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ORIGINAL ARTICLE

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Association analysis of the polymorphisms of the *VDR* gene with bone mineral density and the occurrence of fractures

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Abstract Associations of the FokI, BsmI, ApaI, and TaqI polymorphisms of the vitamin D receptor (*VDR*) gene with the bone mineral density (BMD) of the lumbar part of the spinal column (BMD LS) and the neck of the femur (BMD FN), and with the occurrence of fractures, were studied using the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis on DNA isolated from peripheral blood of 239 women and 40 men from the region of western Poland. Three polymorphisms of the 3′ end of the *VDR* gene (BsmI, ApaI, TaqI) indicated a strong linkage disequilibrium. Association analysis of the *VDR* gene FokI polymorphism with BMD LS showed a dose effect of allele *f*. The association of the *bAT* haplotype of the BsmI, ApaI, and TaqI polymorphisms of the *VDR* gene with BMD FN was statistically significant. The association of the ApaI polymorphism with the occurrence of fractures was observed. Associations were also observed between the occurrence of fractures and the *baT* haplotypes of the *VDR* gene.

Key words osteoporosis · vitamin D receptor (*VDR*) gene · association · haplotype · bone mineral density

Introduction

Osteoporosis affects nearly 40% of women and 12% of men (data for the USA) [1,2], and the resulting fractures, which entail long treatment, frequently end in permanent disability. In cases of fracture of the neck of the femur, it may even lead to death caused by secondary changes in the vascular system [3,4]. Care of patients with fractures is difficult and expensive. Bone fractures are not generally associated with serious trauma, but occur as a result of ordinary daily activities and worsening bone quality [5]. The disease usually develops over many years before the bone is weakened and can no longer carry normal loading.

Numerous studies have demonstrated that a high proportion (up to 85%) of genetic factors are involved in changes in the bone mineral density (BMD) [6–18]. One of the most frequently investigated genes is the vitamin D receptor (*VDR*) gene, but investigations carried out in different populations yielded contradictory results [19–38].

The aim of this investigation was to analyze the associations of the FokI, BsmI, ApaI, and TaqI polymorphisms of the *VDR* gene with the BMD of the lumbar part of the spinal column (BMD LS) and the neck of the femur (BMD FN), and with the occurrence of fractures in a group of 239 women and 40 men from the region of Western Poland. Polymorphism FokI (*ff* or *Mm*), c.2T > C, results in T > C transition in codon 1 and a shortening of the protein by 3 amino acids. Polymorphisms BsmI (*bB*), 1024 + 283G > A, and ApaI (*aA*), 1025 – 49G > T, are located in intron 8, while silent polymorphism TaqI (*Tt*), c.1056T > C, is located in exon 9 (Ile > Ile).

Materials and methods

Patients examined and control group

The experimental material comprised DNA isolated from peripheral blood of 304 consecutive subjects from the region of western Poland, who were treated in the Department of Family Medicine of the University of Medical Sciences at Poznan. The clinical data included the bone mineral density of the neck of the femur, the bone mineral density of the lumbar part of the spinal column (L1–L4), information from the patients and their medical records about the absence or occurrence of fractures, age at densimetric

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Table 1. Characteristics of the patients and the control groups. For quantitative traits, mean values ± standard deviation are given, with minimal and maximal values in parentheses

Characteristics	Women with measurements of the bone mineral density of the neck of the femur (BMD FN)	Women with measurements of the bone mineral density of the lumbar segment of the spinal column (BMD LS)	Women and men investigated for associations of polymorphisms with the occurrence of fractures
Women (No.)	163	99	239
Men (No.)	–	–	40
Age (years)	65.5 ± 9.9 (47.0–89.0)	63.0 ± 9.3 (38.0–84.0)	64.4 ± 10.9 (24.0–89.0)
Body weight (kg)	59.8 ± 10.1 (39.0–95.0)	61.3 ± 9.7 (41.0–95.0)	–
Height (m)	1.589 ± 0.057 (1.440–1.730)	1.591 ± 0.063 (1.405–1.720)	–
BMI (kg/m^2)	23.7 ± 3.9 (16.8–40.6)	24.2 ± 3.7 (17.5–40.6)	–
BMD FN (g/cm^2)	0.711 ± 0.092 (0.517–1.080)	–	–
BMD LS (g/cm^2)	–	0.828 ± 0.143 (0.565–1.306)	–
Subjects in FN group (No.)	–	71	163
Subjects in LS group (No.)	71	–	99
Subjects in group with fractures (No.)	163	99	–

BMD FN, bone mineral density of the neck of the femur; BMD LS, bone mineral density of the lumbar segment of the spine; BMI, body mass index

examination, height, and body weight. The densimetric examinations involved dual-energy X-ray absorptiometry (DEXA). People suffering from diseases which influence the bone mineral density, such as hyperthyroidism, rheumatoid joint inflammation, endocrine and kidney diseases, disorders associated with the pituitary gland after strumectomy, etc., were excluded from the investigations. A total of 279 people (239 women and 40 men) were subjected to the investigations, and their clinical data were subsequently used to analyze any associations with the occurrence of fractures. This group was further subdivided into two groups: 163 women (group A) for whom the BMD FN was found, and a group of 99 women (group B) for whom the BMD LS (L1–L4) was found. Table 1 gives the general characteristics of the groups examined. Since data concerning the fracture occurrence (low energy or strong trauma) were not available in 25 cases, the means of these parameters were not calculated.

Genotyping

Following the isolation of DNA from the peripheral blood by the standard method with guanidinium thiocyanate (GTC), the polymerase chain reaction (PCR) was carried out in 20 µl with 200 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 7.5 pmol of each primer, and 0.5 units of Taq polymerase (Sigma). The reaction was conducted in the following conditions: initial denaturation at 94°C for 4 min; denaturation at 94°C for 40 s; primer annealing for 40 s; elongation at 72°C for 100 s; final incubation at 72°C for 180 s. For FokI polymorphism of the *VDR* gene, 267 bp fragment was amplified using primers F AGC TGG CCC TGG CAC TGA CTC TGC TCT and R ATG GAA ACA CCT TGC TTC TTC TCC CTC, and 31 cycles were performed at the annealing temperature of 60°C [39]. For BsmI polymorphism, 837 bp fragment was amplified at an annealing temperature of 55°C for 35 cycles using primers F GGC AAC CAA GAC TAC AAG TAC C and R TCT TCT CAC CTC TAA CCA GCG. For ApaI and TaqI polymorphisms of the *VDR* gene, 745 bp fragment was amplified at an annealing temperature 64°C for 35 cycles using primers F CAG AGC ATG GAC AGG GAG CAA and R GCA ACT CCT CAT GGC TGA GGT CTC [25]. The PCR product was then subjected to restriction fragment length polymorphism (RFLP) analysis using the following restriction enzymes: TaqI, restrictase *Taq*I (Fermentas); ApaI, restrictase *Bsp*120I (Fermentas); BsmI, restrictase *Mva*1269I (Fermentas); FokI, restrictase *Bse*GI (Fermentas). All analyses were carried out according to the manufacturer's recommendations, and the products of hydrolysis were separated in 1.5% agarose gels.

Statistical analysis

The analysis of the linkage disequilibrium of the polymorphisms examined was performed using the Haploview v.3.11 program [40]. The analysis of the compliance of the distribution of genotypes and haplogenotypes with the distribution in the Hardy–Weinberg equilibrium was carried out with the assistance of the programs available at http://ihg.gsf.de/cgi-bin/hw/hwa1.pl and the STATISTICA 6.0 (StatSoft). Hardy–Weinberg equilibrium was shown for all the polymorphisms and haplogenotypes analyzed.

In order to present the relationships between the bone mineral density and the remaining major clinical parameters, i.e., age, body weight, and height, a correlation analysis was performed. The association analysis was performed for three possible effects: the allele or haplotype dose, the recessive activity, or the dominating action. Apart from the analysis carried out on the raw (uncorrected) BMD, analyses were also carried out for the BMD corrected against age only, or corrected against age, body weight, and height. The significance of the differences for age, body weight, height, and body mass index (BMI) between groups of different genotypes were analyzed using analysis of variance. The impact of the allele or haplotype dosage on the bone mineral

density was analyzed by simple regression (raw BMD) or by multiple regression (corrected BMD). The impact of the effect of recessiveness and dominance on the bone mineral density was analyzed by analysis of variance (raw BMD) or by analysis of covariance (corrected BMD). In addition, an analysis of associations (*case-control* type) with the occurrence of fractures was also performed. The analysis was carried out for the following three possible effects of action, the allele dose (χ^2 Armitage test for trend), and the recessive and dominant action (χ^2 Pearson's and the odds ratio at the significant test value χ^2). In addition, frequencies of alleles were also compared between the groups without and with fractures (χ^2 Pearson's and the odds ratio for the risk alleles of all polymorphisms). The results were treated as statistically significant when P was lower than or equal to 0.05.

Results

From four polymorphisms of the *VDR* gene, the polymorphisms at the 3′ end of the gene (BsmI, ApaI, TaqI) exhibited a strong linkage disequilibrium with respect to one another: D′ ranged from 0.98 to 1.0 (Table 2) forming three frequent haplotypes: *baT*, *BAt*, and *bAT*, which jointly represent nearly 99% of all haplotypes (Fig. 1). The FokI polymorphism does not exhibit linkage disequilibrium with the polymorphisms of the 3′ end of the *VDR* gene from which it is separated by over 33 kbp; D′ ranged from 0.01 to 0.11.

The presence of three frequent haplotypes allowed the determination of haplogenotypes for 276 out of 279 subjects, with very minor errors for a few haplogenotypes. For example, the identification of heterozygosity at the genotyping for all three polymorphisms may be due to the presence in a given person of frequent haplotypes *baT* and *BAt*, or a frequent haplotype *bAT* and rare haplotype *Bat*. However, the probability of a second case is [2 × frequency of the frequent haplotype × frequency of the rare haplotype], which in this case equals $2 \times 0.126 \times 0.002 = 0.000504$. This means that out of a population of 2000 people in Poland, only one person has such a haplogenotype. The identification of haplogenotypes for the polymorphisms of the *VDR* gene made it possible to carry out an analysis of associations for haplotypes later in the study.

Table 2. Distance between polymorphisms and the strength of the linkage disequilibrium (LD) for the FokI, BsmI, ApaI, TaqI polymorphisms of the *VDR* gene

Polymorphism	Distance between polymorphisms	Linkage disequilibrium (LD)	
		r^2	Lewontin's D'
FokI vs. BsmI	33 060 bp	0.0	0.08
FokI vs. ApaI	34 058 bp	0.0	0.01
FokI vs. TaqI	34 138 bp	0.01	0.11
BsmI vs. ApaI	998 bp	0.58	0.98
BsmI vs. TaqI	1 078 bp	0.95	1.0
ApaI vs. TaqI	80 bp	0.57	0.99

The results of the power calculations are given in Table 3 as the smallest changes in bone mineral density, the smallest correlation coefficient, or the smallest relative risk for which the statistical power is 0.8.

Association analysis of the *VDR* gene FokI polymorphism with the BMD of the L1–L4 (BMD LS) showed the dose effect of allele *f* (Table 4, Fig. 2). In *ff* homozygotes, the BMD value (corrected for age, body weight, and height) is lowest, i.e., 0.792 g/cm^2, in *Ff* heterozygotes it reaches 0.826 g/cm^2, while in *FF* homozygotes it is 0.875 g/cm^2, (P = 0.047). This effect is also visible for the raw BMD data and

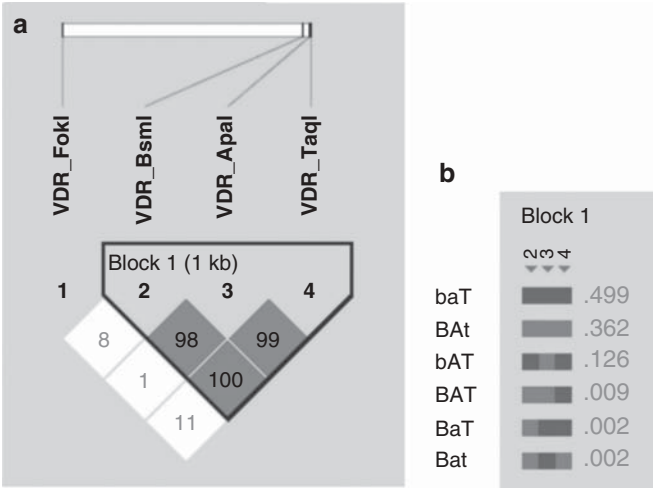

Fig. 1. Linkage disequilibrium analysis between the FokI, BsmI, ApaI, TaqI polymorphisms of the *VDR* gene using the Haploview v.3.11 program. **a** Schematic presentation of the block of polymorphisms in a linkage disequilibrium. Values in individual fields represent the linkage strength as Lewontin's *D′* expressed as a percentage; **b** haplotypes in the group examined and their estimated frequency

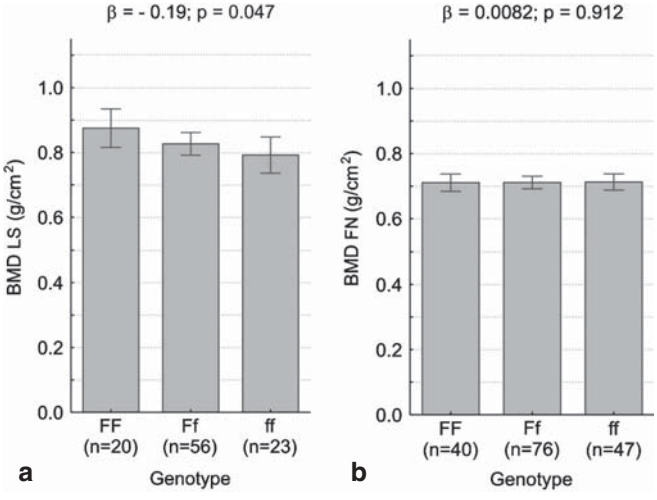

Fig. 2. Comparison of the bone mineral density (*BMD*) between groups of women with a different genotype for the FokI polymorphism of the *VDR* gene. **a** BMD of the lumbar segment of the spinal column. **b** BMD of the neck of the femur. These diagrams give the results of the analysis for allele dose (multiple regressions). The BMD was corrected against age, body weight, and height. *Error bars* represent 95% confidence intervals

Table 3. Results of statistical power tests. The results for individual polymorphisms and haplotypes are presented as the minimal difference of the parameters analyzed, for which the power of the statistical tests equals 0.8. For the dose effect, the minimal deviation of the correlation coefficient from value 0 is given. For domination and recessiveness effects, the minimal difference in the BMD between the groups analyzed is given. In cases where an analysis of the minimal relative risk of an association with the occurrence of fractures was provided, its value exceeded 1. In cases where haplotypes were also given a maximal relative risk value, the value was lower than 1. The lack of a value in one of the fields for the VDR *bAT* haplotype is due to the small number in the group analyzed

Polymorphisms or haplotype analyzed	Bone mineral density						Occurrence of fractures
	Allele dose effect ("min" correlation coefficient)		Dominance – recessiveness 0 + 1 vs 2 (min BMD differences in g/cm^2)		Dominance – recessiveness 0 vs 1 + 2 (min BMD differences in g/cm^2)		Association for allele frequency (min relative risk, and by haplotypes max and min relative risk)
	Neck of the femur	Lumbar segment of the spinal column	Neck of the femur	Lumbar segment of the spinal column	Neck of the femur	Lumbar segment of the spinalcolumn	
VDR FokI	±0.219	±0.280	±0.042	±0.092	±0.043	±0.960	2.105
VDR TaqI	±0.219	±0.280	±0.054	±0.110	±0.038	±0.078	2.262
VDR ApaI	±0.219	±0.280	±0.044	±0.086	±0.045	±0.092	2.105
VDR BsmI	±0.219	±0.280	±0.038	±0.080	±0.054	±0.110	2.242
VDR baT	±0.220	±0.281	±0.044	±0.087	±0.045	±0.091	0.474, 2.111
VDR BAt	±0.220	±0.281	±0.054	±0.114	±0.038	±0.080	0.441, 2.091
VDR bAT	±0.220	±0.281	±0.799	–	±0.044	±0.103	0.232, 2.55
ESR1 px	±0.219	±0.280	±0.044	±0.10	±0.042	±0.083	0.477, 2.111
ESR1 PX	±0.219	±0.280	±0.070	±0.123	±0.038	±0.082	0.437, 2.088
ESR1 Px	±0.219	±0.280	±0.137	±0.399	±0.041	±0.084	0.268, 2.438

for the data corrected for age only (Table 4). However, there is no association of this polymorphism with the BMD in the neck of the femur (BMD FN) (Fig. 2). No significant associations were found for the TaqI, ApaI, BsmI polymorphisms of the *VDR* gene with BMD FN or BMD LS.

Association analysis with BMD FN and BMD LS for the three most frequent haplotypes of the *VDR* gene was also performed. The results obtained were statistically significant for an association of the *bAT* haplotype of the BsmI, ApaI, and TaqI polymorphisms of the *VDR* gene with BMD FN. The effect of the *bAT* haplotype dose was found (Fig. 3). For homozygous *bAT* women, the lowest mean BMD of the neck of the femur was 0.671 g/cm^2, in the case of heterozygotes with only one copy of the *bAT* haplotype, the BMD reached 0.686 g/cm^2, whereas in homozygotes without the *bAT* haplotype it was 0.719 g/cm^2 (P = 0.031). This effect is not significant for the raw BMD data or for the BMD corrected for age only. For this haplotype, it is also possible to see the effect of domination irrespective of whether the BMD is raw or corrected. Women with haplotype *bAT* on one or both chromosomes have a lower BMD (0.685 g/cm^2, corrected for age, body weight, and height) than women without this haplotype (0.719 g/cm^2) (P = 0.031). However, the effect of the *bAT* haplotype is not visible in the lumbar segment of the spinal column, where the trend is reversed (Fig. 3).

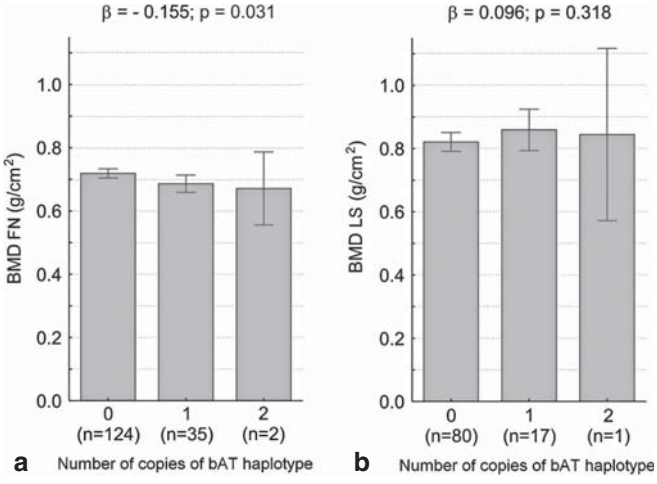

Fig. 3. Comparison of the BMD between groups of women with a different number of copies of the *bAT* haplotype of polymorphisms of the 3′end *VDR* gene. **a** BMD of the neck of the femur; **b** BMD of the lumbar segment of the spinal column. These diagrams give the results of the analysis for haplotype dose (multiple regressions). The BMD was corrected against age, body weight, and height. *Error bars* represent 95% confidence intervals

An analysis of associations (case-control type) was performed with the occurrence of fractures for the polymorphisms of the *VDR* genes examined. An association of the ApaI polymorphism with the occurrence of fractures was observed. This association was linked with the effect of recessiveness for the *a* allele. Fractures occurred in nearly 41% of people with the *aa* genotype, and in only 29% and 26.6% of subjects with the *AA* and *Aa* genotype, respectively, which corresponds with the following odds ratio: OR = 1.83; P = 0.034 (Fig. 4). No significant association was found for the remaining polymorphisms.

An analysis of association (case-control type) was performed with the occurrence of fractures for the 3 most frequent haplotypes of the *VDR* gene. Associations were observed with the occurrence of fractures with the *baT* haplotypes of the *VDR* gene. This effect is linked with the association presented earlier of the allele *a* of the ApaI

Table 4. Analysis of the association of the FokI polymorphism of the *VDR* gene with the BMD LS

VDR FokI, lumbar segment of the spinal column+	Number (*n*)	Age (years)	Body weight (kg)	Height (m)	BMI (kg/m^2)	Noncorrected BMD (g/cm^2)	Age-corrected BMD (g/cm^2)	Age, height, and body weight-corrected BMD (g/cm^2)
Allele dose effect	$n = 99$							
Allele dose								
FF	20 (20.2%)	63.3 (±9.4)	64.4 (±11.2)	1.591 (±0.051)	25.5 (±4.7)	0.884 (±0.146)	0.886 (±0.134)	0.875 (±0.133)
Ff	56 (56.6%)	63.4 (±9.2)	60.6 (±9.0)	1.583 (±0.065)	24.2 (±3.4)	0.823 (±0.134)	0.824 (±0.134)	0.826 (±0.132)
ff	23 (23.2%)	61.8 (±9.8)	60.2 (±10.0)	1.613 (±0.065)	23.1 (±3.0)	0.793 (±0.152)	0.788 (±0.134)	0.792 (±0.134)
Significance		$P = 0.797$	$P = 0.260$	$P = 0.157$	$P = 0.093$	$P = 0.039$	$P = 0.019$	$P = 0.047$
Recessiveness and dominance effect								
Genotype								
FF + Ff	76 (76.8%)	63.3 (±9.2)	61.6 (±9.7)	1.585 (±0.062)	24.5 (±3.8)	0.839 (±0.139)	0.841 (±0.135)	0.839 (±0.133)
ff	23 (23.2%)	61.8 (±9.8)	60.2 (±10.0)	1.613 (±0.065)	23.1 (±3.0)	0.793 (±0.152)	0.788 (±0.136)	0.792 (±0.135)
Significance		$P = 0.500$	$P = 0.561$	$P = 0.063$	$P = 0.095$	$P = 0.181$	$P = 0.105$	$P = 0.152$
Genotype								
FF	20 (20.2%)	63.3 (±9.4)	64.4 (±11.2)	1.591 (±0.051)	25.5 (±4.7)	0.884 (±0.146)	0.886 (±0.134)	0.874 (±0.133)
Ff + ff	79 (79.8%)	62.9 (±9.3)	60.5 (±9.2)	1.591 (±0.066)	23.9 (±3.3)	0.814 (±0.139)	0.814 (±0.134)	0.817 (±0.132)
Significance		$P = 0.885$	$P = 0.101$	$P = 0.977$	$P = 0.072$	$P = 0.049$	$P = 0.035$	$P = 0.087$

polymorphism of the *VDR* gene which constitutes part of the *baT* haplotype. The recessive effect is also visible in this case. Among subjects homozygotic for the *baT* haplotype, people with fractures constitute 41.4% of the group compared with 26.5% and 28.6% in the group of subjects with one or without the *baT* haplotype, respectively, which corresponds to the following odds ratio: OR = 1.9; $P = 0.026$ (Fig. 5). No significant associations were found for the remaining haplotypes. When analyzing haplogenotypes, precisely the same association with the *baT baT* haplogenotype is apparent (Table 5).

Discussion

Research concerning diseases with a complex, multifactorial background, such as osteoporosis, have been aimed at establishing both the environmental factors and the genetic background affecting disease development. Early detection of a genetic predisposition to osteoporosis should allow appropriate prophylaxis, and delay and/or limit unfavourable changes in the bone tissue. The examination of several, or perhaps even several, hundred, polymorphic sites would give a relatively accurate picture of susceptibility, and at the same time allow effective genotype-adjusted prevention. Polymorphisms of the vitamin D receptor gene (*VDR*) have been widely investigated in various populations. The first investigations regarding its associations with osteoporosis were carried out in 1994 on an Australian population of European origin [19]. The widespread interest in the *VDR* gene is due primarily to the significant impact of vitamin D on calcium take-up. Shortages of vitamin D lead to hypocalcemia caused by reduced absorption of calcium. In addition, the vitamin stimulates calcium resorption in the kidneys, and increases calcium release from bones via the induction of the expression of the osteoclast differentiation factor RANKL in osteoblasts [41]. The most commonly analyzed polymorphisms of the *VDR* gene in other populations were also selected for this investigation: FokI, TaqI, ApaI, and BsmI. The FokI polymorphism elongates protein by three amino acids, and may affect the activity of the vitamin D_3 receptor [42,43].

Measurements of the BMD FN or BMD LS or both were performed for the majority of the 279 subjects in our group. The results of genotyping of all subjects, irrespective of their sex, were used for an analysis of the associations with the occurrence of fractures. The investigations included 163 people for FN and 99 people for LS. Women for whom BMD measurements were carried out in the areas of both the FN and the LS were included in both groups. Since age, body weight, and height exert a significant influence on BMD, as well as the analysis of the associations for the raw (uncorrected) BMD, we also carried out an analysis for the BMD corrected in relation to age, as well as the BMD corrected simultaneously for age, body weight, and height.

In the case of the *VDR* gene, a strong linkage disequilibrium was found for the polymorphisms at the end of 3′

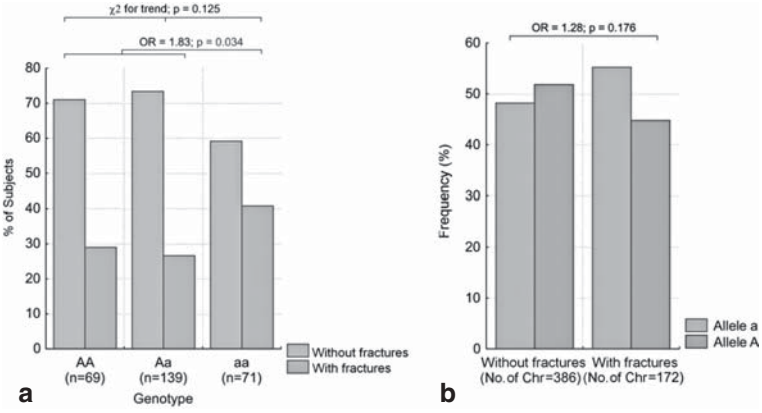

Fig. 4. Graphic representation of the results of the analysis of the association of the ApaI polymorphisms of the *VDR* gene with the occurrence of fractures. **a** The occurrence of fractures relative to the ApaI polymorphism genotype. The *top* diagram shows the results of the analysis for the allele dose effect (χ^2 for the trend) and for the recessive effect of the *a* allele (χ^2 Pearson's and odds ratio (*OR*)). **b** The frequency of the alleles of the ApaI polymorphism in the groups of subjects without and with fractures. *No. of Chr*, number of chromosomes

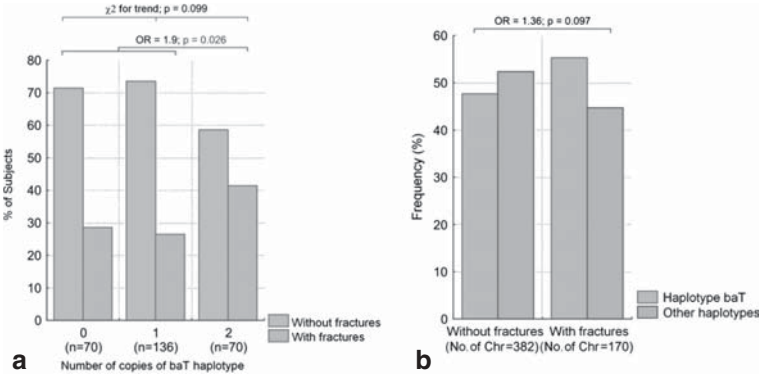

Fig. 5. Graphic representation of the results of the analysis of the association of the *baT* haplotype of the polymorphisms of the 3′end *VDR* gene with the occurrence of fractures. **a** Occurrence of fractures relative to the number of copies of the *baT* haplotype. The *top* diagram shows the results of the analysis for the haplotype dose effect (χ^2 for the trend) and for the recessive effect of the *baT* haplotype (χ^2 Pearson's and OR). **b** Frequency of the *baT* haplotype in comparison with the frequency of the remaining haplotypes in the groups of subjects without and with fractures

Table 5. An analysis of the association of haplogenotypes of the 3′ end polymorphisms of the *VDR* gene with the occurrence of fractures. The percentage of patients with fractures relative to the total number of patients with a given genotype is shown. Haplogenotypes *BAt Bat*, *bAT BAT*, and *baT BaT* were omitted in the statistical analysis because of the lack of patients with fractures for those haplogenotypes

	Number of patients and percentage of patients with fractures relative to the total number of patients with a given genotype				χ^2 analysis for a given genotype against the sum of other genotypes			
	Patients without fractures		Patients with fractures					
	n	*n*	*n*	(%)				
Polymorphisms of the 3′ end of the *VDR* gene		191	85		df = 1			
Haplogenotype								
BAt BAt	42	32	10	(23.8%)	$\chi^2 = 1.14$	$P = 0.286$		
BAt bAT	19	11	8	(42.1%)	$\chi^2 = 1.22$	$P = 0.269$		
baT BAt	98	71	27	(27.6%)	$\chi^2 = 0.75$	$P = 0.386$		
bAT bAT	6	4	2	(33.3%)	$\chi^2 = 0.02$	$P = 0.892$		
bAT baT	37	28	9	(24.3%)	$\chi^2 = 0.84$	$P = 0.359$		
baT baT	70	41	29	(41.4%)	$\chi^2 = 4.97$	$P = 0.026$	OR = 1.9	(1.08–3.34)
BAt Bat	1	1	0	(0.0%)				
bAT BAT	2	2	0	(0.0%)				
baT BaT	1	1	0	(0.0%)				
χ^2 Pearsons		$\chi^2 = 6.96$	df = 5	$P = 0.224$				

gene, BsmI, ApaI, and TaqI. The FokI polymorphism, which is located over 33 kbp upstream from the BsmI, ApaI, and TaqI polymorphisms, fails to show linkage disequilibrium. These results are consistent with investigations carried out for the Danish population [24]. The association is full ($D' = 1.0$) for the BsmI and TaqI polymorphisms, since the r^2 coefficient in this case is 0.95. With some exceptions, the *b* allele of the BsmI polymorphism occurs with the *T* allele of the TaqI polymorphism. In the case of the ApaI polymorphism, the linkage is weaker: $D' = 0.99$ in relation to

TaqI and D′ = 0.98 against BsmI. However, in this case, it is not possible to determine the allele of the ApaI polymorphism accurately on the basis of knowledge of only the allele of TaqI or BsmI polymorphism: $r^2 = 0.57$ and 0.58, respectively. The three haplotypes of polymorphisms at the end of 3′ of the *VDR* gene occurring most frequently in the population examined include *baT*, *BAt*, and *bAT*. Together, they make up nearly 99% of all haplotypes. These results are in agreement with the findings for other European populations [44–46]. On the other hand, in the case of Asian populations, the most frequent haplotypes include *baT*, *bAT*, and *BaT* [46].

Each polymorphism and haplotype was examined with respect to the association with BMD FN and BMD LS, as well as with the occurrence of fractures. Statistically significant associations were discovered for the FokI and ApaI polymorphisms, and for the *bAT* and *baT* haplotype of the *VDR* gene. We observed the *f* allele dose effect of the FokI polymorphisms of the *VDR* gene on the BMD LS. Women with the *ff* genotype have the lowest BMD, those with the *Ff* an intermediate BMD, and those with the *FF* homozygote the highest BMD, irrespective of the analysis of the raw BMD, corrected for age only or corrected jointly for age, body weight, and height. However, the effect is not visible for the BMD of the neck of the femur. The observed association of the FokI polymorphism with the bone mineral density in the lumbar segment of the spinal column only is consistent with the results obtained for the Caucasian American population and the American population of Mexican origin, as well as the Taiwanese and Italian populations [39,47–49]. The association of the *f* allele with the lowest BMD of the lumbar segment of the spinal column was also reported in the Japanese population [42]. However, in those investigations, the BMD of the neck of the femur was not measured. It is possible that the FokI polymorphism exerts an influence on the spongy bone tissue which is more evident in the lumbar segment of the spinal column than in the neck of the femur. However, this assumption is not confirmed by investigations carried out on a Danish population and some American subjects from the Boston area, where the association was apparent for the neck of the femur and much less so (statistically nonsignificant) for the lumbar segment of the spinal column [50,51]. On the other hand, many investigations have failed to confirm the existence of the association of this polymorphism with the bone mineral density [24,31,33,52–54]. Such contradictory results between different populations, and even within the same population reported in different papers, may be due to environmental factors. An example is provided by a study of a Danish population where they reported that the impact of the FokI polymorphism was dependent on the body weight index (BMI) [50]. The association of the *f* allele with the lowest BMD was visible in very slim women only (BMI < 25 kg/m^2). The significance of the *f* allele association with the lower BMD L1–L4 in this study is weakened by a reverse trend which becomes apparent when analyzing the association of the FokI polymorphism with fractures. In this case, the risk allele is the F allele, which occurs more frequently in people with fractures.

Another observed association concerned the *bAT* haplotype of the BsmI, ApaI, and TaqI polymorphisms of the *VDR* gene with the lower bone mineral density of the neck of the femur. Both the impact of the haplotype dose and the effect of the domination of this haplotype are apparent. The impact of the haplotype dose is evident only for the BMD FN corrected in relation to age, body weight, and height. On the other hand, the effect of the domination is significant for both the raw as well as the corrected BMD. This finding is in agreement with that from a large Dutch population, where the association of the *bAT* haplotype with lower BMD FN was reported [34]. However, the association of this haplotype is not visible for the BMD LS. A stronger impact of the polymorphism effect of the 3′ end of the *VDR* gene on the mineral density of the neck of the femur was also observed among young white women in a Canadian population, where subjects with the *bAT bAT* haplogenotype have the lowest BMD FN [38].

In the analysis of fractures, an association with the *baT* haplotype was observed. The same effect was found for the *a* allele of the ApaI polymorphism, which in the Polish population is almost identical with the *baT* haplotype due to a very low frequency of the *BaT* and *Bat* haplotypes. These results are consistent with those obtained for a Dutch population [55]. The *baT* haplotype shows an association in a recessive manner. Subjects with the *baT baT* haplogenotype (or with the *aa* genotype) are characterized by nearly twice as great a risk of fractures than subjects with other haplogenotypes. The above-mentioned observations are, to a considerable extent, consistent with the results from a Greek population [37]. Numerous examples of investigations can be found in literature which either do not report associations, or indicate associations with the *B* allele [21–33]. The association of the *baT* haplotype (or *a* allele of the ApaI polymorphism) with the occurrence of fractures appears to be independent of the impact on the BMD. No significant influence of this haplotype was observed on the BMD of the group examined.

In this study, no significant impact of a single polymorphism from the 3′ end of the *VDR* gene on the bone mineral density was observed. The haplotype analysis is favorable, especially for polymorphisms which do not directly affect the activity or expression of the protein product, and act only as markers in the linkage disequilibrium with other functional polymorphisms. The 3′ end polymorphisms of the *VDR* gene do not change the protein product, and their influence on the gene expression remains open to discussion [56]. The most recent studies performed in Europe with 26 242 participants, involving the FokI, BsmI, ApaI, and TaqI VDR polymorphisms, showed no association with BMD or with fractures. The analysis of the Cdx2 promoter polymorphism may be associated with risk of vertebral fractures [57]. The inconsistencies in the case of the results for the polymorphisms from the 3′ end of the *VDR* gene can be attributed to different linkage disequilibrium in different populations, or the influence of environmental factors. In the Canadian population, interactions were observed between the 3′ end polymorphisms of the *VDR* gene with physical activity and calcium intake on the influence on the

BMD [38]. A similar interaction was also observed for Caucasian women in the American population with the intake of calcium [36] and caffeine [58]. Some interesting studies of relationship between polymorphisms in the vitamin D receptor and estrogen receptor-α genes and BMD, bone mineral content, and markers of bone turnover were conducted by Cusack et al. [59] with a group of 224 Danish girls aged 11–12 years. No significant differences in anthropometrical variables, physical activity, and dietary calcium among genotype groups were observed. *XX* and *PP ERα* genotypes were associated ($P < 0.05$) with reduced levels of urinary pyridinium cross-links, whereas serum osteocalcin was similar among genotypes. These findings suggest that the rate of bone resorption was influenced by ERα genotypes.

In our study, a statistically significant association was observed of the *VDR* gene polymorphisms and haplotypes with the BMD and with the occurrence of fractures. Differences between the association with the BMD FN, BMD LS, and the occurrence of fractures may indicate the existence of very specific interrelations of individual alleles with a given effect. The association of the *baT* haplotype (or the *a* allele of the ApaI polymorphism) with fractures appears to be independent of the impact on the BMD. In prevention, the most important thing is to avoid fractures. It appears that in diagnostics, the most effective and advisable measure would be an analysis of the presence of the *baT* haplotype of polymorphisms from the 3′ end of the *VDR* gene which, for practical purposes, could be limited to an analysis of the *a* allele presence of the ApaI polymorphism only. In order to limit the risk of statistical error, we did not carry out association analyses of the BMD with polymorphisms depending on the age and body weigh index (BMI) of the subjects examined, because further division into subgroups would lead to very small experimental groups, and an additional risk of the occurrence of sampling errors.

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