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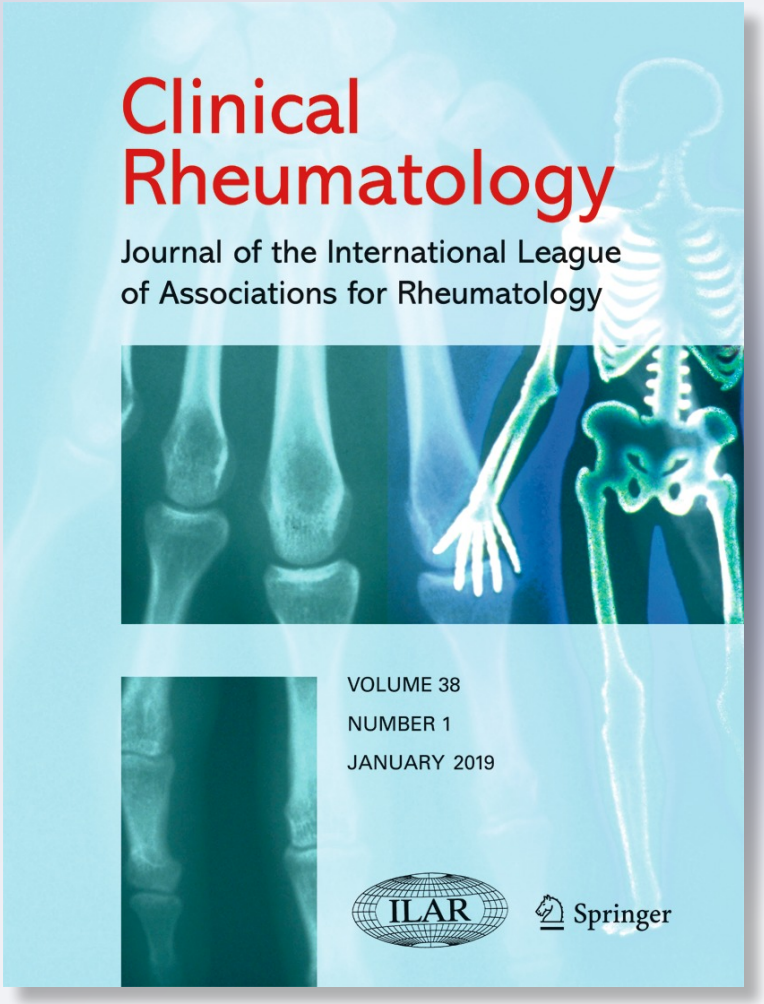
Clinical Rheumatology
Journal of the International League of Associations for Rheumatology

ISSN 0770-3198
Volume 38
Number 1

Clin Rheumatol (2019) 38:117-124
DOI 10.1007/s10067-018-4264-2



Springer

Clinical Rheumatology (2019) 38:117–124
https://doi.org/10.1007/s10067-018-4264-2

ORIGINAL ARTICLE

Vitamin D receptor gene polymorphism influences lipid profile in patients with juvenile idiopathic arthritis

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Received: 21 May 2018 / Revised: 27 July 2018 / Accepted: 15 August 2018 / Published online: 20 August 2018

Abstract

Vitamin D receptor (VDR) gene FokI (rs2228570) polymorphism was postulated to influence outcome of several inflammatory diseases. The aim of this study was to evaluate the influence of rs2228570 polymorphism on lipid profile and on outcome in patients with juvenile idiopathic arthritis (JIA) treated with etanercept. A total of 153 subjects (62 JIA patients and 91 controls) were screened for the rs2228570 using the PCR-RFLP method. Lipid profile (cholesterol, triacylglycerol, HDL-C, and LDL-C) was determined using standard biochemical analysis in controls, while in JIA patients, it was determined prior to and 12 months after anti-TNF (etanercept) therapy. Clinical outcome was assessed using the JIA—American College of Rheumatology (ACR) response criteria. There were significant differences in the distribution of genotypes ($p = 0.024$) and alleles ($p = 0.006$; OR = 2.222, 95% CI 1.136–4.348) of the rs2228570 between patients and controls. Etanercept treatment significantly increased HDL-C levels ($p = 0.006$) in JIA patients with FF genotype in comparison to baseline values. No significant differences were seen in JIA—ACR 30/50/70 responses at month 12 between FF and Ff/ff genotype carriers. This is the first study to demonstrate the protective effect of the VDR FokI FF genotype on lipid profile in JIA patients treated with etanercept. However, this has to be confirmed in a larger cohort of patients.

Keywords Etanercept · FokI polymorphism · Juvenile idiopathic arthritis · Lipid profile

Introduction

Juvenile idiopathic arthritis (JIA) is a heterogeneous inflammatory disease resulting in chronic arthritis that begins before the 16th birthday and persists in one or more joints for at least 6 weeks, with the exclusion of any other possible cause of joint inflammation. According to the International League of Associations for Rheumatology (ILAR) classification criteria, there are seven JIA subtypes, dependent on the number of joints involved and clinical signs and symptoms during the first 6 months of the illness. Polyarticular JIA affects five or more joints during the first 6 months of the disease and is divided into the seropositive and seronegative disease subtype [1].

JIA is developed after multifactorially triggered inflammatory reaction, followed by extreme production of pro-inflammatory cytokines (interleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF-α)), resulting in an increased synthesis of acute phase proteins in the liver, lipolysis and insulin resistance in adipose tissue, activation of the endothelium, which can finally cause endothelial dysfunction, and atherosclerosis [2]. Lipoprotein-related disorders play a key role in the development of atherosclerosis, which can be associated with autoimmune disease. It is known that cardiovascular diseases in patients with rheumatoid arthritis (RA) are associated with an increase in C-reactive protein (CRP) levels, low-density lipoprotein cholesterol (LDL-C) and triacylglycerol (TG) levels, and a decrease in high-density lipoprotein cholesterol (HDL-C) levels, as well as a shortened lifespan [3]. However, there is a growing evidence of the importance of lipid profile assessment in JIA [4–6].

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Vitamin D belongs to the group of liposoluble vitamins. The active form of vitamin D, 1,25-dihydroxyvitamin D (1,25 (OH)2D), plays its effect in target cells after binding to the C terminal fragment of the vitamin D receptor (VDR) in the cytosol, which is present in almost all tissues and immune cells [7]. The resulting hormone receptor complex transfers into the nucleus, complexes with the retinoid X receptor (RXR), and binds to the vitamin D response element (VDRE) in the gene promoter [8]. Vitamin D deficiency has been found as a potential risk factor for increased cardiovascular risk, abnormal HDL-C and LDL-C levels in adolescents, RA, and systemic lupus erythematosus (SLE) patients [9–11].

It has been shown that detection of single nucleotide polymorphisms (SNPs), as genetic biomarkers, plays an important role in diagnosis, prognosis, and treatment response evaluation in RA, which is becoming a trend in personalized medicine [12]. The VDR gene is located on the chromosome 12q12-14; it consists of 11 exons, and so far, 510 of its genetic variations have been discovered [13]. The most commonly studied SNP is FokI C/T (rs2228570) located in exon 2 of the VDR gene. This genetic variation in the VDR gene could influence the receptor structure, function, the binding of vitamin D and its effects. Although different studies have examined the correlation of this polymorphism with the risk to develop RA, showing contradictory results [14–16], its functional significance in JIA has not yet been well-established.

Beneficial effects of TNF-α inhibitors, such as etanercept, on inflammatory process and their clinical efficacy in chronic inflammatory arthritides are well-known [17]. There are still no data about the influence of rs2228570 on lipid profile in JIA patients treated with etanercept. The aim of this study was to evaluate the influence of rs2228570 SNP on lipid profile and on outcome in JIA patients treated with etanercept.

Patients and methods

Ethics and consent

All participants voluntarily agreed to participate in the research, and informed consent was signed by the parents or by the patients if they were aged ≥ 12 years. The Ethical Committee of the Medical Faculty in Niš gave consent for conducting this study. All participants were treated in accordance with the Helsinki Declaration. The research was conducted in the Laboratory for Functional Genomics and Proteomics, at the Faculty of Medicine, University of Niš, Serbia.

Patients

The study involved 153 subjects, 62 JIA patients and 91 healthy controls. JIA was diagnosed according to the ILAR classification criteria [1]. We have included JIA patients with active polyarticular disease course despite standard disease-modifying anti-rheumatic drugs (methotrexate 15–20 mg/m2/week). Polyarticular disease was defined by the presence of five or more joints with active arthritis. Joints were defined as active by the presence of swelling or, if no swelling was present, by the limitation of motion accompanied by pain, tenderness, or both. Etanercept therapy was given at the dosage of 0.4 mg/kg subcutaneously twice a week. JIA patients were diagnosed and treated in the Clinic of Pediatrics, Clinical Centre of Niš and Institute of Rheumatology, Belgrade, Serbia, and all of them were included in the etanercept national registry survey. Paired blood samples were collected (before starting etanercept therapy and 12 months after the continuous therapy). Clinical response to etanercept was assessed after 12 months of therapy according to the JIA—American College of Rheumatology (ACR) response criteria, using six core variables: the physician global assessment of disease activity, on a 10-cm visual analog scale (VAS; 0–100); the parent/patient global assessment of overall well-being, on a 10-cm VAS (0–100); the Childhood Health Assessment Questionnaire (CHAQ; 0–3); the number of joints with a limited range of motions (LOM); the number of joints with active arthritis (AA); and the erythrocyte sedimentation rate. The JIA—ACR 30/50/70 response was defined by international consensus: three or more JIA criteria improved by at least 30%/50%/70% with respect to baseline, and no more than one core set criterion worsened by 30% [18]. Exclusion criteria were conditions and comorbidities that affect lipid profile: cholestatic liver disease, obesity (body mass index > 30 kg/m2), familial hypercholesterolemia, hypertriglyceridemia, diabetes mellitus, thyroid disease, Cushing syndrome, nephrotic syndrome, as well as the use of steroids or lipid-lowering drugs during the study period. The control group consisted of 91 healthy subjects, children or adolescents, matched by age and sex with the patients, without previous history of JIA and other diseases.

Blood sample preparation and DNA isolation

Blood samples were taken in the morning, after fasting. From the blood samples (with EDTA as the anticoagulant), we used 200 μL of blood for DNA isolation. The second test tube (without the anticoagulant) with the blood sample was centrifuged at 3500 rpm for 10 min at + 4 °C, after which the serum was separated and used for lipid profile assessment. The isolation of DNA was performed using a commercial kit for DNA isolation (QIAamp DNA Blood Mini Kit, Qiagen GmbH, Hilden, Germany).

Genotyping methods

We examined the polymorphism rs2228570 in the VDR gene using the polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP) [19]. The fragment of 265 base pairs (bp) was amplified using a forward (5′-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3′) and a reverse primer (5′-ATG GAA ACA CCT TGC TTC TCC CTC-3′). The PCR reaction mixture in a volume of 25 μL contained 12.5 μL of KAPA 2G Fast HS Ready-Mix PCR kit solution (KAPA Biosystems, Germany), 0.5 μL of the primer (10 pmol/μL) (Fermentas GmbH, St. Leon-Rot, Germany), and 20 ng of DNA. The PCR conditions were initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, elongation at 72 °C for 15 s, and termination at 72 °C for 30 s. The amplified PCR products were visualized under UV light after agarose gel (2%) electrophoresis. PCR products were cut into smaller fragments by the FokI restriction enzyme (Fermentas GmbH, St. Leon-Rot, Germany) at 37 °C overnight and analyzed by vertical polyacrylamide gel (8%) electrophoresis. Homozygous for the C allele (wild type) was detected as one fragment of 265 bp (genotype CC or FF), while the polymorphic homozygous (TT or ff) was shown as two fragments of 169 and 96 bp. Heterozygous (CT or Ff) was confirmed by the presence of three fragments on the gel (265, 169, and 96 bp).

Lipid profile assessment

Sera from early morning blood samples were used for the assessment of lipid profile. Total cholesterol (TC), triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were determined in the Olympus AU400 analyzer, using standard biochemical analyses.

Statistical analysis

The frequency of alleles and genotypes in the patients and controls was analyzed and compared using the χ^2 test or Fisher's exact test, but we also determined the possible deviation from the expected values of the Hardy-Weinberg equilibrium. The risk was estimated by the odds ratio (OR) with 95% confidence interval (CI). Univariate logistic regression analysis was used to analyze the association between genetic polymorphism and JIA. The lipid profile parameter levels were expressed as mean (M) ± standard deviation (SD). The statistically significant differences in values between the groups were determined by a *t* test for two independent samples or by a paired *t* test. We performed a χ^2 test to compare clinical response (JIA—ACR 30/50/70) between different genotypes. A $p < 0.05$ value was considered statistically significant. The statistical analysis was conducted using the SPSS software package version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Out of 62 JIA patients, there were 22 males and 40 females. The mean age at the study entry was 14.36 ± 3.49 (range 4–25). The mean age at disease onset was 9.32 ± 3.83 years and a disease duration of 5.08 ± 2.74 years. Ninety-one healthy children and young blood donors were included (44 males and 47 females), with the mean age of 15.28 ± 3.62 years. No significant differences were found between age ($p = 0.119$) and gender ($p = 0.136$) of the patients and controls.

Genotype and allele frequencies of the rs2228570 VDR gene polymorphism

Genotype frequencies of the rs2228570 VDR gene polymorphism did not deviate from the normal distribution of the Hardy-Weinberg equilibrium in patients and control groups ($p > 0.05$). The results shown in Table 1 indicate that the genotype frequency distributions of the rs2228570 VDR SNP in JIA patients were significantly different from those of the controls ($\chi^2 = 7.458$, df = 2, $p = 0.024$). The frequency of the f allele was significantly higher in JIA patients compared to controls ($\chi^2 = 7.563$, df = 1, $p = 0.006$; Fisher's exact test $p = 0.007$). Univariate logistic regression showed that the ff genotype was associated with sixfold risk

Table 1 Genotype and allele frequencies of the rs2228570 VDR gene polymorphism in the studied groups

Genotype	Control $n = 91$	JIA $n = 62$	p value (χ^2 test)	OR (95% CI)	p value
FF	64 (70.3%)	32 (51.6%)	0.024	1	
Ff	25 (27.5%)	24 (38.7%)		1.920 (0.951-3.877)	0.069
ff	2 (2.2%)	6 (9.7%)		6.000 (1.146-31.418)	0.034
Allele	Control $2n = 182$	JIA $2n = 124$	p value (χ^2 test)	OR (95% CI)	
F	153 (84.07%)	88 (70.97%)	0.006	1	
f	29 (15.93%)	36 (29.03%)		2.222 (1.136-4.348)	0.020

OR odds ratio, *95% CI* 95% confidence interval

(95% CI 1.146–31.418, $p = 0.034$) for JIA as compared to the FF genotype, while the presence of the f allele was associated with 2.22-fold higher risk for JIA compared to the F allele (95% CI 1.136–4.348, $p = 0.020$).

Lipid profile in the studied groups

Lipid profile was assessed in 36 JIA patients before starting and 12 months after etanercept therapy, and in 34 controls. The mean values of TC, LDL-C, and TG in patients with JIA either before ($p = 0.003$, $p = 0.016$, $p < 0.001$, respectively) or after treatment were significantly higher than they were in healthy subjects ($p = 0.004$, $p = 0.023$, $p = 0.006$, respectively). On the other hand, HDL-C values in patients were significantly lower in comparison to control ($p < 0.001$). Etanercept treatment significantly increased HDL-C values ($p = 0.007$) and decreased TG values ($p = 0.020$) in JIA patients compared to the values before the introduction of the treatment, while no difference was observed in TC ($p = 0.668$) and LDL-C levels ($p = 0.558$) (Table 2).

The influence of the rs2228570 VDR gene polymorphism on lipid profile

Bearing in mind that the Ff genotype was detected in six JIA patients, we compared the influence of etanercept on lipid status parameters between the FF genotype carriers and the Ff/ff genotype carriers. The results of the study of the rs2228570 SNP effect on the tested parameters in JIA patients before etanercept treatment showed that there were no significant differences in TC levels ($p = 0.576$), LDL-C levels ($p = 0.734$), HDL-C levels ($p = 0.250$), and TG levels ($p = 0.525$) between the FF genotype carriers and the Ff/ff genotype carriers. The differences in TC, LDL-C, HDL-C, and TG levels were not seen in JIA patients after treatment between FF and Ff/ff genotype carriers, either ($p = 0.254$, $p = 0.113$, $p = 0.623$, $p = 0.615$, respectively).

JIA patients carrying the FF genotype had significantly higher HDL-C levels after etanercept treatment compared to values before the treatment ($t = -3.088$, df = 19, $p = 0.006$). Etanercept treatment decreased TG levels in patients with the FF genotype, but the difference did not reach significance ($p = 0.062$). No influence of etanercept treatment was seen in HDL-C and TG levels in comparison to baseline values in Ff/ff genotype carriers ($p = 0.390$, $p = 0.183$, respectively). The analysis of etanercept treatment influence on TC and LDL-C levels revealed no significant differences in JIA patients with the FF genotype ($p = 0.252$; $p = 0.223$, respectively) and patients, carriers of the Ff/ff genotype either ($p = 0.164$; $p = 0.227$, respectively) compared to the values before treatment introduction (Table 3).

The influence of the rs2228570 VDR gene polymorphism on etanercept treatment response

The results of the rs2228570 VDR gene polymorphism influence on the JIA—ACR 30/50/70 response in JIA patients at month 12 are shown in Table 4. Fifty-nine of 62 (95.16%) JIA patients achieved the JIA—ACR 30 response, 88.70% of patients achieved the JIA—ACR 50 response, and 56.45% of JIA patients responded to etanercept treatment achieving the JIA—ACR 70 response. Patients achieved the JIA—ACR 70 response significantly less frequently than the JIA—ACR 50 response ($\chi^2 = 16.209$, df = 1, $p < 0.001$) and the JIA—ACR 30 response ($\chi^2 = 25.328$, df = 1, $p < 0.001$). No significant difference was seen between JIA—ACR 30 and JIA—ACR 50 response achievement ($p = 0.323$). Only three out of 62 patients (4.83%) did not respond to treatment.

At month 12, JIA patients, carriers of the FF genotype achieved the JIA—ACR 30/50/70 response more frequently (96.87%/93.75%/62.5%) in comparison to the Ff/ff genotype carriers (93.33%/83.33%/50%), but the difference was not statistically significant ($p = 0.607$, $p = 0.249$, $p = 0.443$, respectively). One out of 32 patients (3.12%) with the FF genotype, one out of 24 patients (4.15%) with the Ff genotype and one out of 6 JIA patients (16.66%) with the ff genotype did not respond to etanercept treatment.

Table 2 Lipid profile in control and JIA patients before and 12 months after etanercept treatment

Lipid profile	Control M (SD)	JIA before treatment M (SD)	JIA after treatment M (SD)
TC (mmol/L)	3.65 (0.76)	4.25 (0.85)[a]	4.19 (0.72)[a1]
LDL-C (mmol/L)	2.55 (0.65)	2.99 (0.82)[b]	2.91 (0.64)[b1]
HDL-C (mmol/L)	1.20 (0.26)	0.70 (0.33)[c]	0.88 (0.25)[c1,*]
TG (mmol/L)	0.70 (0.27)	1.04 (0.36)[d]	0.87 (0.21)[d1, **]

TC total cholesterol, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *TG* triacylglycerol; [a] $p = 0.003$, [a1] $p = 0.004$ vs. control; [b] $p = 0.016$, [b1] $p = 0.023$ vs. control; [c, c1] $p = <0.001$ vs. control; [d] $p = <0.001$, [d1] $p = 0.006$ vs. control; *$p = 0.007$, **$p = 0.020$ vs. JIA before treatment

Table 3 The influence of the rs2228570 VDR gene polymorphism on lipid profile in JIA patients

Lipid profile	Genotype			
	FF ($n = 20$)		Ff/ff ($n = 16$)	
	Before treatment M (SD)	After treatment M (SD)	Before treatment M (SD)	After treatment M (SD)
TC (mmol/L)	4.32 (0.90)	4.06 (0.77)	4.16 (0.79)	4.34 (0.65)
LDL-C (mmol/L)	3.03 (0.91)	2.76 (0.63)	2.94 (0.72)	3.10 (0.61)
HDL-C (mmol/L)	0.64 (0.29)	0.89(0.25)*	0.78 (0.38)	0.85 (0.24)
TG (mmol/L)	1.07 (0.42)	0.89 (0.16)	0.99 (0.29)	0.85 (0.26)

*$p = 0.006$ vs. before treatment

Discussion

Although the rs2228570 polymorphism is one of the most analyzed VDR gene polymorphisms, there are limited data in regard to the role of this polymorphism in JIA. We showed that this SNP increased susceptibility to polyarticular JIA six times for polymorphic homozygotes (ff) in comparison to FF allele carriers and 2.22 times for f allele carriers compared to carriers of the F allele. These results are consistent with the findings of Masi et al. [20], who included 50 JIA patients in the Italian population. They also observed that patients with the ff genotype had lower bone mineral density and could be at a higher risk of losing bone mass in comparison with FF genotype carriers.

In this study, we observed a disbalance of lipid profile in JIA patients in comparison to control, which is consistent with previous findings [4, 5], while etanercept therapy significantly increased HDL-C levels and decreased TG levels in the patients compared to baseline values. De Sanctis et al. [21] showed pro-inflammatory cytokine level (TNF-α, IL-1β, IL-6, and interferon-γ) decrease and reductions in TC, LDL-C, and TG levels after etanercept treatment, whereas no significant change was found in HDL-C levels. On the other hand, Breda et al. [5] and Yeh et al. [6] did not find significant difference in lipid profile in JIA patients after etanercept treatment. Previous studies have found that hypercholesterolemia in childhood is the most important risk factor for atherosclerosis and cardiovascular disease in adulthood, where elevated TC, LDL-C, and decreased HDL-C levels strongly correlate with the development of early atherosclerosis [22]. Antiatherogenic effect of HDL-C is well-known. Furthermore, another important role of HDL is intravascular degradation of triacylglycerol-rich lipoproteins (chylomicrons and very low-density lipoprotein (VLDL)). Some authors suggest that the decrease in HDL-C levels in inflammatory diseases is a consequence of increased serum amyloid A (SAA) synthesis in the liver. Inflammatory cytokines cause increased production of SAA, which consequently increases its content in HDL, which results in the faster clearance of HDL and the decrease in HDL-C concentration [23]. It is known that there is increased production of TNF-α and other pro-inflammatory cytokines in JIA, with the consequently increased synthesis of acute-phase proteins in the liver [24, 25]. Bearing in mind the role of TNF-α in this disease, dyslipidemia in our study could be a consequence of increased production of pro-inflammatory molecules. It is possible that the increase in HDL-C levels in JIA patients after etanercept treatment, and consequent decrease in TG levels, can be the result of the "inverse phase reaction" and a consequence of inhibition of TNF-α and suppressed pro-inflammatory effects.

To the authors' knowledge and based on the literature search performed, this is the first study to examine the impact of the rs2228570 polymorphism on lipid profile and clinical response to etanercept treatment in JIA. The results of this study showed significant increase in HDL-C levels in the patients, carriers of the FF genotype, after etanercept treatment, while the decrease in TG levels showed only a trend.

Although the results of several studies have shown lower 25-OHD3 levels in JIA patients than in healthy subjects [26, 27], there is still lack of consensus and definition of vitamin D deficiency in pediatric population [28]. Vitamin D binds to VDR in the immune cells, including antigen presenting cells,

Table 4 The influence of the rs2228570 VDR gene polymorphism on the JIA—ACR Pedi 30/50/70 response at month 12

Clinical response	JIA patients	Genotype			
	N (%)	FF *N* (%)	Ff *N* (%)	ff *N* (%)	Ff/ff *N* (%)
JIA—ACR 30	59/62 (95.16)	31/32 (96.87)	23/24 (95.83)	5/6 (83.33)	28/30 (93.33)
JIA—ACR 50	55/62 (88.70)	30/32 (93.75)	20/24 (83.33)	5/6 (83.33)	25/30 (83.33)
JIA—ACR 70	35/62 (56.45)*	20/32 (62.50)	11/24 (45.83)	4/6 (66.66)	15/30 (50.00)

*$p < 0.001$ vs. JIA—ACR 30 and JIA—ACR 50

CD4 + T and CD8 + T cells, with final immunosuppressive and anti-inflammatory effects [14, 28, 29]. The study performed in the mouse brain endothelial cell line showed that the inhibitory effect of 1,25 (OH)2D3 on hypoxia-induced NF-κB activation and consequently IL-1 and TNF-α production was VDR-mediated [30]. Xiang et al. [31] found that intravenous transplantation of genetically modified endothelial progenitor cells overexpressing VDR could increase HDL-C levels and reduce atherosclerotic plaque formation in ApoE −/− mice. Andrukhova et al. [32] reported the importance of intact VDR signaling in the preservation of NO synthesis and normal vascular function. Furthermore, the study results of Chow et al. [33] showed that activation of the VDR increased the expression of hepatic CYP7A1 and reduced levels of plasma and liver cholesterol in mice. By examining the functional significance of the rs2228570 polymorphism, some authors suggest that the presence of F allele leads to the displacement of the start point of translation for three codons and the synthesis of three amino acids shorter protein (F/M4 form) compared to that in f allele carriers (f/M1 form). This shorter (F/M4) form of VDR represents a more active form of the receptor, which more effectively associates with transcription factor (TFIIB), as demonstrated in HeLa and COS-7 cells in vitro [8, 34]. Given the above facts, etanercept-mediated inhibition of TNF-α in our study followed by increase in HDL-C levels in patients with the FF genotype could be a consequence of the presence of a more active VDR form compared to the patients, carriers of the polymorphic f allele. It is possible that the increase in HDL-C levels in JIA patients after etanercept treatment can be the result not only of TNF inhibition and suppressed inflammation, but also of the presence of the rs2228570 polymorphism. However, although etanercept has shown high efficacy in JIA [24, 25, 35–37], the rs2228570 SNP did not affect the JIA—ACR response, as a clinical outcome measure in our study. Research on a larger number of patients is certainly necessary in order to investigate a possible association of this polymorphism with the risk of JIA, its effects on lipid profile and etanercept treatment response, and, especially, the effects of SNP-SNP interactions, which we carried out in patients for different SNPs in our previous studies [38, 39].

The limitations of our study are related to the relatively small sample size. On the other hand, we did not evaluate the potential influences of daily physical activity and diet on HDL-C levels.

Conclusion

This is the first study examining the influence of the VDR gene rs2228570 polymorphism on lipid profile and etanercept treatment response. Etanercept treatment significantly decreased TG values and increased HDL-C values in JIA patients compared to baseline values. Considering the VDR gene polymorphism, significant increase in HDL-C levels was detected in the FF genotype carriers. These findings suggest that TNF-α blockers might reduce atherosclerotic risk in patients with JIA, especially those with the FF genotype. However, future research should focus on testing the underlying mechanisms of potential protective effect of the FF genotype on atherosclerosis development in JIA.

Funding information This work was supported by the Ministry of Education, Science, and Technological Development of the Republic of Serbia (grant number III41018).

Compliance with ethical standards

All participants voluntarily agreed to participate in the research, and informed consent was signed by parents or by the patients if they were aged ≥ 12 years. The Ethical Committee of the Medical Faculty in Niš gave consent for conducting this study. All participants were treated in accordance with the Helsinki Declaration. The research was conducted in the Laboratory for Functional Genomics and Proteomics, at the Medical Faculty, University of Niš, Serbia.

Conflict of interests The authors declare that they have no conflict of interest.

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