *IL-2 +166/G* allele (OR = 0.626). An inverse positive association was found for the alternative alleles for the same cytokine genes: *IL-4 -1998/T* allele (OR = 2.673), and *IL-2 +166/T* allele (OR = 1.599). After the Bonferroni adjustment, only *IL-4 -1998* alleles were significantly associated with DCM (Table I).

Cytokine genotypes

Table II contains results for different cytokine genotypes found in our study.

Analysis of all cytokine genotypes showed that six genotypes were positively associated with DCM. The highest OR was found for *IL-4 -1098/T:T* (OR = 4.279), and the lowest OR for *IL-1RA mspa11100/C:T* (OR = 1.850). Three cytokine genotypes only were negatively associated with DCM: *IL-4 -1098/G:T* (OR = 0.215), *IL-1α +3962/C:C* (OR = 0.376), and *IFNγ utr5644/A:T* (OR = 0.458). After the Bonferroni adjustment, only *IL-4 -1098/T:T, IL-4 -1098/G:T, IL-1α +3962/C:C*, and *IL-1β +3962/T:T* genotypes were significantly associated with DCM (Table II).

Cytokine haplotypes

Using the Heidelberg PCR-SSP kit we were able to detect true haplotypes for several genes with multiple SNPs per gene (*TGF-β1, TNF-α, IL-2, IL-4, IL-6, IL-10*). Cytokine haplotype frequency in patients with DCM and healthy Macedonians, together with the Fisher exact p-value, OR and Wald's 95% CI are shown in Table III.

Significant associations with DCM were observed in two IL-4 haplotypes and one IL-2 haplotype. Positive associations were shown (according the level of susceptibility) for *IL-4/TCC* ($p < 0.001$), OR 2.221 (1.456-3.390); and *IL-2/TT* ($p = 0.013$), OR 1.745 (1.118-2.722). A negative association was found only for *IL-4/GCC* haplotype ($p = 0.003$), OR 0.423 (0.238-0.753). Haplotypes *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 a healthy Macedonian population, while only patients with DCM had *TGF-β1/GG* and *TNF-α/AA* haplotypes. After the Bonferroni adjustment, only two haplotypes (*IL-4/TCC* and *IL-4/GCC*) were significantly associated with DCM (Table III).

Cytokine diplotypes (haplotype zygosity)

Cytokine diplotypes (or haplotype zygosity) are combinations of haplotypes from both parents. Table IV contains results of the cytokine diplotype analysis.

Four haplotype combinations had a positive association [*IL-4/TCC:TTC* (OR = 10.693), *IL4/TCC:TTT* (OR = 2.764), *IL-2/TT:TT* (OR = 2.495), *IL-4/GCC:TCC* (OR = 2.380), *TNF-α/GG:GG* (OR = 2.152), and *IL-6/CA:GG* (OR = 2.001)] with DCM. We found a negative association of two haplotype combinations [*IL-4/GCC:TTC* (OR = 0.071), and *IL-4/GCC:TTT* (OR = 0.156)] with DCM. We found that *TGF-β1/CC:CG, TNF-α/AG:AG, TNF-α/GA:GA, IL-2/GT:GG, IL-2/GT:TT, IL-4/GCT:TTT, IL-4/GTC:TTC, IL-4/GTT:TTC, IL-6/CG:GG, IL-6/GA:GG, IL-10/GCC:GCC, IL-10/ACA:GCC, IL-10/ACA:ATA,* and *IL-10/ATC:GCC* haplotype combinations were present only in a healthy Macedonian population. On the other hand, *TGF-β1/CC:GG, TNF-α/GG:AA,* and *TNF-α/AG:GA* diplotypes were found only in patients with DCM. Only two diplotypes (*IL-4/TCC:TTC* and *IL-4/GCC:TTC*) were significantly associated with DCM, after Bonferroni correction of p-value (Table IV).

Table V presents a summary of all positive (susceptible) and negative (protective) cytokine polymorphisms. The majority of cytokine genotypes (six of them) and cytokine diplotypes (six of them) were positively associated with DCM, while only three cytokine genotypes, and two cytokine diplotypes showed a negative association. Two cytokine alleles and two cytokine haplotypes also showed a positive association with DCM. A negative association with DCM was documented for two cytokine alleles, and only one cytokine haplotype. However, only one allele, one genotype, one haplotype, and one diplotype were positively associated with DCM after Bonferroni correction of p-value. Similarly, only one allele, two genotypes, one haplotype and one diplotype were negatively associated with DCM after Bonferroni correction of p-value.

Discussion

In this study we examined 52 Macedonian patients with DCM, and found several positive and negative associations between cytokine alleles, genotypes, haplotypes, diplotypes and DCM.

The most frequent association was found between *IL-4* gene and DCM. We investigated *IL-4* polymorphism at three positions: *-1098, -590*, and *-33*, and we were able to investigate haplotypes and haplotype combinations (diplotypes) of this cytokine gene. We found that *IL-4 -1098/T* allele, *IL-4 -1098/T:T* genotype, *IL-4/TCC* haplotype as well as *IL-4/TCC:TTC*, *IL-4/TCC:TTT*, and *IL-4/GCC:TCC* haplotype combinations (or diplotypes) were positively associated with DCM. The highest OR was found for *IL-4/TCC:TTC* (10.693), five belongs to *IL-4* polymorphisms (*IL-4 -1098/G* allele, *IL-4 -1098/G:T* genotype, *IL-4/GCC* haplotype, as well as *IL-4/GCC:TTC* and *IL-4/GCC:TTT* haplotype combinations). The cumulative effects of *IL-4 -1098/T* alleles: OR = 2.634 in *IL-4 -1098/T* allele, doubled in *IL-4 -1098/T:T* genotype (OR = 4.729), and four times bigger in *IL-4/TCC:TTC* haplotype combination (OR = 10.693) were documented (Table V). These results are the first ones in the literature. Several SNPs (*IL-1α -889, IL-1β +3962, IL-2 +166, IL-4 -1098, IL-4 -590, IL-4 -33, and IL-10 -592*) in the control group were not in HWP ($p < 0.005$) (12) and we should be very careful about their associations with cardiomyopathy.

After Bonferroni correction of p-values, we found a lower number of alleles, genotypes, haplotypes, and

Table II. Cytokine genotype frequency, Fisher exact p-value, odds ratio and Wald's 95% confidence interval in dilated cardiomyopathy patients and healthy Macedonian population

Polymorphism	Genotype	DCM (n = 52)		Controls (n = 301)		Fisher exact p-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
IL-1α -889	*C:C*	36	0.692	204	0.689	0.003*	0.376	0.218-0.647
	C:T	13	0.250	74	0.250	0	1	0.506-1.975
	T:T	3	0.058	18	0.061	0.931	0.946	0.268-3.332
IL-1β -511	*C:C*	21	0.404	143	0.475	0.342	0.749	0.411-1.362
	C:T	24	0.461	118	0.392	0.345	1.329	0.735-2.404
	T:T	7	0.135	40	0.133	0.973	1.015	0.428-2.406
IL-1β +3962	*C:C*	29	0.558	174	0.578	0.784	0.920	0.509-1.665
	C:T	10	0.192	91	0.302	0.105	0.550	0.264-1.143
	T:T	13	0.250	36	0.120	0.012*	2.454	1.197-5.030
IL-1R psti1970	*C:C*	26	0.500	133	0.442	0.437	1.263	0.701-2.277
	C:T	22	0.423	133	0.442	0.810	0.926	0.511-1.680
	T:T	4	0.077	35	0.116	0.403	0.633	0.215-1.863
IL-1RA mspa11100	*C:C*	4	0.077	30	0.100	0.608	0.753	0.254-2.233
	C:T	29	0.558	122	0.405	0.040	1.850	1.022-3.350
	T:T	19	0.365	149	0.495	0.084	0.587	0.320-1.079
IL-4Rα +1902	*A:A*	38	0.731	212	0.704	0.698	1.140	0.588-2.207
	A:G	13	0.250	78	0.259	0.889	0.953	0.484-1.878
	G:G	1	0.019	11	0.037	0.525	0.517	0.065-4.091
IL-12 -1188	*A:A*	26	0.500	160	0.550	0.506	0.819	0.454-1.478
	A:C	21	0.404	113	0.388	0.833	1.067	0.584-1.948
	C:C	5	0.096	18	0.062	0.362	1.614	0.571-4.556
IFNγ utr5644	*A:A*	19	0.365	64	0.257	0.112	1.664	0.885-3.131
	A:T	14	0.270	111	0.446	0.019	0.458	0.236-0.888
	T:T	19	0.365	74	0.297	0.333	1.362	0.728-2.548
TGF-β1 cdn10	*C:C*	6	0.115	65	0.231	0.061	0.433	0.177-1.061
	C:T	33	0.635	150	0.534	0.180	1.517	0.823-2.795
	T:T	13	0.250	66	0.235	0.814	1.086	0.547-2.155
TGF-β1 cdn25	*C:G*	3	0.058	30	0.107	0.276	0.512	0.150-1.745
	G:G	49	0.942	251	0.893	0.277	1.952	0.573-6.650
	C:C	0	0	0	0	0	&	&
TNF-α -308	*A:G*	8	0.157	66	0.219	0.312	0.662	0.297-1.478
	G:G	43	0.843	231	0.768	0.229	1.629	0.731-3.627
	A:A	0	0	4	0.013	&	&	&
TNF-α -238	*A:G*	3	0.059	23	0.076	0.657	0.755	0.218-2.615
	G:G	48	0.941	276	0.917	0.554	1.449	0.421-4.989
	A:A	0	0	2	0.007	&	&	&
IL-2 -330	*G:G*	5	0.096	27	0.094	0.962	1.024	0.376-2.795
	G:T	23	0.442	137	0.477	0.641	0.868	0.479-1.573
	T:T	24	0.462	123	0.429	0.659	1.143	0.632-2.068
IL-2 +166	*G:G*	24	0.462	162	0.565	0.170	0.661	0.366-1.197
	G:T	18	0.346	98	0.341	0.948	1.021	0.549-1.900
	T:T	10	0.192	27	0.094	0.037	2.293	1.035-5.079
IL-4 -1098	*G:T*	13	0.250	174	0.608	<0.001*	0.215	0.110-0.420
	T:T	38	0.731	111	0.388	<0.001*	4.279	2.218-8.257
	G:G	1	0.019	1	0.004	0.174	5.588	0.344-90.785

*Abbreviations: & – cannot be calculated because expected < 5, χ^2 test, * statistically significant after Bonferroni adjustment (p-value × number of genotypes) < 0.05. Rest of abbreviations as in Table I*

Table II. continued

Polymorphism	Genotype	DCM (n = 52)		Controls (n = 301)		Fisher exact p-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
IL-4 -590	*C:C*	23	0.442	95	0.332	0.125	1.595	0.875-2.906
	C:T	28	0.539	187	0.654	0.112	0.618	0.340-1.122
	T:T	1	0.019	4	0.014	0.773	1.382	0.151-12.621
IL-4 -33	*C:C*	36	0.692	209	0.731	0.568	0.829	0.435-1.579
	C:T	13	0.250	61	0.213	0.556	1.230	0.618-2.448
	T:T	3	0.058	16	0.056	0.960	1.033	0.290-3.680
IL-6 -174	*C:C*	3	0.058	25	0.083	0.532	0.676	0.197-2.325
	C:G	30	0.577	132	0.439	0.064	1.746	0.963-3.167
	G:G	19	0.365	144	0.478	0.131	0.628	0.342-1.153
IL-6 nt565	*A:A*	3	0.058	25	0.083	0.532	0.676	0.197-2.325
	A:G	30	0.577	123	0.409	0.024	1.973	1.087-3.582
	G:G	19	0.365	153	0.508	0.057	0.557	0.303-1.023
IL-10 -1082	*A:A*	8	0.154	70	0.234	0.199	0.595	0.267-1.323
	A:G	44	0.846	212	0.709	0.040	2.257	1.021-4.991
	G:G	0	0	17	0.057	&	&	&
IL-10 -819	*C:C*	29	0.558	155	0.518	0.600	1.171	0.648-2.118
	C:T	21	0.404	125	0.418	0.848	0.943	0.518-1.718
	T:T	2	0.038	19	0.064	0.481	0.590	0.133-2.610
IL-10 -592	*A:A*	2	0.038	28	0.094	0.189	0.387	0.089-1.677
	A:C	21	0.404	117	0.391	0.864	1.054	0.578-1.921
	C:C	29	0.558	154	0.515	0.570	1.187	0.657-2.147

*Abbreviations: & – cannot be calculated because expected < 5, χ^2 test, * statistically significant after Bonferroni adjustment (p-value × number of genotypes) < 0.05. Rest of abbreviations as in Table I*

diplotypes associated with DCM in Macedonians. Several types of multiple testing corrections are used: i) Bonferroni; ii) Bonferroni Step-down (Holm); iii) Westfall and Young Permutation; and iv) Benjamini and Hochberg False Discovery Rate [22, 23]. The methods are listed in the order of their stringency, with the Bonferroni being the most stringent, and the Benjamini and Hochberg FDR being the least stringent. The more stringent the multiple testing correction, the less false positive genes are allowed. The trade-off of a stringent multiple testing corrections is that the rate of false negatives (alleles, genotypes, haplotypes, and diplotypes that are called non-significant when they are) is very high. Inclusion of Bonferroni correction of p-value in our paper means more false negatives with subsequently less significant associations with DCM.

In a paper of Bijlsma et al., 2002 [24] an incidence of rejection was significantly lower in patients who received a donor heart with the *IL-4 -590/T*-positive genotype compared with patients who received a heart from a */T*--negative donor. Patients who had the */T*-negative genotype and received a heart from a */T*-positive donor, suffered significantly less from rejection than */T*-negative patients who received a T-negative donor heart. This indicates that IL-4 production within the donor heart and by cells from the donor is important for reducing the incidence of episodes of rejection [24]. Liu et al., 2006 [25] showed that sCTLA-4 levels of patients with idiopathic DCM were associated with the haplotype and genotype. Patients with *-1772/TC* genotype or *-1772/TC -1661/AA, -1772/TC -1661/AG* haplotypes had higher sCTLA-4 levels than patients with other haplotypes. The frequency of *-1772/TC* genotype was significantly higher in patients with low LV ejection fraction values, whereas the frequency of *-1661/G* allele and *-1661/GG* genotype was lower in idiopathic DCM patients. Levels of IL-4 were increased in this group. It was concluded that patients with idiopathic DCM have an aberrant expression of the CTLA-4 products, and the *-1772 C/T* and *-1661 A/G* polymorphisms. The two SNPs may function as genetic markers for disease susceptibility [25]. We can add that other *IL-4* polymorphisms (especially *IL-4 -1098*) could be also responsible for the aberrant expression of CTLA-4 products in patients with DCM.

We investigated *IL-2* gene polymorphism at two positions: *-330* and *+166*, and were able to investigate haplotypes and haplotype combinations (diplotypes). We found a positive association of four *IL-2* polymorphisms and one negative association of *IL-2* with DCM. However, Bonferroni-corrected p-values were not significant. Huang et al., 2008 [26] investigated whether *-384T/G, -475A/T*, and *-631G/A* polymorphisms in the *IL-2* gene promoter region

were associated with idiopathic DCM in a South-western Chinese Han population. They found that the *T* allele of the *IL-2* gene promoter at position *-384* might increase the risk of developing idiopathic DCM [21]. Our results are the first ones that show the positive association of *IL-2 +166/T* allele, and the opposite – a negative association of *IL-2 +166/G* allele with DCM, if not corrected with Bonferroni. Homozygous combinations of *IL-2* polymorphisms (*IL-2 +166/T:T* genotype, *IL-2/TT* haplotype, and *IL-2/TT:TT* haplotype combinations) are positively associated with DCM.

We have also published for the first time that *IL-1β +3962/T:T* homozygous genotype is positively associated with DCM, while *IL-1β +3962/C:C* homozygous genotype is negatively associated with DCM. The *IL-1RA mspa11100/C:T* heterozygous genotype is positively associated with DCM, while *IFNγ utr5644/A:T* heterozygous genotype is negatively associated with DCM. These results suggest that *IL-1* cluster genes are included in the development of DCM.

Our results showing a positive association of *TNF-α/GG:GG* homozygous combination of haplotypes (diplotype) with DCM are similar to the results of Ito et al., 2000 [8] in Japanese patients. Several other studies were unable to find any association between the *TNF-α* and/or *IL-10* polymorphisms and DCM [5-7, 22].

The presence and absence of certain haplotypes and haplotype combinations (diplotypes) in a healthy Macedonian population and in patients with DCM, could be a result of small frequencies of those cytokine polymorphisms.

The number of patients in our study, as well as in other published papers [6-8, 20], is very small. In the association studies, some positive results might be spurious and some negative findings might be a consequence of low statistical power. It could be due to a small sample size or methodological shortcomings, such as the selection of an inappropriate control group. It is necessary to investigate cytokine gene polymorphisms in our population in well-

Table III. Haplotype frequency of cytokine polymorphism, Fisher exact p-value, odds ratio and Wald's 95% confidence interval in dilated cardiomyopathy patients and healthy Macedonian population

Polymorphism	Haplotype	DCM (n = 52)		Control (n = 301)		Fisher exact p-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
TGF-β1	*CC*	3	0.029	30	0.053	0.290	0.527	0.158-1.759
	CG	42	0.404	250	0.445	0.439	0.845	0.552-1.294
	TG	58	0.558	282	0.502	0.295	1.252	0.822-1.907
	GG	1	0.009	0	0	&	&	&
TNF-α	*AG*	7	0.068	74	0.123	0.112	0.526	0.235-1.176
	GA	2	0.020	26	0.043	0.260	0.443	0.104-1.896
	GG	92	0.902	502	0.834	0.080	1.833	0.922-3.643
	AA	1	0.010	0	0	&	&	&
IL-2	*GG*	32	0.308	178	0.310	0.723	0.921	0.586-1.450
	GT	1	0.009	14	0.024	0.346	0.388	0.051-2.986
	TG	34	0.327	244	0.425	0.061	0.657	0.422-1.022
	TT	37	0.356	138	0.240	0.013	1.745	1.118-2.722
IL-4	*GCC*	15	0.144	163	0.285	0.003*	0.423	0.238-0.753
	GCT	0	0	8	0.014	&	&	&
	GTC	0	0	4	0.007	&	&	&
	GTT	0	0	1	0.002	&	&	&
	TCC	57	0.548	202	0.353	<0.001*	2.221	1.456-3.390
	TCT	2	0.019	4	0.007	0.221	2.784	0.503-15.40
	TTC	13	0.125	110	0.192	0.102	0.600	0.324-1.112
	TTT	17	0.164	80	0.140	0.528	1.202	0.679-2.127
IL-6	*CA*	36	0.346	172	0.286	0.212	1.324	0.852-2.057
	CG	0	0	9	0.150	&	&	&
	GG	68	0.654	420	0.698	0.372	0.819	0.527-1.271
	GA	0	0	1	0.002	&	&	&
IL-10	*ACA*	0	0	12	0.020	&	&	&
	ACC	35	0.337	177	0.296	0.406	1.207	0.775-1.879
	ATA	25	0.240	161	0.269	0.538	0.859	0.529-1.395
	ATC	0	0	2	0.003	&	&	&
	GCC	44	0.423	246	0.411	0.823	1.049	0.688-1.600

Abbreviations: as in Tables I and II

Table IV. Cytokine diplotypes (haplotype zygotes), Fisher exact p-value, odds ratio and Wald's 95% confidence interval in dilated cardiomyopathy patients and healthy Macedonian population

Polymorphism	Genotype	DCM (n = 52)		Control (n = 301)		Fisher exact p-value	Odds ratio	Wald' 95% CI
		N	F	N	F			
TGF-β1	*CC:CG*	0	0	16	0.057	&	&	&
	CC:TG	3	0.058	14	0.050	0.813	1.168	0.324-4.215
	CG:CG	6	0.115	49	0.174	0.293	0.618	0.250-1.526
	CG:TG	29	0.558	136	0.484	0.329	1.344	0.741-2.438
	TG:TG	13	0.250	66	0.235	0.814	1.086	0.547-2.155
	CC:GG	1	0.019	0	0	&	&	&
TNF-α	*AG:GG*	6	0.117	66	0.219	0.096	0.475	0.194-1.161
	GA:GG	1	0.020	24	0.080	0.122	0.231	0.031-1.745
	GG:GG	42	0.823	206	0.684	0.044	2.152	1.007-4.601
	AG:AG	0	0	4	0.013	&	&	&
	GG:AA	1	0.020	0	0	&	&	&
	GA:GA	0	0	1	0.004	&	&	&
	AG:GA	1	0.020	0	0	&	&	&
IL-2	*GG:GG*	5	0.096	27	0.094	0.962	1.024	0.376-2.795
	GG:TG	10	0.192	85	0.296	0.125	0.566	0.271-1.180
	GG:TT	12	0.231	38	0.133	0.066	1.966	0.948-4.079
	GT:TG	1	0.019	11	0.058	0.493	0.492	0.062-3.894
	TG:TG	9	0.174	50	0.174	0.984	0.992	0.455-2.165
	TG:TT	5	0.096	48	0.168	0.194	0.530	0.200-1.401
	TT:TT	10	0.192	25	0.087	0.022	2.495	1.118-5.567
	GT:GG	0	0	1	0.003	&	&	&
	GT:TT	0	0	2	0.007	&	&	&
IL-4	*GCC:GCC*	1	0.019	1	0.003	0.174	5.588	0.344-90.785
	GCC:TCC	10	0.192	26	0.091	0.029	2.380	1.071-5.293
	GCC:TTC	2	0.039	103	0.360	< 0.001*	0.071	0.017-0.298
	GCC:TTT	1	0.019	32	0.112	0.038	0.156	0.021-1.165
	TCC:TCC	12	0.231	68	0.238	0.913	0.962	0.478-1.937
	TCC:TTC	11	0.211	7	0.025	< 0.001*	10.693	3.923-29.148
	TCC:TTT	12	0.231	28	0.098	0.006	2.764	1.301-5.875
	TTT:TTT	1	0.019	4	0.014	0.773	1.382	0.151-12.621
	GCT:TTT	0	0	8	0.028	&	&	&
	GTC:TTC	0	0	4	0.014	&	&	&
	TCT:TTT	2	0.039	4	0.014	0.219	2.82	0.503-15.809
	GTT:TTC	0	0	1	0.003	&	&	&
IL-6	*CA:CA*	3	0.058	25	0.083	0.532	0.676	0.197-2.325
	CA:GG	30	0.577	122	0.405	0.021	2.001	1.102-3.632
	CG:GG	0	0	9	0.030	&	&	&
	GG:GG	19	0.365	144	0.479	0.131	0.628	0.342-1.153
	GA:GG	0	0	1	0.003	&	&	&
IL-10	*ACC:ACC*	4	0.077	21	0.070	0.863	1.103	0.363-3.355
	ACC:ATA	2	0.038	21	0.070	0.393	0.530	0.120-2.329
	ACC:GCC	25	0.481	114	0.381	0.176	1.503	0.831-2.716
	ATA:ATA	2	0.038	19	0.064	0.481	0.590	0.133-2.610
	ATA:GCC	19	0.366	93	0.311	0.438	1.275	0.689-2.360
	GCC:GCC	0	0	17	0.057	&	&	&
	ACA :GCC	0	0	3	0.010	&	&	&
	ACA :ATA	0	0	9	0.030	&	&	&
	ATC :GCC	0	0	2	0.007	&	&	&

Abbreviations: as in Tables I and II

Table V. Summary of all susceptible and protective cytokine polymorphisms for dilated cardiomyopathy in Macedonian population

Cytokine	Susceptible (positive) association			Protective (negative) association		
	Polymorphism	p	Odds ratio	Polymorphism	p	Odds ratio
Alleles	*IL-4 -1098/T*	< 0.001*	2.637	*IL-4 -1098/G*	< 0.001*	0.379
	IL-2 +166/T	0.036	1.599	*IL-2 +166/G*	0.036	0.626
Genotypes	*IL-4 -1098/ T:T*	< 0.001*	4.279	*IL-4 -1098/G:T*	< 0.001*	0.215
	IL-1? +3962/T:T	0.012*	2.454	*IL-1β +3962/C:C*	0.003*	0.376
	IL-2 +166/ T:T	0.037	2.293	*IFNγ utr5644/A:T*	0.019	0.458
	IL-10 -1082/A:G	0.040	2.257			
	IL-6 nt565/A:G	0.024	1.973			
	IL-1RA mspa11100/C:T	0.040	1.850			
Haplotypes	*IL-4/TCC*	< 0.001*	2.221	*IL-4/GCC*	0.003*	0.423
	IL-2/TT	0.013	1.745			
Diplotypes (haplotype combinations)	*IL-4/TCC:TTC*	< 0.001*	10.693	*IL-4/GCC:TTC*	< 0.001*	0.071
	IL-4/TCC:TTT	0.006	2.764	*IL-4/GCC:TTT*	0.038	0.156
	IL-2/TT:TT	0.022	2.495			
	IL-4/GCC:TCC	0.029	2.380			
	TNF-α/GG:GG	0.044	2.152			
	IL-6/CA:GG	0.021	2.001			

** statistically significant after Bonferroni adjustment (p-value × number of alleles, genotypes, haplotypes or diplotypes) < 0.05*

-defined subgroups of phenotypes with more participants in order to have more precise conclusions for genetic background of DCM development in Macedonians. Multicentric studies and/or meta-analysis of the patients with DCM and an association with cytokine polymorphisms should be very useful.

It can be concluded that, at least in Macedonian patients with DCM, some cytokine polymorphisms contribute to susceptibility/protection to the disease. Ethnic factors might play a role in the variability of results in different populations. Therefore, additional studies are needed to clarify this issue.

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Związek pomiędzy występowaniem polimorfizmu genu 22 cytokiny a kardiomiopatią rozstrzeniową w populacji chorych z Macedonii

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Streszczenie

Wstęp: Zapalenie jest jednym z istotnych czynników patogenetycznych wielu chorób układu sercowo-naczyniowego i częstym mechanizmem powstawania kardiomiopatii. W wielu badaniach analizowano związki pomiędzy polimorfizmem genu cytokiny a kardiomiopatią rozstrzeniową (DCM), ale wyniki były rozbieżne.

Cel: Zbadanie związku pomiędzy występowaniem polimorfizmu genu 22 cytokiny a DCM w populacji macedońskiej.

Metody: Grupa badana składała się z 301 zdrowych ochotników i 52 chorych z DCM. Badania genetyczne wykonano techniką łańcuchowej reakcji polimerazy (ang. *polymerase chain reaction*, PCR).

Wyniki: Po zastosowaniu poprawki Bonferroniego okazało się, że występowanie *IL-4 -1098/T, IL-4 -1098/T:T, IL-4/TCC*, i *IL-4/TCC:TTC* było pozytywnie związane z obecnością DCM, podczas gdy występowanie *IL-4 -1098/G, IL-4 -1098/G:T, IL-1β +3962/C:C, IL-4/GCC*, i *IL-4/GCC:TTC* było negatywnie związane z obecnością tej choroby.

Wnioski: Powyższe wyniki wskazują, że niektóre polimorfizmy genu cytokiny 22 są związane z występowaniem DCM w populacji macedońskiej.

Słowa kluczowe: kardiomiopatia rozstrzeniowa, polimorfizm genu cytokiny, Macedonia

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