Association of Gene Variants in *TLR4* and *IL-6* Genes with Perthes Disease

Sanja Srzentić[1], Vesna Spasovski[1], Duško Spasovski[2,3], Zorica Živković[4], Dragana Matanović[2,5], Zoran Baščarević[2,3], Zorica Terzić Šupić[2], Maja Stojiljković[1], Teodora Karan-Djurašević[1], Biljana Stanković[1], Sonja Pavlović[1], Gordana Nikčević[1], Zoran Vukašinović[2,3]

[1]Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia;
[2]Faculty of Medicine, University of Belgrade, Belgrade, Serbia;
[3]Institute for Orthopedic Surgery "Banjica", Belgrade, Serbia;
[4]Clinical Hospital Center "Dr Dragiša Mišović", Belgrade, Serbia;
[5]Clinic for Physical Medicine and Rehabilitation, Clinical Center of Serbia, Belgrade, Serbia

SUMMARY

Introduction Perthes disease is idiopathic avascular osteonecrosis of the hip in children, with unknown etiology. Inflammation is present during development of Perthes disease and it is known that this process influences bone remodeling.

Objective Since genetic studies related to inflammation have not been performed in Perthes disease so far, the aim of this study was to analyze the association of frequencies of genetic variants of immune response genes, toll-like receptor 4 (*TLR4*) and interleukin-6 (*IL-6*), with this disease.

Methods The study cohort consisted of 37 patients with Perthes disease and 50 healthy controls. Polymorphisms of well described inflammatory mediators: *TLR4* (Asp299Gly, Thr399Ile) and *IL-6* (G-174C, G-597A) were determined by polymerase chain reaction restriction fragment length polymorphism method.

Results *IL-6* G-174C and G-597A polymorphisms were in complete linkage disequilibrium. A statistically significant increase of heterozygote subjects for *IL-6* G-174C/G-597A was found in controls in comparison to Perthes patient group (p=0.047, OR=2.49, 95% CI=1.00-6.21). Also, the patient group for *IL-6* G-174C/G-597A polymorphisms was not in Hardy-Weinberg equilibrium. No statistically significant differences were found between patient and control groups for *TLR4* analyzed polymorphisms. A stratified analysis by the age at disease onset also did not reveal any significant difference for all analyzed polymorphisms.

Conclusion Our study revealed that heterozygote subjects for the *IL-6* G-174C/G-597A polymorphisms were significantly overrepresented in the control group than in the Perthes patient group. Consequently, we concluded that children who are heterozygous for these polymorphisms have a lower chance of developing Perthes disease than carriers of both homozygote genotypes.

Keywords: gene variants; inflammation; Perthes disease

INTRODUCTION

Perthes disease is idiopathic avascular necrosis of the femoral head in childhood. It affects children between 2 and 14 years old, the male-to-female ratio is approximately 4:1, and bilateral disease occurs in 10 to 20% of patients [1, 2]. This disease belongs to a group of rare diseases with the prevalence ranging from 5.1 to 16.9 per 100,000 in various regions of the world [3, 4, 5]. Treatment depends on the age of the child and the severity of the condition, and may include surgery [6, 7].

Perthes disease remains one of the most controversial conditions in pediatric orthopedics, since both the etiology and pathophysiology of the disease remain unclear. The prevailing view is that Perthes disease is multifactorial, caused by a combination of environmental and genetic factors. A number of possible causes have been proposed, including repetitive microtrauma, skeletal retardation, inflammation and vascular insufficiency [1].

There is not much research about the role of inflammatory factors in the pathophysiology of Perthes disease, despite the fact that inflammatory changes were noticed in magnetic resonance studies long ago [8]. The majority of those changes are marked during the initial phase, as well as during fragmentation phase when bone resorption and bone formation are uncoupled [8, 9, 10]. The link between bone remodeling and cytokine level came from studies of other systemic and joint bone diseases, like rheumatoid arthritis [11], osteoporosis [12] and osteonecrosis of the femoral head [13], illustrating that high levels of inflammatory mediators in affected joint can inhibit bone formation.

OBJECTIVE

We assumed that the development of Perthes disease could be associated with the presence of polymorphisms in immune response genes, which also has the key role in bone metabolism. Therefore, the objective of this study was to analyze the frequency of single nucleotide polymorphisms (SNPs) of well described inflammatory mediators, toll-like receptor 4 (*TLR4*) and interleukin-6 (*IL-6*) in Perthes patients.

Correspondence to:
Gordana NIKČEVIĆ
Institute of Molecular Genetics and Genetic Engineering
University of Belgrade
Vojvode Stepe 444a
11010 Belgrade
Serbia
gordnik@imgge.bg.ac.rs

METHODS

Patients and control subjects

Our study was conducted according to the guidelines of the Helsinki Declaration and was also approved by the Ethics Committee of the Institute for Orthopedic Surgery "Banjica", Belgrade, Serbia. Written informed consent was obtained for all patients.

This study enrolled 37 patients and 50 healthy controls, from the same geographic area. The patient group consisted of 29 males and 8 females with a median age at disease onset being 5 (range: 2-11) years. Blood collection was performed at the time of surgery for 17 patients, and for the rest of them during control examination. The healthy control group was sex matched and all controls were older than 14 years, therefore could not develop Perthes disease in future. The control group included 35 male and 15 female voluntary donors, with a median age 31.5 (range: 16-53) years.

All patients were diagnosed with Perthes disease from 1998-2012, at the Institute of Orthopedic Surgery "Banjica", Belgrade. The diagnosis was established according to the standard clinical criteria (onset of groin pain, disturbed stance on affected leg and gait, limitation of hip joint movements, especially internal rotation, absence of clinical signs suggesting trauma or infection), ultrasonographic examination (verification of homogenous hip joint effusion) and radiographic signs (condensation or fragmentation of epiphyseal ossification center, loss of femoral head sphericity). The left hip was affected in 19, right in 16 and both in 2 patients. They were all treated in hospital by containment method; five were treated conservatively by skin traction, and other were operated on using various methods (Salter osteotomy in 28 patients including two operated on bilaterally, Chiari osteotomy in three patients and triple pelvic osteotomy in one patient), depending on their age as well as the duration and severity of disease.

DNA isolation

Genomic DNA was isolated from peripheral blood with QIAampDNA Mini Kit (Qiagen GmbH, Hilden, Germany), and stored at -20°C.

Gene variants detection

All analyzed polymorphisms and primers used for their detection, are shown in Table 1. Polymorphisms are denoted by both, common nomenclature (used through the entire article), and HGVS nomenclature.

Toll-like receptor 4 Asp299Gly and Thr399Ile detection

Polymorphisms Asp299Gly (rs4986790) and Thr399Ile (rs4986791) in *TLR4* gene were detected by a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method as previously described [14]. In both cases, the forward primer sequences were altered to generate a restriction site in the minor allele. The bases that are underlined indicate altered nucleotides that create *Nco* I (Asp299Gly) or *Hinf* I (Thr399Ile) restriction sites, respectively. The amplification program consisted of a denaturation step at 94°C for 5 min followed by 35 cycles (94°C for 30 sec, 55°C for 30 sec and 72°C for 45 sec) and an elongation step of 10 min at 72°C. Digestion with *Nco* I (MBI Fermentas, Vilnius, Lithuania) gave fragments of the following length (in base pairs): Asp/Asp-275, Asp/Gly- 275+249+26, Gly/Gly- 249+26, while digestion with *Hinf* I (MBI Fermentas, Vilnius, Lithuania) gave for Thr/Thr - 407, Thr/Ile- 407+379+29 and Ile/Ile- 379+29.

Interleukin-6 G-174C and G-597A detection

The genotyping of the polymorphisms in *IL-6* promoter G-174C (rs1800795) and G-597A (rs1800797) was performed using PCR-RFLP technique as previously described [15]. A 527-bp fragment containing both polymorphic sites (-597 and -174) was amplified using one primer set. The PCR conditions were: denaturation step at 94°C for 5 min, 35 cycles of amplification (94°C for 30 sec, 57°C for 30 sec and 72°C for 45 sec) and elongation step of 10 min at 72°C. The obtained PCR product was digested with *Hin1* II and *Fok* I (MBI Fermentas, Vilnius, Lithuania). Digestion with *Hin1* II (G-174C) gave the following pattern (in base pairs): 331+167+29 for GG genotype, 331+167+29+122+45 for GC genotype and 331+122+45 for CC genotype. Digestion with *Fok* I (G-597A) gave fragments of the following length (in base pairs): 527 for

Table 1. Polymorphisms and primers for genotyping

Gene	Polymorphism		Primers: in 5′-3′ direction
	Common nomenclature	HGVS nomenclature	
TLR4	Asp299Gly	NP_612564.1:p.Asp299Gly/ NM_138554.4:c.896A>G	F: GATTAGCATACTTAGACTACTACCTCCATG R: GATCAACCTTCTGAAAAGCATTCCCAC
	Thr399Ile	NP_612564.1:p.Thr399Ile/ NM_138557.2:c.596C>T	F: GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA R: ACCTGAAGACTGGAGAGTGAGTTAAATGCT
IL-6	G-174C	NC_000007.13:g.22766645C>G/ NM_000600.3:c.-116-121C>G	F: GGAGTCACACACTCCACCT R: CTGATTGGAAACCTTATTAAG
	G-597A	NC_000007.13:g.22766221A>G/ NM_000600.3:c.-116-545A>G	

F – forward; R – reverse

Table 2. Genotype and allele distributions among Perthes patients and controls (statistical method: Pearson chi-squared test)

Gene variant	Genotype	Perthes patients n=37 (frequency)	Controls n=50 (frequency)	OR (95% CI)*	p*
TLR4 Asp299Gly	Asp/Asp	30 (0.81)	45 (0.90)	2.10 (0.61–7.24)	0.23
	Asp/Gly	6 (0.16)	5 (0.10)		
	Gly/Gly	1 (0.03)	0 (0.00)		
Allele frequency	Asp	0.89	0.95	2.30 (0.72–7.35)	0.15
	Gly	0.11	0.05		
TLR4 Thr399Ile	Thr/Thr	29 (0.78)	45 (0.90)	2.48 (0.74–8.33)	0.13
	Thr/Ile	7 (0.19)	5 (0.10)		
	Ile/Ile	1 (0.03)	0 (0.00)		
Allele frequency	Thr	0.88	0.95	2.63 (0.84–8.20)	0.09
	Ile	0.12	0.05		
IL-6 G-174C	GG	19 (0.51)	18 (0.36)	0.69 (0.23–2.05)	0.50
	GC	10 (0.27)	24 (0.48)		
	CC	8 (0.22)	8 (0.16)		
Allele frequency	G	0.65	0.60	0.81 (0.43–1.51)	0.51
	C	0.35	0.40		
IL-6 G-597A	GG	19 (0.51)	18 (0.36)	0.69 (0.23–2.05)	0.50
	GA	10 (0.27)	24 (0.48)		
	AA	8 (0.22)	8 (0.16)		
Allele frequency	G	0.65	0.60	0.81 (0.43–1.51)	0.51
	A	0.35	0.40		

n – number of patients; OR – odds ratio; CI – confidence interval

* Shown OR and p-values refer to the following comparisons: *TLR4* Asp299Gly- Asp/Asp vs. Asp/Gly + Gly/Gly, *TLR4* Thr399Ile- Thr/Thr vs. Thr/Ile + Ile/Ile, *IL-6* G-174C- GG+GC vs. CC, *IL-6* G-597A- GG+GA vs. AA

the GG genotype, 527+461+66 for the GA genotype and 461+66 for the AA genotype.

Statistical analysis

The differences in genotype and allele distributions between patients and controls, and groups stratified within the patient group, were analyzed with the Pearson chi-squared test or Fisher's exact test, when appropriate. The consistency of genotype distributions with Hardy-Weinberg equilibrium was tested using Pearson chi-squared test, for each cohort. All of the tests were 2-sided and differences were considered to be significant in all cases when $p<0.05$. Data was analyzed using the SPSS for Windows 20.0 software (SPSS, Inc, Chicago, IL, USA). Values of linkage disequilibrium (D', LOD, r^2) between polymorphisms were tested using the Haploview software, Version 4.2 [16].

RESULTS

Incidence of polymorphisms among patient and control groups

Genotype and allele distributions of all analyzed genes in the patient and the control groups are presented in Table 2.

TLR4

The comparison of genotype and allele frequencies among patients and controls did not reveal any significant differences for *TLR4* analyzed polymorphisms. Also, genotype distributions for patient and control groups were in Hardy-Weinberg equilibrium (data not shown). *TLR4* polymorphisms Asp299Gly and Thr399Ile were in complete linkage disequilibrium in both patient and control groups (D'=1.0, LOD=15.2, r^2=0.92).

IL-6

Complete linkage disequilibrium was observed between the G-174C and G-597A polymorphisms (D'=1.0, LOD=39.92, r^2=1.0). All the subjects homozygous -174GG were homozygous -597GG, and all the subjects homozygous -174CC were homozygous -597AA; consequently, heterozygote subjects at position -174 were invariably heterozygous at -597. Therefore, *IL-6* G-174C and G-597A genotypes and alleles occurred with identical frequencies.

Frequencies of alleles -174G/-597G and -174C/-597A were similar in patient and control groups, thus no significant difference was detected (p=0.51). Also, comparison of genotypes according to the presence of allele G (-174GG/-597GG and -174GC/-597GA vs. -174CC/-597AA) among patients and controls did not show any significant difference (p=0.50). However, the frequency of *IL-6* heterozygotes (-174GC/-597GA) was higher, while the frequencies of both homozygotes (-174GG/-597GG and -174CC/-597AA) were lower in controls compared with patients and this difference was statistically significant (p=0.047) (Table 3). Thus, according to these results, carriers of heterozygote genotype *IL-6* -174GC/-597GA have 2.49 fold decreased risk of developing Perthes dis-

Table 3. Heterozygote/homozygote ratio for *IL-6* G-174C/G-597A

Gene variant of *IL-6*	Perthes patients n=37 (frequency)	Controls n=50 (frequency)	OR (95% CI)	p
GC/GA	10 (0.27)	24 (0.48)	2.49 (1.00-6.21)	0.047
GG/GG + CC/AA	27 (0.73)	26 (0.52)		

Statistical method: Pearson chi-squared test

Table 4. Genotype and allele distributions among Perthes patients with early and late onset

Gene variant	Genotype	Age at onset ≤5 n=20 (frequency)	Age at onset >5 n=17 (frequency)	OR (95% CI)*	p*	Statistical method
TLR4 Asp299Gly	Asp/Asp	16 (0.80)	14 (0.82)	1.17 (0.22–6.14)	1.00	Fisher's exact test
	Asp/Gly	3 (0.15)	3 (0.18)			
	Gly/Gly	1 (0.05)	0 (0.00)			
Allele frequency	Asp	0.87	0.91	1.48 (0.33–6.69)	0.72	Fisher's exact test
	Gly	0.13	0.09			
TLR4 Thr399Ile	Thr/Thr	15 (0.75)	14 (0.82)	1.56 (0.31–7.75)	0.70	Fisher's exact test
	Thr/Ile	4 (0.20)	3 (0.18)			
	Ile/Ile	1 (0.05)	0 (0.00)			
Allele frequency	Thr	0.85	0.91	1.82 (0.41–7.92)	0.50	Fisher's exact test
	Ile	0.15	0.09			
IL-6 G-174C	GG	10 (0.50)	9 (0.53)	2.36 (0.47–11.82)	0.43	Fisher's exact test
	GC	7 (0.35)	3 (0.18)			
	CC	3 (0.15)	5 (0.29)			
Allele frequency	G	0.67	0.62	0.77 (0.30–2.05)	0.6	Pearson chi-squared test
	C	0.33	0.38			
IL-6 G-597A	GG	10 (0.50)	9 (0.53)	2.36 (0.47–11.82)	0.43	Fisher's exact test
	GA	7 (0.35)	3 (0.18)			
	AA	3 (0.15)	5 (0.29)			
Allele frequency	G	0.67	0.62	0.77 (0.30–2.05)	0.6	Pearson chi-squared test
	A	0.33	0.38			

* Shown OR and p-values refer to the following comparisons: *TLR4* Asp299Gly- Asp/Asp vs. Asp/Gly + Gly/Gly, *TLR4* Thr399Ile- Thr/Thr vs. Thr/Ile + Ile/Ile, *IL-6* G-174C- GG+GC vs. CC, *IL-6* G-597A- GG+GA vs. AA

ease (OR=2.49, 95% CI=1.00-6.21) (Table 3). Moreover, the patient group was not in Hardy-Weinberg equilibrium for *IL-6* G-174C/G-597A polymorphisms. There were more both homozygotes (-174GG/-597GG and -174CC/-597AA) and less heterozygotes (-174GC/-597GA) observed than expected in this group (p=0.01). In contrast to these results, the control group was in complete Hardy-Weinberg equilibrium, i.e. the observed values were identical to those expected. The fact that only the patient group was in Hardy-Weinberg disequilibrium, due to heterozygote under representation, supports our finding that heterozygotes have a lower chance for the development of Perthes disease. These results suggest protective role of well-balanced plasma levels of IL-6 protein in the development of Perthes disease.

Incidence of polymorphisms and age of disease onset

Perthes patients were stratified into two groups: one, with early disease onset (younger than five years) and the other, with late disease onset (older than five years). Comparison of genotype and allele frequencies among these two groups did not show significant difference for any of the analyzed genes (Table 4).

DISCUSSION

The initiating insult triggering the onset of Perthes disease remains unknown. It is generally accepted that the disruption of blood supply to the femoral head is a key pathogenic event, followed by pathological and subsequent structural changes to the growing femoral head [17]. Well accepted stages of the pathogenesis of the disease are: synovitis, condensation, fragmentation (resorption), reossification and healed femoral head [18]. During the fragmentation phase, there is a lot of necrotic bone resorption and not enough new bone formation. Imbalance between bone resorption and bone formation plays an important role in the development of severe deformity of the affected hip [1]. The inflammatory changes in Perthes disease are most marked during the fragmentation phase when bone remodeling is unbalanced [9, 10].

Based on the knowledge gained from rheumatoid arthritis and osteoporosis research, that inflammatory cytokines inhibit bone formation and stimulate resorption [19, 20], it is possible that the same mechanism could be responsible for disturbed bone formation seen in Perthes disease. For that reason, the aim of this study was to investigate the association of Perthes disease with the occurrence of different genetic variants of immune response genes *TLR4* and *IL-6*.

TLR4 is one of the most important pathogen recognition receptors that is activated by lipopolysaccharide (LPS) of Gram-negative bacteria [21]. Using the rat model system, it has been shown that femoral head osteonecrosis can be induced by treatment with LPS and methylprednisolone, through TLR4 signaling pathway, which includes the regulation of cytokine IL-6 [22].

The *TLR4* gene has two important non-synonymous polymorphisms that lead to impaired TLR4 response and the altered expression of cytokines regulated by this receptor [23]. These are an A/G transition causing an aspartic acid/glycine substitution at amino acid position 299 (Asp299Gly), and a C/T transition causing a threonine/isoleucine change at 399 (Thr399Ile). These two polymorphisms cosegregated in our patient and control groups, which is consistent with the results of other studies [24, 25].

We proposed that *TLR4* Asp299Gly and Thr399Ile polymorphisms, and their effects on immune gene expression might change bone turn-over against bone formation and influence the development of Perthes disease, but we did not find any significant association of these two polymorphisms with this disease.

IL-6 is a cytokine with diverse functions, including the major role in the regulation of bone metabolism [19]. Dysregulated IL-6 production is implicated in the pathology of several diseases. Increased IL-6 levels are characteristic of systemic onset juvenile chronic arthritis [26], rheumatoid arthritis [27] and osteoporosis [28].

IL-6 is not constitutively expressed, but is highly inducible and is produced in response to a number of inflammatory stimuli such as IL-1, platelet-derived growth factor, TNF-a, bacterial products and viral infection [29]. Circulating levels of IL-6 are mainly regulated at the level of gene expression [30] that is tightly controlled by co-ordination among various transcription factors [31]. Polymorphisms in the promoter region of *IL-6* gene, in particular SNPs at positions G-174C and G-597A that are involved in the transcriptional regulation of the *IL-6* gene, have been reported to be associated with plasma levels of IL-6 and with the risk of several bone and joint diseases [26, 32, 33].

Analyzed polymorphisms in *IL-6* gene (G-174C and G-597A) were in linkage disequilibrium in both the patient and control groups, which is consistent with the results of other studies [15, 32, 33].

We found that *IL-6* heterozygotes were significantly overrepresented in the controls compared to the patients. Also, Perthes patients were not in Hardy-Weinberg equilibrium, in contrast to the control subjects, which were in complete equilibrium. The genotype distribution of our control population resembled that of white control populations investigated by others [26, 33]. Thus, our results suggest that carriers of heterozygote genotypes *IL-6* -174GC/-597GA have a smaller chance of developing Perthes disease than carriers of both types of homozygote genotypes (-174GG/-597GG and -174CC/-597AA).

The heterozygote advantage of the *IL-6* G-174C/G-597A polymorphisms has not been described before. Controversial data came from the studies that correlated *IL-6* -174 homozygote GG genotype and/or presence of G allele and homozygote CC genotype with IL-6 plasma levels [26, 34-36], as well as with the bone quality [33, 37]. Dual roles of IL-6 could be a possible explanation for heterozygote advantage, since both the proinflammatory and antiinflammatory effects of IL-6 have been well described [38]. Because IL-6 is a pleiotropic cytokine involved in immune response and bone remodeling, heterozygote advantage might be the result of the better balancing of these processes.

CONCLUSION

To the best of our knowledge, this is the first report regarding the genetic aspect of inflammatory mediators in Perthes patients. Comparison of genotype distribution between the control and Perthes patient groups revealed that heterozygote subjects for the *IL-6* G-174C/G-597A polymorphisms were significantly overrepresented in the control group. Consequently, we concluded that children who are heterozygous for these polymorphisms have a lower chance of developing Perthes disease than carriers of both homozygote genotypes. This finding points to the importance of well-balanced IL-6 protein level in the regulation of inflammatory process in Perthes disease. Although our results need to be confirmed in larger groups, they represent new and interesting findings and we believe that they could contribute to the understanding of the association of Perthes disease with genetic heterogeneity in immune response genes.

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Повезаност варијанти у генима *TLR4* и *IL-6* с Пертесовом болешћу

Сања Срзентић[1], Весна Спасовски[1], Душко Спасовски[2,3], Зорица Живковић[4], Драгана Матановић[2,5], Зоран Башчаревић[2,3], Зорица Терзић Шупић[2], Маја Стојиљковић[1], Теодора Каран-Ђурашевић[1], Биљана Станковић[1], Соња Павловић[1], Гордана Никчевић[1], Зоран Вукашиновић[2,3]

[1]Институт за молекуларну генетику и генетичко инжењерство, Универзитет у Београду, Београд, Србија;
[2]Медицински факултет, Универзитет у Београду, Београд, Србија;
[3]Институт за ортопедско-хируршке болести „Бањица", Београд, Србија;
[4]Клиничко-болнички центар „Др Драгиша Мишовић – Дедиње", Београд, Србија;
[5]Клиника за физикалну медицину и рехабилитацију, Клинички центар Србије, Београд, Србија

КРАТАК САДРЖАЈ

Увод Пертесова болест је идиопатска аваскуларна остеонекроза проксималне епифизе фемура која се јавља код деце. Етиологија ове болести је непозната. Током развоја Пертесове болести заступљен је процес запаљења, за који је показано да утиче на ремоделовање коштаног ткива.

Циљ рада С обзиром на то да генетички фактори који утичу на процес запаљења досад нису испитивани код Пертесове болести, циљ овог истраживања је био да се утврди повезаност учесталости варијанти у генима који учествују у инфламаторном одговору, *TLR4* (енгл. *toll-like receptor 4*) и *IL-6* (интерлеукин 6), и ове болести.

Методе рада Испитано је 37 деце с Пертесовом болешћу и 50 здравих особа. Методом *PCR-RFLP* одређени су полиморфизми медијатора запаљења *TLR4* (*Asp299Gly*, *Thr399Ile*) и *IL-6* (*G-174C*, *G-597A*).

Резултати Показано је да су полиморфизми *IL-6 G-174C* и *G-597A* у нашем испитивању били у потпуној гаметској неравнотежи везаности. У контролној групи је било статистички значајно више носилаца хетерозиготног генотипа *IL-6 G-174C/G-597A* у поређењу са групом испитаника с Пертесовом болешћу (p=0,047; *OR*=2,49; *95% CI*=1,00–6,21). Такође, група болесника није била у Харди–Вајнберговој равнотежи за полиморфизме *IL-6 G-174C/G-597A*. Није примећена статистички значајна разлика у расподели генотипова за полиморфизме анализиране у *TLR4* гену. Расподела генотипова међу групама болесника формираних на основу узраста када се болест појавила није показала статистички значајну повезаност с анализираним полиморфизмима.

Закључак Наше истраживање је показало да су носиоци хетерозиготног генотипа за *IL-6 G-174C/G-597A* полиморфизме били значајно чешћи у контролној групи него у групи деце оболеле од Пертесове болести. На основу тога закључили смо да је код деце која су носиоци хетерозиготног генотипа за ове полиморфизме мања вероватноћа за развој Пертесове болести него код носилаца оба хомозиготна генотипа.

Кључне речи: генске варијанте; инфламација; Пертесова болест

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