ISSN 1814-6023 (Print)
ISSN 2524-2350 (Online)
UDC 616.71-007.234-056.7
https://doi.org/10.29235/1814-6023-2020-17-4-480-492 Received 03.06.2020

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VITAMIN D RECEPTOR GENE POLYMORPHISM, BONE MINERAL DENSITY AND 25(OH)D LEVEL IN WOMEN WITH OSTEOPOROSIS

Abstract. Vitamin D plays an important role in bone metabolism and pathology. Although the *VDR* gene is one of the most studied determinants of bone mineral density (BMD) and osteoporosis (OP), its exact effects have yet to be established. Prediction of OP and/or fracture risk, based on individual genetic profile, is of high importance. The aim of our study was to develop prognostic model for postmenopausal OP individual risk evaluation in Belarusian women, based on the analysis of *VDR* gene variants.

Case group included women with postmenopausal OP (n = 350), the control group comprised of women with normal BMD and without previous fragility fractures (n = 243). *VDR* gene ApaI rs7975232, BsmI rs1544410, TaqI rs731236, FokI rs2228570 and Cdx2 rs11568820 variants were determined using TaqMan genotyping assays.

We revealed a significant association of single ApaI A/A (p = 0.045), BsmI T/T (p = 0.015) and TaqI G/G (p = 0.005) variants and their A-T-G-haplotype (OR = 4.6, p = 0.003) with increased OP risk. Together with Cdx2 rs11568820, these variants correlated with BMD ($p < 0.05$ in all cases). For the bearers of non-favorable alleles of *VDR* gene variants, the serum 25(OH)D level was significantly increased. The constructed from informative *VDR* gene variants model of individual OP risk evaluation possessed a good prognostic value (AUC = 0.79) with high sensitivity level (82.9 %) and average specificity (69.4 %). Our findings highlight the importance of analyzed *VDR* gene variants for personalized OP risk prediction.

Keywords: vitamin D, genetic risk, predictive model, gene variants, haplotype, postmenopausal osteoporosis

For citation: Rudenka A. V., Rudenka E. V., Samokhovec V. Yu., Kobets K. V., Marozik P. M. Vitamin D receptor gene polymorphism, bone mineral density and 25(OH)D level in women with osteoporosis. *Vestsi Natsyyanal'nai akademii navuk Belarusi. Seriya meditsinskikh navuk = Proceedings of the National Academy of Sciences of Belarus. Medical series*, 2020, vol. 17, no. 4, pp. 480–492. https://doi.org/10.29235/1814-6023-2020-17-4-480-492

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СВЯЗЬ ПОЛИМОРФИЗМА ГЕНА РЕЦЕПТОРА ВИТАМИНА D С ПОКАЗАТЕЛЯМИ МИНЕРАЛЬНОЙ ПЛОТНОСТИ КОСТНОЙ ТКАНИ И УРОВНЕМ 25(OH)D У ЖЕНЩИН С ОСТЕОПОРОЗОМ

Аннотация. Витамин D является секостероидным гормоном, который реализует свои многочисленные клеточные эффекты, инициируя транскрипцию витамин D-зависимых генов. Несмотря на большое количество проведенных исследований, точная роль вариантов гена *VDR* с уровнем минеральной плотности костей (МПК) и риском остеопороза (ОП) до сих пор не установлена. Разработка и внедрение методики прогнозирования индивидуального риска ОП и/или костных переломов на основании генетического тестирования имеет большое значение для своевременной профилактики заболевания. Целью настоящего исследования являлась разработка прогностической модели оценки индивидуального риска развития ОП у женщин Беларуси, включающей анализ полиморфных вариантов гена *VDR*.

В исследование были включены женщины в постменопаузе с ОП (n = 350), контрольную группу составили женщины с нормальной МПК, без низкотравматических переломов (n = 243). Генотипирование вариантов ApaI rs7975232, BsmI rs1544410, TaqI rs731236, FokI rs2228570 и Cdx2 rs11568820 гена *VDR* осуществляли методом ПЦР с использованием проб TaqMan.

По результатам исследования была выявлена значимая ассоциация генотипов ApaI A/A (p = 0,045), BsmI T/T (p = 0,015) и TaqI G/G (p = 0,005), а также их гаплотипа A-T-G (OR = 4,6; p = 0,003) с повышенным риском ОП. Эти же

варианты, а также Cdx2 rs11568820 коррелировали с уровнем МПК ($p < 0,05$ во всех случаях). Показано, что среди носителей неблагоприятных аллелей исследованных вариантов гена *VDR* уровень 25(OH)D в сыворотке был существенно повышен ($\beta = 4,1$; $p = 0,007$). Построенная из информативных вариантов гена *VDR* модель оценки индивидуального риска ОП имела хорошую прогностическую ценность (AUC = 0,79) с высоким уровнем чувствительности (82,9 %) и средней специфичностью (69,4 %). Полученные нами результаты подчеркивают важность проанализированных вариантов гена *VDR* для прогнозирования индивидуального риска ОП.

Ключевые слова: витамин D, генетический риск, прогнозирующая модель, варианты гена, гаплотип, постменопаузальный остеопороз

Для цитирования: Связь полиморфизма гена рецептора витамина D с показателями минеральной плотности костной ткани и уровнем 25(OH)D у женщин с остеопорозом / Е. В. Руденко [и др.] // Вес. Нац. акад. навук Беларусі. Сер. мед. навук. – 2020. – Т. 17, № 4. – С. 480–492 (*на англ.*). https://doi.org/10.29235/1814-6023-2020-17-4-480-492

Introduction. Vitamin D is a secosteroid hormone that implements its numerous cellular effects by initiating transcription of vitamin D-dependent genes. Studies of the last two decades have established that diverse biological effects of the active metabolite of vitamin D-1.25-dihydroxyvitamin D (calcitriol) are carried out by modulating the expression of genes that are mediated by interaction with the intracellular vitamin D receptor (VDR) [1]. Activation of VDR through direct interaction with $1.25(OH)_2D$ causes fast binding of the receptor to the regulatory regions of target genes, which initiates the transcription and synthesis of new mRNA molecules, translation of mRNA, synthesis of new proteins and the implementation of specific biological reactions. These reactions are specific to various tissues and range from very complex mechanisms necessary for homeostatic control of mineral metabolism to focal effects that regulate growth, differentiation, proliferation, apoptosis, adaptive and innate immune responses, and the functional activity of many types of cells [2]. One of the main functions of vitamin D is the regulation of calcium-phosphorus metabolism in the intestine and kidneys. Calcitriol promotes active cellular absorption and transport of calcium in intestine, in renal tubules it controls its own homeostasis (suppression of 1-α-hydroxylase and 24-hydroxylase stimulation), potentiates the effects of parathyroid hormone (PTH) on calcium reabsorption, and induces trans epithelial calcium transfer [1]. By binding to VDR, which is present in all types of bone cells – osteoblasts, osteocytes and osteoclasts, calcitriol has a direct effect on bone metabolism [1].

VDR is the product of the corresponding gene, the *VDR* gene, which determines its structure and functional activity. The human *VDR* gene is located on the 12th chromosome (12q12-14) and consists of 14 exons spanning about 75 kb: eight protein-coding exons (2-9), six untranslated exons (1A-1F), located on the non-coding 5′ region, and several promoter regions – DNA sequences recognized by RNA polymerase as a launching pad for the initiation of specific transcription [3]. Several substitutions in the *VDR* gene were identified, which are single nucleotide variations (SNVs). Most variants in the *VDR* gene are found in regulatory regions, such as the promoter region and the 3′-untranslated region. Changes in the regulatory region of the gene can determine the amino acid sequence of the synthesized protein and lead to functional effects such as a change in ligand affinity or DNA binding [3].

Cdx2 G-to-A (alternatively C/T, rs11568820) substitution is located in the promoter region 1e of the *VDR* gene. It is suggested that this site plays an important role in the specific transcription of the *VDR* gene in the intestine and determines the regulatory role of vitamin D in intestinal calcium absorption. Some studies have demonstrated that A-allele has greater transcriptional activity than the G-allele [4]. Allele A causes increased intestinal expression of VDR and can increase the transcription of calcium-transporting proteins, such as calbindin, channel-forming proteins of the transient receptor potential (TRP) protein superfamily, the most selective for calcium – TRPV5, TRPV6 [5]. Thus, the presence of A-allele contributes to increased absorption of calcium in the intestine and can contribute to gain in BMD. The *VDR* FokI variant (rs2228570, c.2T(A, f) > C(G, F), p.Met1Arg) is located in the coding region of the *VDR* gene (exon 2) and leads to an alternative transcription initiation site due to the replacement of thymine (T) with cytosine (C) and affects the activity of the receptor, which depends on the length of the amino acid sequence: the protein synthesized by the F allele (ACG variant) is three amino acids shorter than the product of the f allele (ATG variant) and is 1.7 times more active [6]. It was found that Caucasian postmenopausal women with FokI F/f-genotype demonstrated lower BMD compared to bearers of F/F-homozygotes [7]. The *VDR* gene BsmI and ApaI (in intron 8) and TaqI (in exon 9) variants are located at the 3′-untranslated end and are in close linkage disequilibrium (LD). These SNVs do not alter

the amino acid sequence of the encoded protein but influence gene expression, regulating mRNA stability [8]. The BsmI (rs1544410, 1024+443C>T, alternatively b>B) and TaqI (rs731236, c.1056A>G, alternatively T>t) haplotype frequencies are associated with an increased level of VDR receptor. ApaI (rs7975232, c.1025-49C>A, or a>A) variant has also been shown to be associated with the activity or expression of VDR. Though the results of the multiple studies on *VDR* gene variants association with osteoporosis (OP) risk are controversial, some studies on different populations revealed their association with BMD [9–15].

The results of the above studies indicate the important role of the vitamin D-endocrine system in the regulation of bone metabolism, though many issues in this area are not fully understood. Determination of *VDR* gene polymorphisms associated with low BMD will allow to identify individuals with high hereditary risk of early reduction in BMD long before the development of OP, and thus to conduct timely set of preventive measures in target risk groups, and also to evaluate effectiveness of therapy [16].

The aim of our study was to develop prognostic model for postmenopausal osteoporosis (PMO) risk evaluation in Belarusian women, based on the analysis of *VDR* gene variants.

Materials and methods. This study was a cross-sectional cohort study, conducted at out-patient department and inpatient clinic. Patients were recruited at Minsk City Center for Osteoporosis and Bone-Muscular Diseases Prevention and rheumatologic department of 1st Minsk city clinic (Minsk, Belarus). The study protocol was approved by the Local Research Ethics Committee of Belarusian Medical Academy of Postgraduate Education. White Caucasian women were screened for participation. Inclusion criteria were: wiliness to participate in the study, female sex, established diagnosis of OP according to WHO Diagnostic Criteria [17]. Exclusion criteria: presence of other metabolic bone diseases (such as Paget's disease and osteomalacia), diseases, affecting bone metabolism (such as endocrine osteopathies, renal failure, Cron's disease, rheumatic diseases etc.), malignant tumors, using of medications likely to influence BMD (except bisphosphonates, calcium and vitamin D supplementation). After assessing compliance with inclusion and exclusion criteria all the enrolled women signed written informed consent for participation in the study in accordance with the declaration of Helsinki (as revised in 2013). Participants of the study have filled out questionnaires to identify risk factors for OP (age of menopause, history of fractures etc.).

BMD was evaluated by DEXA (GE Lunar, Madison, WI, USA). Calibration of the devise was performed daily using a standard spine phantom provided by the manufacturer. Lumbar spine (LS, L1–L4) and femoral neck (FN) BMD (g/cm^2) was measured on the same machine. Diagnosis of OP was established on the basis of T-criteria for Caucasian women [17].

Determination of serum total vitamin D (25(OH)D) concentration was performed by electrochemiluminescence immunoassay on the Cobas e411 analyzer (Roche Diagnostic), fasting blood samples were obtained from the cubital vein in the morning, not earlier than 10–12 hours after the last meal, into a sterile vacuum Vacutainer tube without additives. In accordance with international recommendations, level of vitamin D was considered appropriate at 25(OH)D value > 30 ng/ml, deficiency was diagnosed at rates of 20–30 ng/ml, 25(OH)D concentration less than 20 ng/ml was considered as vitamin D deficiency [18–20].

For genetic analysis, venous blood samples were taken from the cubital vein using the Vacutainer system with EDTA (Beckton-Dickinson, USA). DNA was isolated using the standard phenol-chloroform extraction. The quantity of DNA samples was checked using Qubit 2 Fluorimeter (Thermo Scientific, USA), the quality and purity were checked using NanoDrop 8000 spectrophotometer (Thermo Scientific, USA). Information on *VDR* gene variants was obtained from Entrez Gene database (www.ncbi.nlm.nih.gov/gene). SNVs were selected according to the following criteria: minor allele frequency (MAF) higher than 5 %; and reported associations with BMD or BMD-related risk factors in previous studies. Selected variants (ApaI rs7975232, BsmI rs1544410, TaqI rs731236, FokI rs2228570 and Cdx2 rs11568820) were determined using the quantitative polymerase chain reaction (PCR) with TaqMan Probes (Thermo Fisher Scientific, USA) in the CFX96™ Real-Time PCR Detection Systems (Bio-Rad©, USA) as previously described [21, 22]. The whole reacting volume in PCR tubes was 10 μL, including 5 μL iTaq™ Universal Probes Supermix BioRad©, 3.75 μL of mQ water, 0.25 μL×40 TaqMan™ SNP Genotyping Assay, 1 μL of genomic DNA (15 ng). The reactions were performed with an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and synthesis at 60 °C for 30 s. The final extension was performed at 72 °C for 1 min. The genotypes were analyzed on the basis of the melting curve Negative and positive

controls were randomly included across each PCR run, several samples were randomly re-genotyped for quality control purposes.

Statistical analysis was performed using the programming language R. The data was presented as median (25 %, 75 % interquartile range) and compared using Mann–Whitney *U*-test. The deviation from Hardy-Weinberg equilibrium was assessed by the chi-square (χ^2) test. The genetic risk of OP was estimated using odds ratios, with 95 % confidence intervals (CI) and calculated in comparison to reference (major homozygous) genotype. Codominant model was defined and tested for all SNVs. Logistic regression models were used to assess difference between the characteristics of analyzed groups for categorical data and for comparison of genotype frequencies between these groups. Multivariate Linear Regression model was used to adjust for confounding factors, such as age at menopause, weight, height, BMI, BMD, and for analysis of genotypes association with quantitative traits. Beta (β) measures difference in quantitative trait between genotypes. Pairwise linkage disequilibrium (LD) and haplotype analysis were performed using the R-packages "haplo.stats" (v.1.7.9) and "SNPassoc" (v.1.9-2), the programs used likelihood ratio tests in a generalized linear model and the expectation-maximization algorithm. The efficacy of designed model for predicting risk of disease was analyzed using multivariate logistic regression with the construction of ROC-curve (Receiver Operating Characteristics) using R-package "pROC" (v.1.16.2). The differences between the groups were considered statistically significant at $p < 0.05$. *p*-values corrected for multiple testing using the False Discovery Rate (FDR) with Benjamini and Hochberg procedure ($n = 5$, multiple comparisons).

Results and discussion. Genomic DNA was extracted from blood samples of 350 patients with PMO and 243 individuals from control group. The participants within both groups were matched for age and sex – no statistically significant differences were found (Tab. 1).

T a b l e 1. **Clinical characteristics of study subjects**

Clinical characteristic	Patients with PMO	Control	*p*-value
Number (%)	350 (59.1)	243 (40.9)	–
Age, years	62.8 (57.1; 69.4)	62.2 (58.2; 68.0)	0.61
Age at menopause, years	48.9 (47.0; 52.0)	50.3 (48.0; 53.0)	0.03
Weight, kg	66.4 (57.8; 74.0)	80.8 (72.0; 91.0)	<0.0001
Height, cm	160.2 (155.0; 165.0)	161.5 (157.0; 166.0)	0.04
BMI	25.9 (22.5; 28.8)	31.1 (28.0; 34.5)	<0.0001
Baseline LS BMD, g/cm^2	0.87 (0.79; 0.94)	1.28 (0.17; 1.35)	<0.0001
Baseline LS T-score	–2.5 (–3.2; –2.0)	0.7 (–0.2; 1.1)	<0.0001
Baseline LS Z-score	–1.1 (–1.7; –0.5)	1.38 (0.5; 2.1)	<0.0001
Baseline FN BMD, g/cm^2	0.79 (0.72; 0.87)	1.1 (0.99; 1.12)	<0.0001
Baseline FN T-score	–1.7 (–2.4; –1.1)	0.6 (–0.1; 1.0)	<0.0001
Baseline FN Z-score	–0.7 (–1.2; –0.1)	1.1 (0.5; 1.7)	<0.0001
Fractures in history	46 (13.1 %)	5 (2.1 %)	<0.0001
β-CrossLaps (β-CTx), ng/ml	2.6 (0.3; 0.5)	0.9 (0.2; 0.5)	0.009
Osteocalcin (BGLAP), ng/ml	26.0 (16.3; 28.8)	30.4 (19.3; 34.1)	0.46
Parathyroid hormone (PTH), pg/ml	54.5 (33.6; 69.0)	48.3 (32.9; 56.2)	0.63
25-hydroxyvitamin D (25(OH)D), ng/ml	29.8 (20.7; 36.8)	20.5 (15.4; 25.2)	$2.3 \cdot 10^{-7}$

N o t e. The data is presented as mean (25 %; 75 % interquartile range). LS, lumbar spine, FN, femoral neck, BMD, bone mineral density, BMI, bone mass index.

The comparison of PMO group with control group using Mann–Whitney *U*-test revealed a slightly significant difference in age at menopause ($p = 0.03$) and height ($p = 0.04$). A strong difference between groups was revealed for weight, BMI, lumbar spine and femoral neck BMD, T- and Z-scores. These factors were considered to potential confounding factors and were adjusted in association analysis. In the study cohort, 46 patients had a fracture history (at least one), compared to 5 individuals from control group. There was no statistically significant difference in osteocalcin and parathormone levels between analyzed groups. However, we did find differences in β-CTx and 25(OH)D levels (Tab. 1).

Five the most commonly analysed polymorphic loci of *VDR* gene were selected from key publications and studied as candidate markers of PMO. These SNVs with previously established involvement in vitamin D and bone tissue metabolisms were included to the study to validate their effect by analysis of combinations of genetic variants on independent cohort. A detailed description of the *VDR* gene variants and their frequencies in control population is presented in Tab. 2.

T a b l e 2. **SNV information of VDR gene in control population**

SNV	Placements GRCh38.p12	Minor allele	MAF	GnomAD MAF	Heterozygosity		HWE *p*-value
					observed	predicted	
rs7975232	g.47845054 C>A	C	0.45	0.47	0.47	0.49	0.44
rs1544410	g.47846052 C>T	T	0.41	0.38	0.44	0.48	0.23
rs731236	g.47844974 A>G	G	0.40	0.38	0.44	0.48	0.14
rs2228570	g.47879112 A>G	A	0.42	0.37	0.51	0.49	1
rs11568820	g.47908762 C>T	T	0.19	0.18	0.29	0.31	0.5

N o t e. MAF – minor allele frequency, HWE – Hardy-Weinberg equilibrium.

The genotype frequencies of all analyzed SNVs were not significantly different from Hardy-Weinberg equilibrium at 5 % level in control group (Tab. 2) and in PMO patients ($p > 0.05$). MAF in control group was calculated and were not significantly different from those taken from to GnomAD data (gnomad.broadinstitute.org) and were close to those of Caucasian subjects reported meta-analysis [7].

All patients were genotyped in the study. The distribution of genotype frequencies of *VDR* gene variants together with the *p*-values are shown in Tab. 3.

T a b l e 3. **The distribution of genotype frequencies of VDR gene variants in patients with postmenopausal osteoporosis (PMO) and control (CON) groups**

Gene variant	Genotype	PMO, %	CON, %	OR (95 % CI)	*p*-value	
					Raw*	FDR**
VDR ApaI rs7975232	C/C	23.8	31.3	1	**0.027**	**0.045**
	C/A	45.1	46.9	1.3 (0.8–1.9)		
	A/A	31.1	21.8	**1.9 (1.2–3.0)**		
VDR BsmI rs1544410	C/C	23.3	37.0	1	**0.006**	**0.015**
	C/T	46.6	44.4	**1.7 (1.1–2.5)**		
	T/T	30.1	18.5	**2.6 (1.6–4.2)**		
VDR TaqI rs731236	A/A	24.4	37.9	1	**0.001**	**0.005**
	A/G	47.8	46.6	**1.7 (1.2–2.5)**		
	G/G	27.9	18.5	**2.3 (1.5–3.7)**		
VDR FokI rs2228570	G/G	26.6	32.3	1	0.45	0.45
	A/G	49.7	50.8	4.5 (2.4–8.7)		
	A/A	23.7	16.9	29.3 (3.6–241.0)		
VDR Cdx2 rs11568820***	C/C	70.7	66.2	1	0.3	0.375
	C/T + T/T	29.3	33.8	0.8 (0.6–1.2)		

N o t e. * – raw *p*-values; ** – *p*-values corrected for multiple testing using the False Discovery Rate ($n = 5$); *** – dominant model of inheritance used due to low minor allele frequency. OR – odds ratio, CI – confidence interval.

The most frequent homozygous genotype was taken for reference. Comparing the genotype frequencies between PMO and CON groups, statistically significant differences after FDR correction for multiple testing were observed for ApaI rs7975232, BsmI rs1544410 and TaqI rs731236 variants of *VDR* gene. The BsmI T/T genotype was significantly over-represented in PMO patients (30.1 %) compared to control

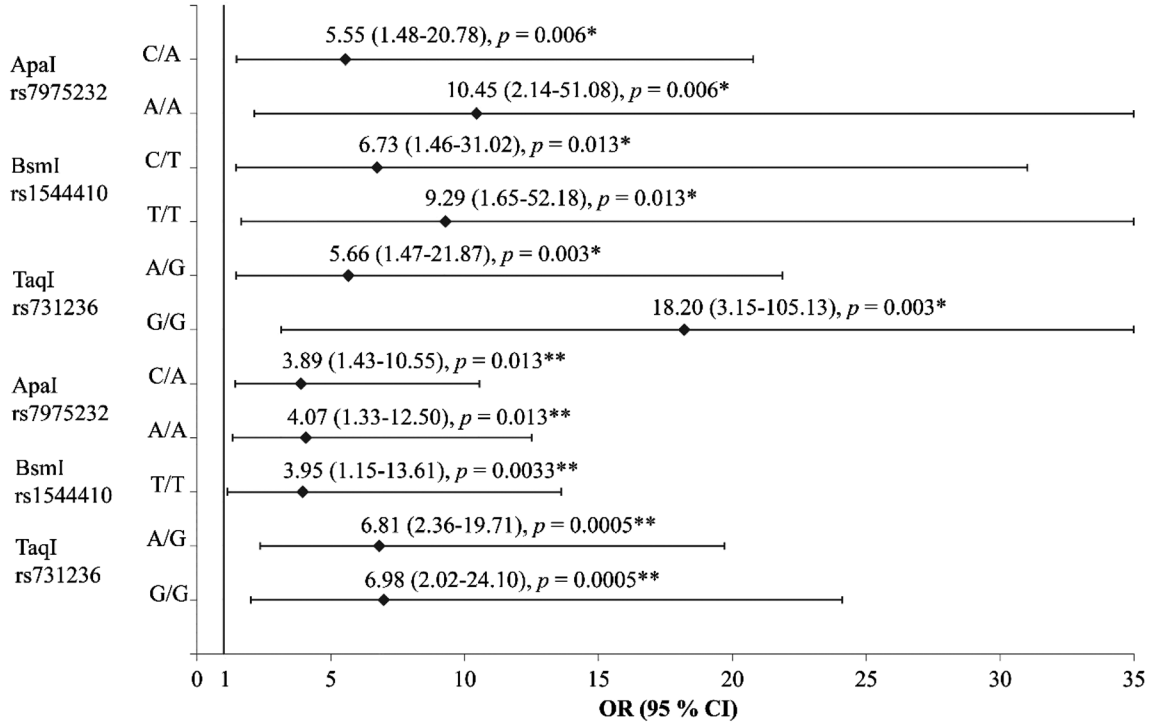

Fig. 1. Significant distribution of specified genotypes of ApaI, BsmI and TaqI variants of *VDR* gene between women with PMO and control group, adjusted by age at menopause, height, weight and (*) LS or (**) FN BMD. *p*-values corrected for multiple testing using the FDR (n = 5, multiple comparisons)

group (18.5 %, OR = 2.6, 95 % CI 1.6–4.2, p_{FDR} = 0.015). For the bearers of TaqI rs731236 G/G homozygous genotype, the risk of osteoporosis was increased (OR = 2.3, 95 % CI 1.5–3.7, p_{FDR} = 0.005). Increased risk of PMO was also revealed for the bearers of heterozygous genotypes of *VDR* BsmI and TaqI variants. The PMO group individuals were more likely to carry *VDR* ApaI A/A genotype (31.1 %), compared to the CON group (21.8 %, OR = 1.9, 95 % CI 1.2–3.0, p_{FDR} = 0.045).

As mentioned above, between the study groups there were significant differences in some clinical characteristics revealed, including age at menopause, height, weight, BMI, LS and FN BMD and others (Tab. 1). To reduce the potential impact of these confounding factors on the results of the analysis, we performed logistic analysis using codominant model of inheritance, adjusted by age at menopause, height, weight, LS and FN BMD. Revealed significant association for adjusted analysis is presented in a forest plot (Fig. 1).

When adjusted by confounding factors, the risk of PMO became much higher for the bearers of ApaI A/A (OR = 10.5, 95 % CI 2.1–51.1, p_{FDR} = 0.006), BsmI T/T (OR = 9.3, 95 % CI 1.7–52.2, p_{FDR} = 0.013) and TaqI G/G (OR = 18.2, 95 % CI 3.2–105.1, p_{FDR} = 0.003) genotypes. In addition, increased risk for PMO was also revealed for the bearers of heterozygous ApaI C/A genotype, which was absent in non-adjusted analysis.

There was no any statistically significant difference revealed in distribution of *VDR* FokI rs2228570 and Cdx2 rs11568820 variants between PMO and CON groups. Since Cdx2 rs11568820 T-allele frequency was very low, we used dominant model of inheritance and merged C/T+T/T genotypes. Despite the absence of significant association, it can be noted that the frequency of Cdx2 AA-genotype is significantly higher in the CON group (4.4 %) compared to PMO patients (1.1 %).

The sample size calculation for separately analyzed genotype frequencies was performed for a significance level of 5 %, post-hoc power value, calculated for *VDR* ApaI rs7975232, BsmI rs1544410, TaqI rs731236, FokI rs2228570 and Cdx2 rs11568820, were 75.2, 82.3, 82.4, 58.3, 24.6 %, respectively.

In a further work, the pairwise linkage disequilibrium between the SNVs within *VDR* gene was estimated in terms of D' and r^2. LD plot was constructed using combined genotype data from both groups of individuals (Fig. 2).

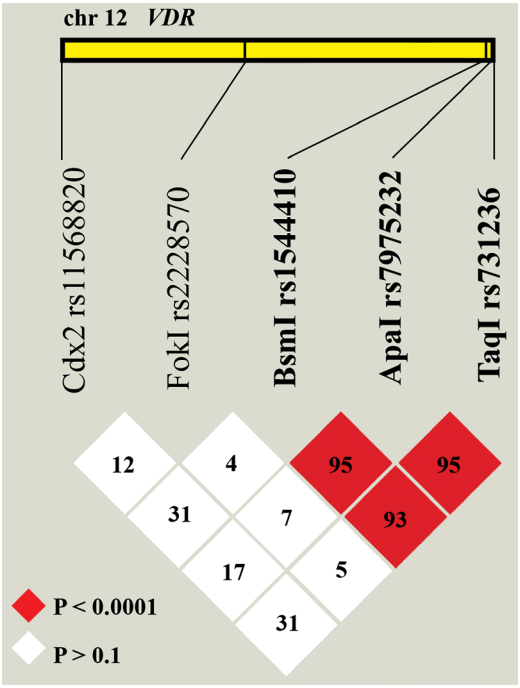

Fig. 2. Linkage disequilibrium plot for ApaI rs7975232, BsmI rs1544410, TaqI rs731236, FokI rs2228570 and Cdx2 rs11568820 of *VDR* gene. LD is displayed as pairwise D' values multiplied by 100 and given for each SNV combination within each cell. Red cells correspond to a very strong LD; ApaI, BsmI and TaqI variants are in the same LD block

By the LD analysis, we identified one haplotype block, composed of ApaI, BsmI and TaqI variants (the measure D' was very close to 1, $p << 0.01$). The positive coefficient of correlation r^2 suggests that major alleles of *VDR* ApaI, BsmI and TaqI gene variants are likely to be inherited together, as well as minor alleles. No significant LD was found for Cdx2 and FokI variants. For further analysis, based on LD data, we combined three *VDR* gene variants ApaI, BsmI, TaqI and performed the haplotype analysis (Tab. 4). FokI and Cdx2 variants were removed from further analysis due to the absence of significant association. Haplotypes were constructed from all possible allelic combinations of three *VDR* markers and compared between the PMO and CON groups, adjusted by age at menopause, weight, height and LS BMD.

Six haplotypes (C-C-A, A-T-G, A-T-A, C-C-G, A-C-A, A-C-G) of the possible eight combinations were inferred at a frequency greater than 1 %. They were present in 97.7 % of study participants. Statistically significant differences between analyzed groups were revealed in the global distribution of allelic combinations (global *p*-value <0.0001), suggesting an association of analyzed haplotypes with the risk of PMO. Statistically significant difference was revealed in distribution of most frequent C-C-A and A-T-G haplotypes between the PMO and control groups even after FDR correction for multiple testing (Tab. 4). The C-C-A haplotype, constructed from three wild-type alleles, was the most frequent (total frequency 40.7 %). This haplotype frequency was significantly higher among controls (46.8 %) than among cases (36.0 %, $p_{FDR} = 0.049$). The negative haplotype score value of –3.39 suggested that this combination is associated with decreased risk of PMO. The total frequency of A-T-G haplotype was 36.6 %, it was significantly under-represented in CON group (31.7 %) compared to PMO group (40.0 %, $p_{FDR} = 0.008$), suggesting that this allelic combination might confers a greater susceptibility to OP. Compared to the most frequent reference (wild-type) haplotype C-C-A, for the bearers of A-T-G haplotype, the risk of PMO was significantly increased (OR = 4.6, 95 % CI 1.7–12.5, $p = 0.003$, haplotype score 3.06). No significant association was found for other revealed haplotypes. A similar picture was observed for FN BMD association with haplotypes (the data is not presented).

Table 4. **Haplotype analysis of VDR gene ApaI, BsmI and TaqI variants in patients with postmenopausal osteoporosis (PMO) and control (CON) groups, adjusted by age at menopause, weight, height and LS BMD**

Haplo-type	Frequency		*p*-value		Haplotype score	Logistic regression		Global score test
	PMO	CON	Raw	FDR		OR (95 % CI)	*p*-value	
C-C-A	36.0	46.8	**0.0007**	**0.049**	–3.39	1	–	Global-stat = 18.75, df = 7, $p = 0.009$
A-T-G	40.0	31.7	**0.0022**	**0.008**	3.06	**4.6 (1.7–12.5)**	**0.003**	
A-T-A	10.5	8.2	0.42	0.74	0.81	1.9 (0.4–9.2)	0.44	
C-C-G	7.1	9.1	0.89	0.89	1.06	1.8 (0.1–25.6)	0.68	
A-C-A	3.8	1.9	0.67	0.78	–1.47	1.9 (0.1–76.9)	0.75	
A-C-G	1.6	1.2	0.55	0.77	–0.59	0.4 (0.1–5.8)	0.49	
rare	1.0	1.1	0.14	0.33	–0.43	2.81 (0.8–9.7)	0.54	

A haplotype is an allelic combination of several gene variants, that are in a strong LD and inherited together. Haplotypes distribution analysis is very important for investigating the genetic aspects of multifactorial diseases, when the power of separate gene analysis is very weak due to correction for multiple testing. Thus, determination of association between genetic polymorphism and phenotype based on haplotype

score seems more critical compared to genotypes and alleles analysis, as it helps to reduce the impact of type I errors (false positive).

Previous studies on Caucasian women with PMO reported that ApaI-BsmI-TaqI A-T-G haplotype was significantly associated with similarly increased risk of OP (OR = 4.2, 95 % CI 2.2–8.1, $p < 0.001$) [23]. Very close haplotype frequency distribution was reported for Dutch [9] and Italian [14] women. However, relatively few studies have been reported to date with significant association of *VDR* gene haplotypes association with PMO risk in Caucasian women.

The analysis of genotype and haplotype distribution between patients with PMO and control group allowed us to identify several informative markers within *VDR* gene, associated with the risk of pathology. A huge interest presents analysis of association of *VDR* gene variants with the level of BMD in lumbar spine and femoral neck, as fractures of this regions are the main clinical manifestation of osteoporosis. The association analysis of quantitative data is usually more objective, will complement qualitative studies and possibly provide with new informative genetic markers. The association analysis between ApaI rs7975232, BsmI rs1544410, TaqI rs731236, FokI rs2228570 and Cdx2 rs11568820 variants of *VDR* gene and BMD level was performed using linear regression on the combined PMO and control groups (Fig. 3).

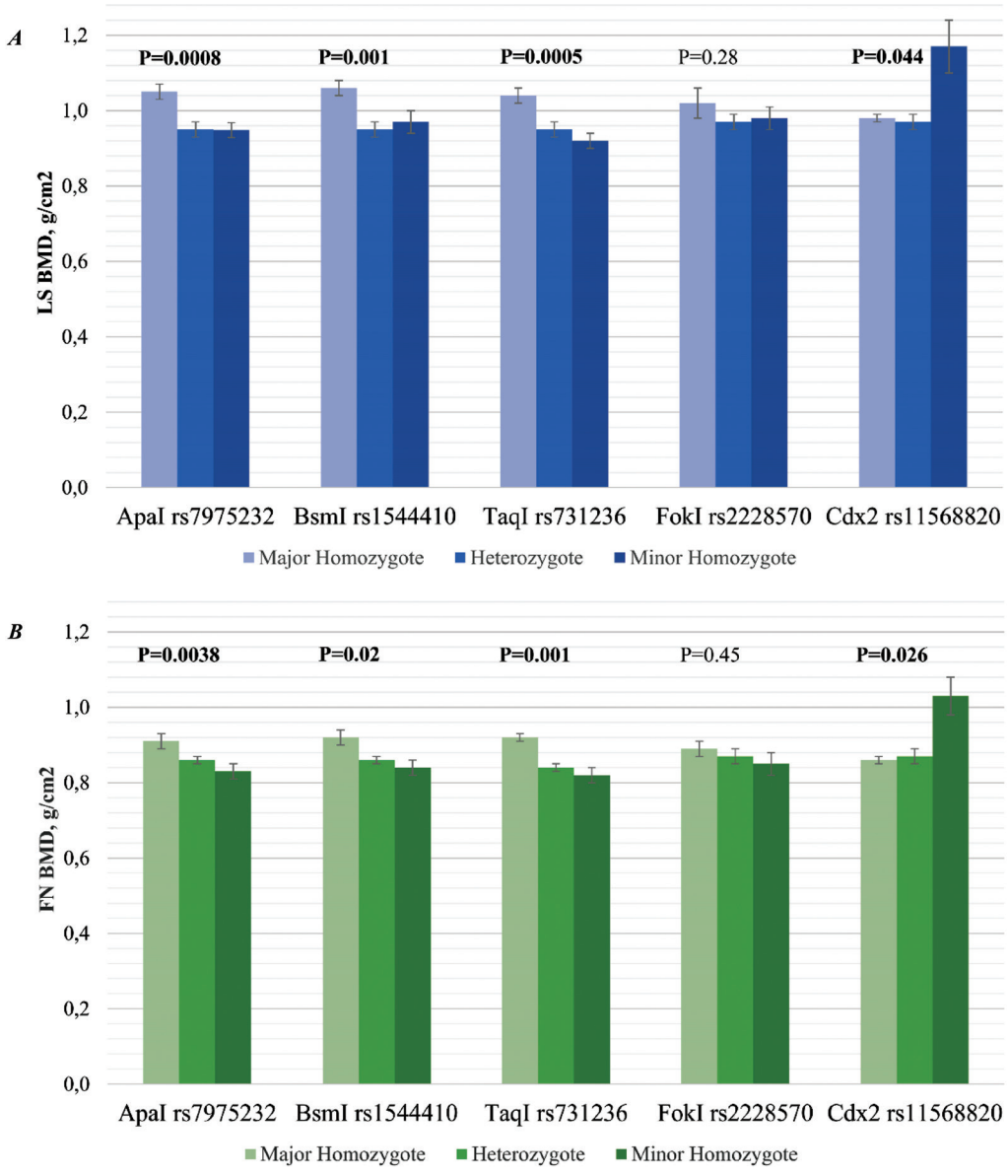

Fig. 3. Bone mineral density measured in lumbar spine (LS, *A*) and femoral neck (FN, *B*) in relation to *VDR* gene variants. *p*-values corrected for multiple testing using the FDR ($n = 5$, multiple comparisons)

Linear regression analysis indicated that different genotypes of four *VDR* gene variants are significantly associated with LS and FN BMD level (Fig. 3). The most significant association with BMD was revealed for TaqI 731236 variant (p_{FDR} = 0.0005 for LS and p_{FDR} = 0.001 for FN). Interestingly, for TaqI marker, there is a gene/dose response: the highest level of LS and FN BMD was found for the bearers of homozygous wild-type genotype A/A (1.04 ± 0.02 and 0.92 ± 0,01 g/cm^2, respectively), intermediate BMD was found in heterozygotes (0.95 ± 0.02 and 0.84 ± 0.01 g/cm^2), and the lowest BMD was found in homozygous for the minor alleles G/G genotype (0.92 ± 0.02 and 0.82 ± 0.02 g/cm^2). ApaI rs7975232 and BsmI rs1544410 variants were also associated with both LS and FN BMD, with highest BMD level in wild-type homozygotes and lowest – in minor homozygotes (ApaI C/C 1.05 ± 0.02 *vs.* A/A 0.95 ± 0.02 g/cm^2, BsmI C/C 1.06 ± 0.02 *vs.* 0.97 ± 0.03 g/cm^2 (Fig. 3, *A*); ApaI C/C 0.91 ± 0.02 *vs.* A/A 0.83 ± 0.02 g/cm^2, BsmI C/C 0.92 ± 0.02 *vs.* 0.84 ± 0.02 g/cm^2 (Fig. 3, *B*).

Interestingly, in quantitative analysis of Cdx2 rs11568820 variant association with LS and FN BMD, a statistically significant association was revealed. Substitution of C to T was associated with much higher LS BMD level (β = 0.19, 95 % CI 0.04–0.33, p_{FDR} = 0.044 (Fig. 3, *A*), as well as higher FN BMD (β = 0.18, 95 % CI 0.05–0.3, p_{FDR} = 0.026 (Fig. 3, *B*). This means that the substitution (T-allele) of Cdx2 rs11568820 has a protective effect (the only one from 5 markers, included in analysis). In previous comparison of PMO patients and control group, no statistically significant association was found in genotypes distribution (Tab. 3). As for group analysis, no significant association for FokI rs2228570 was found for both LS and FN BMD.

Since the first report, showing an association of *VDR* gene with BMD [24], several studies have been performed to investigate the relationship of various *VDR* variants with BMD in various populations. The widespread interest in the *VDR* gene may be explained by its main function to maintain the balance of serum calcium and phosphates in the human body. In general, obtained results are comparable with the results of other studies conducted on the European population [7]. However, in some studies, inconsistent results were observed. In a study on Polish population, authors observed dose effect of a single *VDR* FokI variant on the LS BMD, which was not found in present study [10]. Such inconsistency, observed in different populations, can be largely explained environmental factors and ethnicity, suggesting the importance of research on various nations.

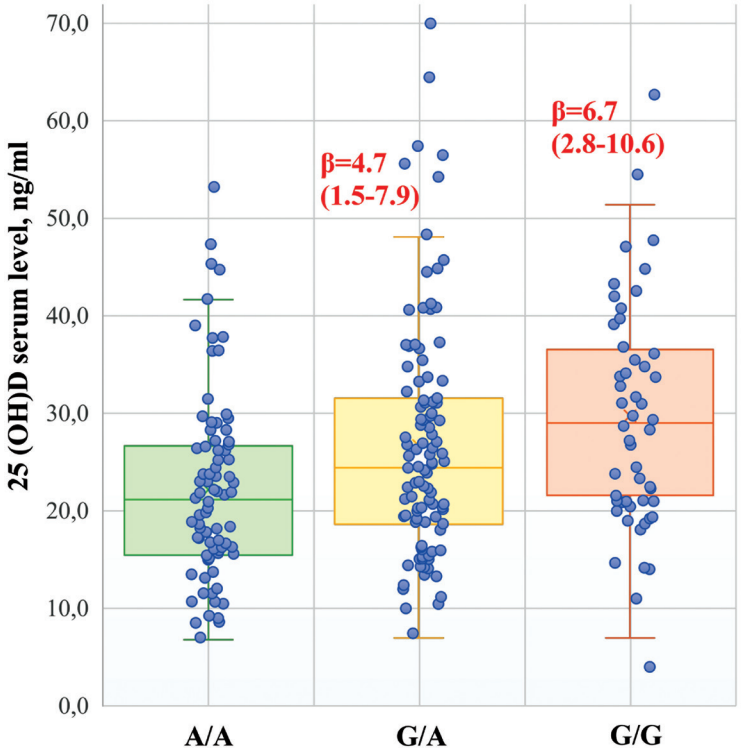

Fig. 4. Serum level of 25-hydroxyvitamin D in bearers of different *VDR* TaqI genotypes (p_{FDR} = 0.008). β – difference in 25(OH)D levels between reference (G/G) and corresponding genotype, error bars represent 25 % and 75 % quartiles

Of particular interest is the correlation analysis of the serum level of 25-hydroxyvitamin D with *VDR* gene variants. This analysis was performed to explore weather *VDR* variants has any influence on circulating 25(OH)D level. Multiple regression analysis indicated revealed association of 25(OH)D serum level with *VDR* TaqI 731236 genotypes (Fig. 4), but not with ApaI rs7975232, BsmI rs1544410, FokI rs2228570 and Cdx2 rs11568820.

The genetic effects of *VDR* TaqI on serum 25(OH)D were gene-dose dependent (Fig. 4): the lowest level was found in reference homozygotes (22.34 ± 1.07 ng/ml), intermediate – in heterozygotes (27.04 ± 1.19 ng/ml) and the highest – in bearers of homozygous G/G genotypes (29.05 ± 1.63 ng/ml, p_{FDR} = 0.008). A tendency for a gene-dose response was found between circulating 25(OH)D and *VDR* BsmI rs1544410 (C/C 23.58 ± 1.18 ng/ml, C/T 26.69 ± 1.23 ng/ml, T/T 27.78 ± 1.59 ng/ml, β = 4.21, 95 % CI 0.3–8.1, p_{FDR} = 0.088) and Cdx2 rs11568820 (C/C 26.85 ± 0.92 ng/ml, C/T 23.15 ± 1.38 ng/ml, T/T 19.43 ± 2.34 ng/ml, β = –3.70, 95 % CI –7.08…–0.32, p_{FDR} = 0.07). No association with 25(OH)D level for ApaI rs7975232 and FokI rs2228570 was found. In order to increase the statistical power, we analyzed association of serum 25(OH)D level with *VDR* ApaI, BsmI, TaqI and Cdx2 haplotypes (Tab. 5).

T a b l e 5. **Haplotype analysis of VDR gene ApaI, BsmI, TaqI and Cdx2 variants association with serum 25(OH)D level**

Haplotype	Frequency	25(OH)D, ng/ml (mean ± SE)	Linear regression	
			β (95 % CI)	p_{FDR}
C-C-A-C	39.4	23.38 ± 1.49	0	–
A-T-G-C	27.3	27.51 ± 1.27	**4.1 (1.6–6.5)**	**0.007**
C-C-G-C	7.3	29.22 ± 2.50	**6.3 (1.7–10.8)**	**0.02**
A-T-G-T	6.9	20.76 ± 2.21	–2.5 (–6.8…–1.8)	0.3
A-T-A-C	6.8	23.66 ± 2.57	0.0 (–4.7…– 4.7)	1
C-C-A-T	6.5	20.33 ± 2.55	–3.9 (–8.0…–1.9)	0.33
Rare	5.8	27.07 ± 5.65	5.1 (0.3–9.9)	0.07
Global haplotype association *p*-value 0.037				

Statistically significant differences were revealed in the global haplotypes distribution (global *p*-value = 0.037), suggesting an association of analyzed haplotypes with 25(OH)D. Interestingly, the highest level of serum 25-hydroxyvitamin D was found in bearers of non-favorable alleles of *VDR* gene variants (A-T-G-C), which reduce vitamin D receptor expression, suggesting that *VDR* gene variants are crucial for circulating 25(OH)D level. For the bearers of A-T-G-C haplotype, 25(OH)D level was significantly higher compared to reference haplotype (β = 4.1, 95 % CI 1.6–6.5, p_{FDR} = 0.007). The increased level of circulating 25(OH)D level may be explained by the fact that most patients with osteoporosis performed vitamin D supplementation, and in patients bearing unfavorable genotypes this vitamin was metabolized less efficiently. This hypothesis is confirmed by the fact that the level of *VDR* mRNA was remarkably reduced in bearers of BsmI T/T-genotype compared to individuals with C/C genotype [25]. Located at 3′-end of the *VDR* gene, ApaI, BsmI and TaqI variants are associated with the different length poly-adenylate sequence and affect the stability of mRNA, while Cdx2 variant could change the transcription activity of the promoter region of the gene [26]. The FokI variant, located in second exon, forms second methionine start site, producing a shorter protein receptor, which displayed higher transcriptional activity. 25(OH)D concentration was higher in bearers of the G/G-genotype compared to individuals with A/A-genotype [27]. Thus, differential activity of the receptor could alter the pattern of vitamin D-mediated gene activation, and thus impact on a wide range of enzymes involved in the production and elimination of 25(OH)D.

The association of serum 25(OH)D with *VDR* variants, revealed in present study, corresponds to previous research on vitamin D supplementation depending on *VDR* genotype [28], where various degrees of metabolic improvements from vitamin D supplementation were observed due to *VDR* gene variation, but the concentration of the active ligand 1.25(OH)D may be suboptimal. In the bearers of non-favorable homozygous ApaI A/A, BsmI T/T, TaqI G/G, FokI A/A genotypes, the baseline 25(OH)D level was higher compared to reference (wild-type) homozygous genotypes. This tendency remained

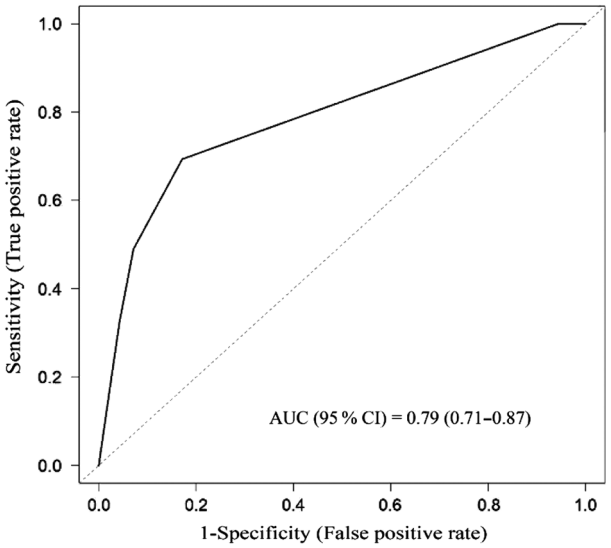

Fig. 5. Area under the receiver operating characteristic curve for a model of PMO risk evaluation

and the difference even increased after vitamin D intervention of 2000IU per day for 12 months (except for *VDR* FokI variant) [28]. Our data is consistent also with other prior reports [29], when higher circulating 25(OH)D was found in volunteers, carrying minor allele at a *VDR* BsmI rs1544410, previously associated with reduced fracture risk. It was hypothesized that the presence of ApaI C-, BsmI C-, TaqI A- and Cdx2 C-alleles of *VDR* gene provides higher expression level of vitamin D receptor, resulting in a better 25(OH)D hydroxylation following vitamin D supplementation. The current study adds the strength to the previous research on association of *VDR* gene functional variants with serum 25(OH)D level.

Thus, according to the performed analysis of the distribution of *VDR* gene variants in patients with PMO and control group, as well as their association with clinical parameters, we have identified four informative genetic markers. The integration of previously identified associations in the combined cohort of both patients and controls allowed to increase the statistical power of the study, as well as to identify a number of new associations, providing with new knowledge for understanding the molecular pathogenesis of osteoporosis and vitamin D metabolism. But it is well known that simple measure of OR association is not essential to reveal prognostic value of genetic variant [30]. To determine the diagnostic and prognostic value of the developed complex clinical and genetic model for evaluation of individual osteoporosis risk, we performed analysis of ROC-curve with area under the curve (AUC) calculation. The AUC in interval of 0.6–0.7 indicate average, 0.7–0.8 – good, and 0.8–0.9 – very good diagnostic level. The ROC analysis will help to separate individuals with high risk of PMO from those with low risk. The results of the ROC-analysis performed for *VDR* ApaI rs7975232, BsmI rs1544410, TaqI rs731236, FokI rs2228570 and Cdx2 rs11568820 variants is presented in Fig. 5.

The model of osteoporosis risk prediction possesses a good prognostic value, AUC = 0.79 (95 % CI 0.71–0.87 (Fig. 5). The model was characterized by high sensitivity level (82.9 %), with average specificity (69.4 %) and good accuracy (77.3 %). Thus, PMO risk assessment model, constructed only from genetic predictors, has shown good predictive value. Nevertheless, in present study we were unable to analyze different genetic markers, associated with bone turnover. The inclusion of other genetic markers, as well as clinical, biochemical, anthropometric, demographic, behavioral factors in this model will significantly increase its prognostic value.

As vitamin D status is linked not only to osteoporosis, but also to many other conditions, understanding the genetic variants that are responsible for vitamin variation is vital and may help us to better understand complex relationship between genetic and environmental factors in multifactorial pathology.

Conclusion. The major finding of this study was the development of model for evaluation of osteoporosis risk, constructed from informative genetic markers of *VDR* gene, which may have a prognostic importance. Our results suggest that the *VDR* ApaI rs7975232, BsmI rs1544410 and TaqI rs731236 variants and their haplotypes are associated with PMO risk in Belarusian women. Together with Cdx2 rs11568820, these markers are associated with BMD variation, and their haplotypes – with serum 25(OH)D level. We consider that revealed informative genetic markers have important clinical significance and our study makes significant contribution to the establishment of the molecular pathogenesis of postmenopausal osteoporosis.

Conflict of interests. The authors declare no conflict of interests.

Acknowledgements. This study was funded by a grant 6.3 of the Union State Program "DNA Identification". The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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