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Association of vitamin D receptor genetic variants with bone mineral density and inflammatory markers in rheumatoid arthritis

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ABSTRACT

Background and aims: Vitamin D receptor (VDR) genetic variants are considered to have a role in the pathogenesis of rheumatoid arthritis (RA). This study examines an association of FokI, BsmI, ApaI and TaqI with RA, as well as with bone mineral density (RA with normal bone mineral density, RA-NBMD; RA with associated osteopenia, RA-OSTP; and RA with associated osteoporosis, RA-OP) and inflammatory markers.
Materials and methods: VDR genetic variants were tested in 248 subjects using the PCR-RFLP method.
Results: Significant differences were observed in the distribution of FokI genotypes between RA patients ($p < 0.001$), or subgroups (RA-NBMD, RA-OSTP, RA-OP) ($p = 0.035$, $p = 0.02$, $p < 0.001$, respectively) and controls. Prevalence of FokI f allele was significantly higher in RA group ($p < 0.001$) and subgroups ($p = 0.003$, $p = 0.021$, $p < 0.001$, respectively) compared to controls. An increased susceptibility to RA-OSTP was revealed in BsmI/ApaI Ba (AC) haplotype carriers ($p = 0.012$). A significantly higher erythrocyte sedimentation rate values were obtained in FokI FF compared to Ff + ff carriers (54.57 ± 23.73 vs. 22.83 ± 12.42; $p < 0.001$) within the RA-NBMD subgroup.
Conclusion: The results of the study indicate an association of RA with FokI genetic variant and increased susceptibility to RA in f allele carriers, as well as to RA-OSTP in BsmI/ApaI Ba (AC) haplotype carriers.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by a complex multifactorial pathogenesis leading to a production of autoantibodies associated with the chronic inflammation of synovial membrane, subsequent cartilage erosion and the progressive destruction of affected joints [1]. Chronic inflammation and disease activity are the most important factors responsible for bone loss, one of the main features and extra-articular complications in RA patients. Additionally, reduced motility, glucocorticoid usage and menopausal status in women may contribute, as well [2]. Rheumatoid factor (RF) positive patients are at higher risk for osteoporosis development and reduced bone mass [3]. Moreover, vitamin D deficiency, which is very common in RA, correlates with increased risk of bone loss [4].

Biological effects of vitamin D are the result of its binding to the vitamin D receptor (VDR) and subsequent regulation of the transcription of target genes [5]. Discovery of VDR expression and local production of vitamin D active form (1,25-dihydroxycholecalciferol; 1,25$(OH)_2D_3$) in cells and tissues different from those involved in calcium and phosphorous metabolism (e.g. mast cells, macrophages, dendritic, natural killer cells, T and B cells), elucidated its "non-classical" roles, such as immunomodulatory and anti-inflammatory role, including inhibition of Th1 and Th17 response, decreased production of pro-inflammatory cytokines (tumor necrosis factor–α, interleukin–1, interleukin–6), inhibition of B cells' proliferation, immunoglobulin class switching, and reduced autoantibody production [6]. Activated macrophages are

Abbreviations: 1, 25$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; BMD, Bone mineral density; CI, Confidence interval; DXA, Dual-energy X-ray absorptiometry; ESR, Erythrocyte sedimentation rate; HWE, Hardy, Weinberg equilibrium; OR, Odds ratio; PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; RA, Rheumatoid arthritis; RA-NBMD, RA with normal bone mineral density; RA-OP, RA with associated osteoporosis; RA-OSTP, RA with associated osteopenia; RF, Rheumatoid factor; SD, Standard deviation; SNPs, Single nucleotide polymorphisms; VDR, Vitamin D receptor.

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Table 1
Distribution of the VDR genotypes and alleles in RA patients and controls.

VDR variant rs number	Genotype or Allele	RA N = 143 (%)	RA subgroup NBMD N = 41 (%)	OSTP N = 59 (%)	OP N = 43 (%)	Control N = 105 (%)	$p_{RA\ vs.\ C}$ (OR; 95% CI)	$p_{NBMD\ vs.C}$ (OR; 95% CI)	$p_{OSTP\ vs.\ C}$ (OR; 95% CI)	$p_{OP\ vs.\ C}$ (OR; 95% CI)	$p_{OSTP\ vs.\ NBMD}$ (OR; 95% CI)	$p_{OP\ vs.\ NBMD}$ (OR; 95% CI)	$p_{OP\ vs.\ OSTP}$ (OR; 95% CI)
FokI	FF (CC)	63 (44.06)	20 (48.78)	29 (49.15)	14 (32.56)	71 (67.62)	1	1	1	1	1	1	1
(rs2228570)	Ff (CT)	67 (46.85)	15 (36.59)	27 (45.77)	25 (58.14)	32 (30.48)	**0.002** (2.36; 1.374–4.052)	0.204 (1.664; 0.756–3.662)	**0.04** (2.066; 1.057–4.037)	**<0.001** (3.962; 1.823–8.609)	0.67 (1.241; 0.53–2.905)	0.102 (2.381; 0.934–6.071)	0.146 (1.918; 0.829–4.435)
	ff (TT)	13 (9.09)	6 (14.63)	3 (5.08)	4 (9.3)	2 (1.90)	**0.005** (7.325; 1.591–33.724)	**0.004** (10.65; 1.994–56.884)	0.164 (3.672; 0.583–23.137)	**0.013** (10.143; 1.691–60.843)	0.274 (0.345; 0.077–1.543)	1 (0.952; 0.226–4.011)	0.234 (2.762; 0.543–14.057)
	F (C)	193 (67.48)	55 (67.07)	85 (72.03)	53 (61.63)	174 (82.86)	1	1	1	1	1	1	1
	f (T)	93 (32.52)	27 (32.93)	33 (27.97)	33 (38.37)	36 (17.14)	**<0.001** (2.329; 1.506–3.601	**0.003** (2.373; 1.323–4.254)	**0.021** (1.876; 1.095–3.216)	**<0.001** (3.009; 1.713–5.288)	0.451 (0.791; 0.429–1.457)	0.462 (1.268; 0.673–2.389)	0.117 (1.604; 0.887–2.899)
BsmI	bb (GG)	52 (36.36)	14 (34.15)	23 (38.98)	15 (34.88)	38 (36.19)	1	1	1	1	1	1	1
(rs1544410)	Bb (AG)	65 (45.46)	21 (51.22)	23 (38.98)	21 (48.84)	47 (44.76)	0.971 (1.011; 0.576–1.773)	0.636 (1.213; 0.545–2.699)	0.562 (0.809; 0.394–1.659)	0.758 (1.132; 0.514–2.491)	0.371 (0.667; 0.274–1.623)	0.886 (0.933; 0.362–2.406)	0.564 (1.4; 0.581–3.373)
	BB (AA)	26 (18.18)	6 (14.63)	13 (22.04)	7 (16.28)	20 (19.05)	0.889 (0.95; 0.464–1.947)	0.714 (0.814; 0.271–2.444)	0.872 (1.074; 0.45–2.562)	0.822 (0.887; 0.311–2.528)	0.644 (1.319; 0.408–4.264)	0.899 (1.089; 0.293–4.041)	0.739 (0.826; 0.268–2.545)
	b (G)	169 (59.09)	49 (59.76)	69 (58.47)	51 (59.3)	123 (58.57)	1	1	1	1	1	1	1
	B (A)	117 (40.91)	33 (40.24)	49 (41.53)	35 (40.7)	87 (41.43)	0.908 (0.979; 0.682–1.406)	0.853 (0.952; 0.566–1.601)	0.986 (1.004; 0.635–1.587)	0.908 (0.970; 0.582–1.616)	0.856 (1.054; 0.594–1.871)	0.952 (1.019; 0.550–1.887)	0.906 (0.966; 0.549–1.7)
ApaI	aa (CC)	33 (23.08)	7 (17.07)	13 (22.04)	13 (30.24)	21 (20.00)	1	1	1	1	1	1	1
(rs7975232)	Aa (AC)	60 (41.96)	18 (43.9)	27 (45.76)	15 (34.88)	49 (46.67)	0.462 (0.779; 0.401–1.515)	0.851 (1.102; 0.401–3.031)	0.785 (0.89; 0.386–2.054)	0.123 (0.495; 0.201–1.218)	0.702 (0.808; 0.27–2.415)	0.167 (0.449; 0.143–1.412)	0.245 (0.556; 0.205–1.502)
	AA (AA)	50 (34.96)	16 (39.02)	19 (32.20)	15 (34.88)	35 (33.33)	0.789 (0.909; 0.453–1.825)	0.551 (1.371; 0.485–3.88)	0.772 (0.877; 0.36–2.133)	0.432 (0.692; 0.276–1.735)	0.438 (0.639; 0.206–1.988)	0.244 (0.505; 0.159–1.607)	0.651 (0.789; 0.283–2.199)
	a (C)	126 (44.06)	32 (39.02)	53 (44.91)	41 (47.67)	91 (43.33)	1	1	1	1	1	1	1
	A (A)	160 (55.94)	50 (60.98)	65 (55.09)	45 (52.33)	119 (56.67)	0.873 (0.971; 0.678–1.391)	0.503 (1.195; 0.71–2.021)	0.782 (0.938; 0.596–1.477)	0.495 (0.839; 0.507–1.389)	0.407 (0.785; 0.443–1.392)	0.258 (0.702; 0.38–1.297)	0.696 (0.895; 0.513–1.563)
TaqI	TT (CC)	59 (41.26)	17 (41.46)	23 (38.98)	19 (44.19)	38 (36.19)	1	1	1	1	1	1	1
(rs731236)	Tt (CT)	60 (41.96)	20 (48.78)	23 (38.98)	17 (39.53)	50 (47.62)	0.362 (0.773; 0.444–1.345)	0.776 (0.894; 0.413–1.935)	0.452 (0.76; 0.372–1.554)	0.33 (0.68; 0.312–1.481)	0.713 (0.85; 0.357–2.023)	0.559 (0.76; 0.303–1.908)	0.803 (0.895; 0.374–2.142)
	tt (TT)	24 (16.78)	4 (9.76)	13 (22.04)	7 (16.28)	17 (16.19)	0.802 (0.909; 0.432–1.912)	0.301 (0.526; 0.154–1.8)	0.606 (1.263; 0.52–3.072)	0.714 (0.824; 0.292–2.326)	0.174 (2.402; 0.665–8.675)	0.526 (1.566; 0.389–6.298)	0.445 (0.652; 0.217–1.961)
	T (C)	178 (62.24)	54 (65.85)	69 (58.47)	55 (63.95)	126 (60.00)	1	1	1	1	1	1	1
	t (T)	108 (37.76)	28 (34.15)	49 (41.53)	31 (36.05)	84 (40.00)	0.613 (0.91; 0.632–1.311)	0.355 (0.778; 0.456–1.826)	0.787 (1.065; 0.673–1.685)	0.526 (0.845; 0.503–1.422)	0.292 (1.37; 0.763–2.459)	0.796 (1.087; 0.577–2.049)	0.429 (0.794; 0.448–1.407)

VDR – vitamin D receptor; N – number of individuals; RA – rheumatoid arthritis; NBMD – RA with normal bone mineral density; OSTP – RA with associated osteopenia; OP – RA with associated osteoporosis; C – control; CI – confidence interval; OR – odds ratio; significant *p*-values are given in bold

capable to produce $1,25(OH)_2D_3$ in synovial membrane affected by the inflammatory process [7]. Furthermore, VDR expression is also shown in the synovial inflammatory cells, chondrocytes and synovial stromal cells in the rheumatoid lesions [8], suggesting a potential role of VDR in RA pathogenesis.

Even the genetic background of complex diseases, including RA, is still not completely understood, recent studies suggest that single nucleotide polymorphisms (SNPs) might be useful biomarkers for RA management, and together with classic biomarkers important for early diagnosis and treatment of RA [9]. Human VDR gene, located at the 12q13-14 position of the 12th chromosome, is characterized by the presence of a large number of genetic variants; however, four of them, FokI (rs2228570, exon 2; T to C substitution), BsmI (rs1544410, intron 8; G to A substitution), ApaI (rs7975232, intron 8; C to A substitution), and TaqI (rs731236, exon 9; C to T substitution), are among the most studied variants. Nevertheless, their role is still not completely understood. In vitro study by Uitterlinden et al. [10] showed higher activity of VDR protein synthesized in the presence of FokI F allele, while BsmI may influence mRNA stability [11]. Some studies also suggested the association of Bat haplotype with higher mRNA expression in comparison to baT haplotype [10].

Although the association of VDR variants with RA and/or bone mineral density (BMD) is previously studied in different populations, results remain inconsistent. VDR FokI was previously studied in Serbian patients with juvenile idiopathic arthritis by our group [12], but to the best of our knowledge not in RA. Therefore, the aim of this study is to examine the possible associations of VDR genetic variants (FokI, BsmI, ApaI, and TaqI) with RA, as well as their association with BMD and inflammatory markers in Serbian patients with RA.

2. Materials and methods

2.1. Patients

A total of 248 subjects, inhabitants of south-east Serbia, were enrolled in this study: 143 patients with RA (29 male and 114 female; mean age 57.04 ± 12.48) and 105 unrelated healthy volunteers (34 male and 71 female; mean age 56 ± 16.59). All RA patients were recruited from the Institute "Niška Banja", Niš, Republic of Serbia. The disease was diagnosed according to the 2010 ACR/EULAR classification criteria. Healthy individuals with no personal and family history of any chronic inflammatory, autoimmune or rheumatic disease were involved in this study as a control group.

Informed consent has been obtained from the subject involved in the study. The study was approved by the Ethics Committee of the Faculty of Medicine, University in Niš, Republic of Serbia and complies with the ethical principles of the Declaration of Helsinki and European Medicines Agency Guidelines for Good Clinical Practice.

Table 2
Distribution of the BsmI/ApaI haplotypes in RA patients and controls.

VDR variant	Haplotype	RA N (%)	RA-NBMD N (%)	RA-OSTP N (%)	RA-OP N (%)	Controls N (%)
BsmI/ApaI	ba (GC)	113 (39.51)	31 (37.81)	46 (38.99)	36 (41.87)	89 (42.38)
	bA (GA)	56 (19.58)	18 (21.95)	23 (19.49)	15 (17.44)	34 (16.19)
	Ba (AC)	13 (4.55)	1 (1.22)	7 (5.93)*	5 (5.81)	2 (0.95)
	BA (AA)	104 (36.36)	32 (39.02)	42 (35.59)	30 (34.88)	85 (40.48)

VDR – vitamin D receptor; N – number of individuals; RA – rheumatoid arthritis; RA-NBMD – RA with normal bone mineral density; RA-OSTP – RA with associated osteopenia; RA-OP – RA with associated osteoporosis; *$p = 0.012$ vs. controls after correction for multiple comparison (cut-off value for statistical significance $p = 0.0125$)

Table 3
Laboratory parameters in RA patients and RA subgroups.

Inflammatory marker	RA (N = 98)	RA-NBMD (N = 26)	RA-OSTP (N = 41)	RA-OP (N = 31)
ESR [mm/h]*	43.94 ± 25.6	39.92 ± 24.92	45.48 ± 28.83	45.25 ± 21.77
Fibrinogen [g/l]*	5.01 ± 1.2	4.68 ± 1.10	5.21 ± 1.18	5.02 ± 1.29
RF [IU/ml]**	48 (0–192.0)	48 (0–192.0)	48 (0–144.0)	48 (0–192.0)

ESR – erythrocyte sedimentation rate; RA – rheumatoid arthritis; RA-NBMD – RA with normal bone mineral density; RA-OSTP – RA with associated osteopenia; RA-OP – RA with associated osteoporosis; RF – rheumatoid factor
* mean ± standard deviation; ** median (25th – 75th percentile)

The research was conducted in the Laboratory for Functional Genomics and Proteomics, Faculty of Medicine, University of Niš, Republic of Serbia.

2.2. Samples

Three venous blood samples were collected: sample collected into the test tube with EDTA as an anticoagulant was used for DNA isolation (0.2 ml) and plasma separation; sample collected into the test tube containing sodium citrate was used for determination of erythrocyte sedimentation rate (ESR), while sample collected into the test tube without an anticoagulant was used for the serum separation. Plasma and serum separation was performed by centrifugation at 3500 rpm for 10 min at 4 °C.

2.3. Assessment of biochemical parameters

Inflammatory parameters (fibrinogen, ESR and RF) were determined in 98 patients with RA at the time of establishing the diagnosis. ESR (mm/h) was determined using the Westergren method. Fibrinogen concentration (g/l) was measured in plasma samples using a steel ball coagulometer (Thrombotrack® Solo, Axis-Shield PoC AS, Oslo, Norway), while serum samples were used for determination of RF IgM (IU/ml) by means of latex agglutination test (HumaTex RF, Human, Wiesbaden, Germany).

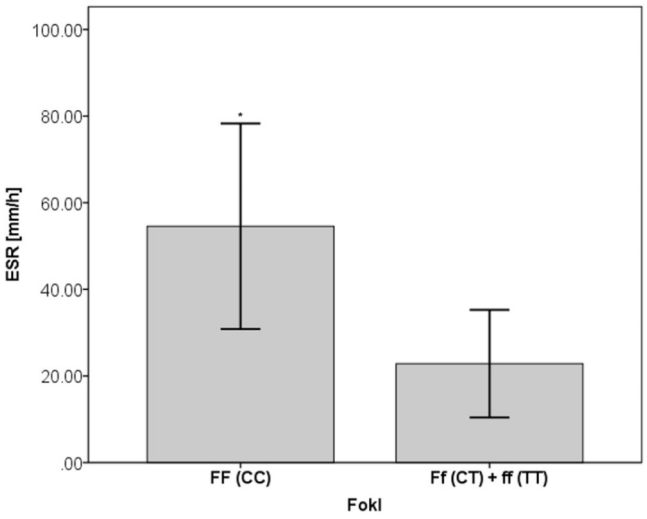

Fig. 1. Erythrocyte sedimentation rate in RA-NBMD patients according to the FokI genotypes ESR – erythrocyte sedimentation rate; RA-NBMD – rheumatoid arthritis associated with normal bone mineral density; *$p < 0.001$.

2.4. Assessment of bone mineral density

According to the BMD, measured using dual-energy X-ray absorptiometry (DXA) of the lumbar spine (L1-L4) and the hip, all patients were classified into 3 groups: I – RA patients with normal BMD (RA-NBMD; T-score ≥ -1); II – RA patients with associated osteopenia (RA-OSTP; T-score −1 to −2.5) and III – RA patients with associated osteoporosis (RA-OP; T-score ≤ -2.5).

2.5. DNA isolation and VDR genotyping

DNA was isolated from the whole blood samples using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The DNA concentration was measured using UV/Vis spectrophotometer (BioSpec-nano, Shimadzu Corporation, Japan) and the purity of isolated DNA was determined by calculating the A260/A280 ratio. All samples were stored at −20 °C until the genotyping experiments were performed.

Four VDR genetic variants (FokI, BsmI, ApaI and TaqI) were determined using the polymerase chain reaction - restriction length polymorphism (PCR-RFLP) method as previously described in [13].

2.6. Statistical analysis

In order to determine differences in genotype and allele frequencies between the groups, chi-square (χ^2) or two-tailed Fisher's test was used. Obtained values of genotype and allele frequencies were compared to those predicted by the Hardy–Weinberg equilibrium (HWE) using the χ^2 test. Odds ratio (OR) and 95% confidence interval (95% CI) were used for the assessment of genetic risks.

Fibrinogen and ESR values were expressed as mean ± standard deviation (SD), while RF values were expressed by median and 25th to 75th percentiles.

The correlations of VDR genotypes with the values of inflammatory parameters were determined using the analysis of one-way variance (ANOVA), if normality and homogeneity of variance assumptions were satisfied; or the Kruskal-Wallis test, in case normality and homogeneity of variance assumptions were not satisfied. Post hoc analysis was performed using the Mann-Whitney *U* test.

The values of $p < 0.05$ were considered statistically significant. Bonferroni adjustments were applied for multiple comparisons.

Statistical analyses were performed using the SPSS version 20.0 software package (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. VDR genotyping results

The obtained genotype frequencies for FokI, BsmI, TaqI, and ApaI did not significantly deviate from those predicted by HWE ($p > 0.05$), except for ApaI in RA-OP subgroup where the deviation was significant ($p = 0.04$).

The distributions of VDR genotypes in RA patients and controls are shown in Table 1.

The obtained results showed significant differences in the distribution of FokI genotypes between RA patients and controls ($p < 0.001$, df = 2), as well as between RA-NBMD and RA-OP subgroups and controls ($p = 0.005$, df = 2 and $p < 0.001$, df = 2; respectively). Difference in the distribution of FokI genotypes between RA-OSTP subgroup and controls reached *p*-value of 0.05 (df = 2). An increased risk for RA and RA-OP is found in Ff (CT) and ff (TT) genotype carriers, while Ff (CT) alone was associated with increased risk for RA-NBMD and ff (TT) alone with increased risk for RA-OP when compared to healthy FF (CC) carriers (Table 1). Since the frequencies of ff (TT) genotype were low in RA subgroups and control group, testing was also performed using dominant model (FF vs. Ff + ff). The obtained results showed significant difference between RA patients and controls ($p < 0.001$, $\chi^2 = 13.534$, OR = 0.377, 95%CI = 0.223–0.638), as well as between all RA subgroups and controls (RA-NBMD vs. controls: $p = 0.035$, $\chi^2 = 4.457$, OR = 0.456, 95%CI = 0.218–0.952; RA-OSTP vs. controls: $p < 0.02$, $\chi^2 = 5.413$, OR = 0.463, 95%CI = 0.241–0.890; RA-OP vs. controls: $p = 0.001$, $\chi^2 = 15.339$, OR = 0.231, 95%CI = 0.108–0.493). However, no differences were observed between RA subgroups, indicating no association of FokI genotypes with BMD in RA patients. The distributions of BsmI, ApaI, and TaqI genotypes did not show differences between RA patients or RA subgroups compared to controls, as well as between RA subgroups (Table 1).

The allele distributions of the studied VDR variants are shown in Table 2.

FokI f (T) allele showed significantly higher prevalence in RA patients ($\chi^2 = 14.874$, $p < 0.001$) and RA subgroups (RA-NBMD vs. controls: $\chi^2 = 8.683$, $p = 0.003$; RA-OSTP vs. controls: $\chi^2 = 5.328$, $p = 0.021$; RA-OP vs. controls: $\chi^2 = 15.382$, $p < 0.001$) compared to healthy controls, but there was no difference between RA subgroups (Table 1). The distributions of BsmI, ApaI, and TaqI alleles did not reveal significant differences between the studied groups (Table 1).

Haplotype analysis showed difference in the distribution of BsmI/ApaI Ba (AC) haplotype in RA group ($p = 0.03$), as well as RA-OSTP ($p = 0.012$) and RA-OP ($p = 0.024$) subgroups compared to controls. However, after applying Bonferroni correction for multiple comparisons (cut-off value for statistical significance $p = 0.0125$), only the difference between RA-OSTP and controls remained significant (Table 2). Distribution of haplotypes that included FokI variants, all four studied VDR variants, or ApaI/TaqI haplotypes didn't show significant differences between the studied groups and subgroups (data not shown).

3.2. Correlation between VDR genotypes and inflammatory markers in RA patients

The values of inflammatory parameters did not differ significantly between RA subgroups (Table 3). However, when VDR variants were taken into consideration significantly higher ESR values were obtained in homozygous FokI FF (CC) carriers compared to heterozygous Ff (CT) carriers within the RA-NBMD subgroup (54.57 ± 23.73 vs. 22.22 ± 9.81; $p = 0.02$). Since only 3 patients in RA-NBMD group were carriers of ff (TT) genotype, the analysis was also performed by grouping f (T) allele containing genotypes (Ff (CT) + ff (TT)), which further increased significance between the groups (54.57 ± 23.73 vs. 22.83 ± 12.42; $p < 0.001$; Fig. 1).

4. Discussion

Although genetic factors are considered as important in the pathogenesis of RA and near 100 genetic loci were reported to be associated with this disease [14], an exact genetic pattern involved in RA development is still not clear. Elucidation of immunomodulatory and anti-inflammatory effects of vitamin D has led to intensive research of VDR genetic variants in association with different diseases, including RA. Among the most studied VDR variants are FokI, BsmI, ApaI and TaqI, showing diverse and conflicting results in different populations.

In this study, we examined for the first time a possible association of VDR variants with BMD (RA-NBMD, RA-OSTP and RA-OP) and inflammatory markers (ESR, fibrinogen and RF) in Serbian patients with RA. Our results showed significant differences in the distribution of FokI genotypes and alleles in RA patients and RA subgroups compared to healthy controls. The distribution of BsmI/ApaI Ba (AC) haplotype between RA-OSTP and healthy controls was significantly different. ESR was significantly higher in FF (CC) carriers of the RA-NBMD subgroup.

Results obtained in the previous studies in the Serbian population showed an association of FokI variant with bronchial asthma and juvenile idiopathic arthritis, but not with Graves' disease [12,13,15]. In bronchial asthma or juvenile idiopathic arthritis patients, susceptibility

to disease development was 2.2-fold higher in f (T) compared to F (C) allele carriers [12,13]. Similar results were also obtained in this study indicating 2.3-fold increased susceptibility to RA and RA-NBMD development in f (T) compared to F (C) allele carriers, as well as 1.9- and 3-fold increased susceptibility to RA-OSTP and RA-OP, respectively. Additionally, FF (CC) genotype was more frequent in healthy controls compared to RA patients and RA subgroups in the present study. However, in the French population FF genotype was more frequent in RA patients compared to healthy subjects [1]. Furthermore, the *meta*-analysis by Lee et al. showed a significant association of FF genotype with RA in the European population [16]. The association of FokI variant was also demonstrated in Tunisians [17] and North Americans [18], but not in German, Turkish and Egyptian populations [19–21].

The association of FokI and osteoporosis was previously reported in postmenopausal women of Asian origin [22], while no relation between FokI genotypes and BMD was found in European female population [23]. In the study by Masi et al., lower lumbar BMD, independent of corticosteroid therapy, was linked to FokI ff genotype in juvenile idiopathic arthritis patients [24], while Ff genotype was correlated with osteoporosis development in RA Egyptian patients [25]. However, no association between VDR genotypes and bone turnover was found in German RA patients [19], which is in accordance with the results obtained in our study that show no difference in genotype and allele distributions between RA subgroups. Since different environmental factors (e.g. exercise, smoking and alcohol consumption, metabolic syndrome, menopausal status in women), therapy (e.g. administration of glucocorticoids) and genetic factors (e.g. estrogen receptor, calcitonin receptor, collagen type I α 1 gene, vitamin D receptor) are implicated in BMD homeostasis, with potential influence on BMD and osteoporosis development in RA patients, additional studies that will include large number of factors and different populations are necessary to elucidate these mechanisms in RA.

Inconsistent results were also reported for BsmI, ApaI and TaqI VDR variants. Genotype bb was associated with less severe disease in the study by Gómez-Vaquero et al. [26]. Lower scores of DXA of the lumbar spine were detected in Bb carriers with RA [25], while BsmI B and TaqI t alleles were associated with an accelerated bone loss especially in female RA patients [27,28]. In the study by Quintana-Duque et al. BB and Bb genotypes were associated with lower 25(OH)D levels and susceptibility to more severe disease [29]. However, the study by Saad et al. revealed a protective effect of BsmI AA genotype and A allele, as well as TaqI CC genotype [21]. ApaI aa genotype was significantly associated with increased BMD in the femoral neck [22]. On the contrary, in the present study, we failed to prove any association of BsmI, ApaI and TaqI genotypes and alleles with RA, as well as with BMD in RA patients, which is in accordance with the results of several other studies [30,1,19] including the *meta*-analyses by Lee et al. and Song et al. [16,31].

Knowing that haplotype-based association analysis may give more powerful information than allele- or genotype-based one, some studies also included linkage disequilibrium analysis of BsmI, ApaI and TaqI variants. In relation to RA, BBtt haplotype was weakly associated with an early onset of disease in females [30], while our study revealed a significant association of BsmI/ApaI Ba (AC) haplotype with RA-OSTP, but not with RA-NBMD and RA-OP.

Available literature results still do not give a clear picture of the role of VDR variants in RA pathophysiology. The importance of studying these genetic variants in association with disease is closely related to the fact that some of them are functional, and thus, they can influence protein synthesis, as it is previously shown for FokI. This variant influences the type of VDR protein synthesized depending on the present allele in the first ATG site. Long (427 amino acids) and less active form is synthesized in the presence of f (T), while 3 amino acids shorter (423 amino acids) and more active form is synthesized in the presence of F (C) allele. It is associated with a higher affinity for $1,25(OH)_2D_3$ binding and higher transcriptional activity [10]. In line with this is lower VDR expression after the exposure to calcitriol in the presence of f allele [32]. These data support the results obtained in our study showing the association of f (T) allele with increased susceptibility to RA. Additionally, a significantly higher presence of F (C) allele in healthy subjects compared to RA patients may partially be explained by the hypothesis of evolutionary adaptation in humans related to the higher affinity of shorter VDR protein to $1,25(OH)_2D_3$ and higher transcriptional activity in the presence of FF genotype. Intron 8 variants (BsmI and ApaI) and silent base change within the exon 9 (TaqI) are not affecting the structure of VDR protein. However, BsmI and ApaI were extensively studied due to their potential influence on mRNA stability, but the previous study by Durrin et al. failed to confirm this assumption [33]. It was suggested that homozygosity for ApaI a and TaqI t alleles was associated with the lower mRNA levels [34], while the study by Uitterlinden et al. showed an association of Bat haplotype with higher mRNA expression in comparison to baT haplotype [10]. Since the results remain inconclusive, further studies are needed to explain the potential role of intron 8 and exon 9 variants in VDR signaling, as well as their clear association with diseases.

Since the main biological effects of vitamin D are the result of its interaction with VDR, it is assumed that VDR genetic variants may also have influence on inflammatory markers in RA. The associations of BsmI bb genotype, as well as b allele, with higher RF titer and increased disease severity, are previously shown by Rass et al. and Ranganathan [7,35]. Mosaad et al. also observed increased levels of RF, CRP and anti-CCP in ApaI aa carriers compared to AA or Aa carriers [25]. In our study, no association of BsmI, ApaI and TaqI VDR variants with inflammatory markers (RF, ESR and fibrinogen) was observed. However, we showed significantly higher ESR levels in RA-NBMD patients with FokI FF (CC) genotype in comparison to f (T) allele containing genotypes (Ff (CT) + ff (TT)). Similarly, an association of FokI with ESR was previously observed in male patients with ankylosing spondylitis [36]. Even if we showed a protective effect of F (C) allele, surprisingly, significantly higher ESR levels were observed in FF (CC) carriers within the RA-NBMD subgroup. The ESR is an inflammatory marker that is widely used in disease activity monitoring in RA. However, its values are influenced by different physiological and environmental factors (age, sex, anemia, stage of menstrual cycle in women, etc.), including a concentration of other acute-phase proteins, mainly fibrinogen [37]. Although a strong positive correlation was observed between ESR and fibrinogen values in the RA-NBMD group, there was no association between FokI genotypes and fibrinogen levels. Additionally, some previous studies showed an association between low vitamin D levels and increased values of inflammatory markers and disease activity [38,39]. Since vitamin D deficiency is common among RA patients, and in our population [40], we assume that increased ESR levels observed in this study might be rather influenced by vitamin D status than the genetic variant itself. However, since the vitamin D status of patients included in the study was not determined at the time of disease diagnosis, it is not included in the analyses.

Limitations of this study are related to a limited number of inflammatory markers used in the study and deviation of ApaI from HWE in the RA-OP subgroup, likely influenced by a subject selection. However, to the best of our knowledge, this is the first study that examined the association of VDR variants (FokI, BsmI, ApaI, and TaqI) in relation to BMD and inflammatory markers in Serbian patients with RA, thus, contributing to more comprehensive research on VDR variants in RA.

Although an extensive research has been made in the past in order to explain the role of VDR genetic variants and their association with different complex diseases, including RA, the exact genetic patterns are still not clear. Great discrepancies in available results might be influenced by different ethnicity of the subjects involved in these studies, as well as by clinical heterogeneity, study design, or relatively small sample size. Differences in study design and clinical parameters evaluated in these studies resulted in a limited number of studies included in available *meta*-analyses which further contributed to the inconsistency of obtained results. Therefore, further studies of a similar design,

performed on larger sample size are needed to explain the association of VDR variants with RA and the specific mechanisms involved in this association, as well as to elucidate interindividual differences, thus leading to a better understanding of vitamin D signaling in RA pathogenesis.

5. Conclusions

The results obtained in this study indicate an association of RA with VDR FokI variant and increased susceptibility to disease in f (T) allele carriers, as well as to RA with associated osteopenia in BsmI/ApaI Ba (AC) haplotype carriers.

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