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Association analysis of vitamin D receptor gene polymorphisms with bone mineral density in young women with Graves' disease

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Graves' (GD) hyperthyroidism induces accelerated bone turnover that leads to decreased bone mineral density (BMD). The role of the *VDR* gene in predisposition to primary osteoporosis has been recognized. Recent studies show associations between the *VDR* gene polymorphisms and susceptibility to autoimmune diseases. Here we analyzed if *VDR* gene polymorphisms: *Bsm*I, *Apa*I, *Taq*I, and *Fok*I may predispose women with Graves' hyperthyroidism to BMD reduction or to disease development. The subjects were 75 premenopausal female Polish patients with GD and 163 healthy women. The genotyping was performed by the use of the restriction fragment length polymorphism analysis (RFLP). We studied the association of the *VDR* polymorphisms and their haplotypes with patients' BMD and also SNPs and haplotypes association with Graves' disease. We found a strong linkage disequilibrium for the *Bsm*I, *Apa*I, and *Taq*I polymorphims that formed three most frequent haplotypes in Graves' women: baT (47.9%), BAt (34.9%), and bAT (16.4%). We did not show statistically significant association of analyzed *VDR* polymorphisms or haplotypes with decreased bone mineral density in Graves' patients. However, the presence of F allele had a weak tendency to be associated with Graves' disease (with OR = 1.93; 95% CI: 0.97–3.84; p=0.058). In conclusion: *VDR* gene polymorphisms do not predict the risk of decreased BMD in Polish women with Graves'. It may be speculated that the F allele carriers of the *VDR-Fok*I polymorphism are predisposed to Graves' disease development.

Keywords: Graves' disease, bone mineral density, *VDR* polymorphisms

INTRODUCTION

Hyperthyroidism concerns 2% of adult people with women's predominance. One of its most common forms is Graves' disease (GD) that tends to affect women between the second and fourth decade of life. The etiology of this autoimmune disorder results from the presence of thyroid stimulating antibodies which bind to the TSH receptor (TSH-R). The genetic background of GD was investigated in several studies that showed increased disease susceptibility associated with polymorphisms in the *HLA* and *CTLA-4* (cytotoxic T lymphocyte associated 4) genes (Donner *et al.*, 1997).

Hyperthyroidism affects bone metabolism and is an important reason for secondary osteoporosis. An excess of thyroid hormones induces accelerated bone turnover due to an increased number of active osteoclasts and increased osteoblasts activity. However, bone resorption predominates over bone formation. Activation of bone resorption results in the elevated level of calcium that inhibits PTH and $1,25(OH)_2D_3$ synthesis and then calcium absorption. In consequence these processes lead to calcium

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Abbreviations: BMD, bone mineral density; FN, femoral neck; GD, Graves' disease; LS, lumbar spine; PTH, parathormone; RFLP, restriction fragment length polymorphism; SNPs, single nucleotide polymorphisms; TSH, thyroid-stimulating hormone; VDR, vitamin D receptor; $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol.

deficit and secondarily stimulate PTH synthesis to restore calcium balance. Mineralization time is also reduced, which additionally decreases bone quality (Kisakol *et al*., 2003). A decreased bone mineral density (BMD) both in the lumbar spine and femoral neck as an effect of overproduction of thyroid hormones has been observed in previous studies (Greenspan & Greenspan, 1999; Skowrońska-Jóźwiak *et al*., 1999). The changes in bones caused by hyperthyroidism are mostly reversible and depend on the hormonal state of the thyroid. Normalization of thyroid function requires time to restore normal bone quality.

Vitamin D_3 regulates calcium and phosphate homeostasis. Vitamin D_3 receptor gene (*VDR* gene) is considered to contribute to the genetic background of the disturbances observed in bone metabolism (Chen *et al*., 2001; Deng *et al*., 2002; Colin *et al*., 2003; Horst-Sikorska *et al*., 2005). In the etiology of GD the association of the following *VDR* polymorphisms: *Bsm*I, *Apa*I, *Taq*I and *Fok*I with disease development has been studied (Ban *et al*., 2000a; 2000b). However, there is still no answer which polymorphic variants can predispose GD patients to decreased bone density. The main problem is the large number of metabolic processes connected with bone quality that are difficult to identify. The associations of the *VDR* gene allelic variations with bone metabolism in GD women have been investigated in different populations. Japanese females homozygous for allele F of *VDR-Fok*I polymorphism, being in disease remission for over 5 years, have a higher risk of osteoporosis (Ban *et al*., 2000b). This observation suggests that predisposition to bone loss in GD patients can be associated with allelic variants of the *VDR* gene.

The aims of this study were to test the association of *VDR* gene polymorphisms with:

1. bone mineral density of young women with GD diagnosis;
2. the predisposition to Graves' disease.

MATERIALS AND METHODS

Seventy-five premenopausal women with GD were recruited from the Endocrinology Outpatient Clinic of Juraszow Hospital (Poznan, Poland). The patients underwent a clinical and hormonal examination. The diagnosis of GD was made on the basis of the presence of clinical symptoms of hyperthyroidism, elevated fT4 (free thyroxin) with decreased TSH (thyroid-stimulating hormone) concentrations in the serum together with an elevated level of antiperoxidase antibodies (aTPO). The average age of the studied group was 37 years (range: 23–46), the average body mass: 64 kg (range: 51–98) and average height: 164 cm (range: 150–180). The control group consisted of 163 healthy unrelated women aged 47–89 years (average 65 years), with no personal or family history of GD. The advanced age of the control subjects was intentional due to the very low risk of GD and other autoimmune disease development in the elderly. The controls were not evaluated for BMD values.

The studied group was divided into two subgroups on the basis of thyroid function. The first subgroup comprised 36 women with active hypertyroidism or with normalized TSH level lasting less than 12 months (subgroup A). The second subgroup of the patients (subgroup B) comprised 39 asymptomatic women whose serum TSH level remained within the normal range for longer than 12 months.

BMD values were evaluated only in the GD patients by use of densitometry equipment (DPX-Plus, Lunar). Measurements were done for the femoral neck (FN) and lumbar spine (LS) in L1–L4 region. All BMD values were referred to standard peak bone mass of the population of 20–45 year-old women. Results over 0.980 g/cm^2 for the femur and 1.202 g/cm^2 for L1–L4 were regarded as required bone density.

DNA analysis. The analysis of *VDR* gene polymorphisms was done for all the women, both from the studied group and the controls. DNA was extracted from the whole blood leukocytes by means of guanidine isothiocyanate (GTC). The target DNA was amplified by polymerase chain reaction (PCR) and subjected to restriction fragment length polymorphism (RFLP) analysis with *Apa*I, *Bsm*I, *Taq*I, *Fok*I restriction endonucleases (Fermentas). Products of enzymatic digestion reaction were separated in 1.5% agarose gels stained with ethidium bromide. All analyses were performed according to the manufacturer's guidelines. Analyzed *VDR* polymorphisms and enzymes used to detect the base changes are shown in Table 1.

The study was approved by a local research ethics committee and all the subjects gave a written informed consent.

Statistical analysis. An analysis of relationships between bone mineral density and clinical parameters, i.e. age, body mass and height was performed with the use of the Spearman's rank correlation (Statistica v. 6.0, StatSoft). Student's *t*-test was used to compare the means of the two samples. Association analyses of each studied polymorphism with bone mineral density were performed and considered: the dose effects of particular alleles or haplotypes, and the effects of recessiveness or dominance. Those analyses were done for non-adjusted BMD and for BMD adjusted by age, body mass, and height. The significance was calculated by analysis of variance or covariance (Statistica v. 6.0, StatSoft). The impact of allele or haplotype dose on BMD was

Table 1. Polymorphisms in the *VDR* gene and the methods of their genotyping.

Methodical nomenclature recommended by Human Genome Variation Society (www.hgvs.org).

Gene	Analyzed polymorphisms		Method used to genotyping
	Common nomenclature used in paper (alleles)	Methodical nomenclature	
VDR	*Taq*I (T t)	c.1056T>C	RFLP (restriction endonuclease *Taq*I)
	*Apa*I (a A)	1025-49G>T	RFLP (restriction endonuclease *Bsp*120I)
	*Bsm*I (b B)	1024+283G>A	RFLP (restriction endonuclease *Mva*1269I)
	*Fok*I (f F)	c.2T>C	RFLP (restriction endonuclease *Bse*GI)

analyzed by simple regression (non-adjusted BMD) or multiple regression (adjusted BMD) (Statistica v. 6.0, StatSoft). A case-control analysis of the association of *VDR* gene polymorphisms with the development of Graves' disease was also performed. This analysis was done for the following effects: effect of allele dose (calculated by Armitage's trend test), and effects of recessiveness and dominance (Person's $\chi 2$ test followed by calculation of odds ratio in case of a significant value of the $\chi 2$ test). The comparison of allele frequencies between the controls and GD's patients was done with the Person's $\chi 2$ test.

The analysis of the linkage disequilibrium (LD) of studied polymorphisms was performed with the use of Haploview v. 3.11 program available on www.broad.mit.edu/mpg/haploview/index.php. This method was used to provide a D prime (D') value. A D' value of 0 indicated no LD between different polymorphisms, and D' value of 1 indicated complete LD.

RESULTS

Mean BMD values for the femoral neck (FN) and lumbar spine (LS) were compared in women with GD. In subgroup A the bone density values were decreased as compared to standard peak bone mass, particularly in LS. DPX parameters were also analyzed according to the time of TSH level normalization. The BMD values were lower in the group of patients with decreased TSH or euthyreosis lasting shorter than 12 months (subgroup A) in comparison with the women with TSH normalized for longer than or at least 12 months (subgroup B, see Table 2), but no significant influence of the time of hyperthyroidism remission on the increase of BMD value was observed. Results, given below, suggest only a tendency to a bone mass increase in the lumbar spine and in the femoral neck depending on the duration of the time of hyperthyroidism symptoms' regression.

Table 2. Comparison of mean BMD values in subgroups

	mean BMD for FN (g/cm^2) ± S.D.	mean BMD for LS (g/cm^2) ± S.D.
Subgroup A (n=36)	0.971 ± 0.134	1.154 ± 0.175
Subgroup B (n=39)	1.004 ± 0.025	1.198 ± 0.139
p-value	0.178	0.529

An analysis of Spearman's rank correlation coefficient indicated an influence of both body mass and height on BMD values both for the lumbar spine and femoral neck in the studied group of patients. Age was significantly correlated with the FN BMD value only (Table 3).

Table 3. Analysis of correlation regarding age, body mass, height and time between making a diagnosis of hyperthyroidism and bone densitometry in women with GD

	BMD value for FN		BMD value for LS	
	Spearman's rank correlation coefficient	p level	Spearman's rank correlation coefficient	p level
Age	r = –0.235	***p = 0.043***	r = –0.124	p = 0.290
Body mass	r = 0.282	***p = 0.014***	r = 0.333	***p = 0.004***
Height	r = 0.308	***p = 0.007***	r = 0.262	***p = 0.023***
Period between diagnosis of hyperthyroidism and BMD measurement : <12 months	r = –0.007	p = 0.966	r = 0.095	p = 0.575
Period between diagnosis of hyperthyroidism and BMD measurement : ≥12 months	r = –0.115	p = 0.492	r = –0.030	r = 0.858

Table 4. Distribution of *VDR* gene polymorphisms and association analysis of certain polymorphisms with FN and LS BMD

VDR BsmI		Number of subjects (n)	Non-adjusted BMD value for FN (g/cm^2)	Adjusted BMD value for FN (g/cm^2)	Non-adjusted BMD value for LS (g/cm^2)	Adjusted BMD value for LS (g/cm^2)
Allele dose effect		n = 75				
Allele dose	BB	7 (9.3%)	0.955 (± 0.181)	0.974 (± 0.136)	1.171 (± 0.258)	1.211 (± 0.150)
	Bb	40 (53.3%)	0.975 (± 0.159)	0.977 (± 0.133)	1.160 (± 0.149)	1.161 (± 0.147)
	bb	28 (37.3%)	0.997 (± 0.115)	0.988 (± 0.134)	1.185 (± 0.152)	1.173 (± 0.148)
Significance			p = 0.437	p = 0.736	p = 0.642	p = 0.788
Effects of recessiveness and dominance						
Genotype	BB+Bb	47 (62.7%)	0.972 (± 0.161)	0.977 (± 0.132)	1.161 (± 0.166)	1.169 (± 0.147)
	bb	28 (37.3%)	0.997 (± 0.115)	0.988 (± 0.133)	1.185 (± 0.152)	1.173 (± 0.148)
Significance			p = 0.479	p = 0.729	p = 0.545	p = 0.913
Genotype	BB	7 (9.3%)	0.955 (± 0.181)	0.974 (± 0.135)	1.171 (± 0.258)	1.211 (± 0.149)
	Bb+bb	68 (90.7%)	0.984 (± 0.142)	0.982 (± 0.131)	1.170 (± 0.150)	1.166 (± 0.145)
Significance			p = 0.625	p = 0.885	p = 0.993	p = 0.448
VDR ApaI						
Allele dose effect		n = 75				
Allele dose	AA	18 (24.0%)	0.979 (± 0.163)	0.989 (± 0.133)	1.184 (± 0.185)	1.199 (± 0.145)
	Aa	42 (56.0%)	0.976 (± 0.151)	0.981 (± 0.132)	1.143 (± 0.148)	1.145 (± 0.144)
	Aa	15 (20.0%)	0.998 (± 0.110)	0.972 (± 0.136)	1.230 (± 0.153)	1.206 (± 0.148)
Significance			p = 0.737	p = 0.716	p = 0.487	p = 0.980
Effects of recessiveness and dominance						
Genotype	AA+Aa	60 (80.0%)	0.977 (± 0.153)	0.983 (± 0.131)	1.155 (± 0.160)	1.161 (± 0.145)
	Aa	15 (20.0%)	0.998 (± 0.110)	0.972 (± 0.135)	1.230 (± 0.153)	1.206 (± 0.149)
Significance			p = 0.620	p = 0.781	p = 0.107	p = 0.303
Genotype	AA	18 (24.0%)	0.979 (± 0.163)	0.989 (± 0.132)	1.184 (± 0.185)	1.200 (± 0.145)
	Aa+aa	57 (76.0%)	0.982 (± 0.140)	0.979 (± 0.131)	1.166 (± 0.153)	1.161 (± 0.144)
Significance			p = 0.955	p = 0.765	p = 0.675	p = 0.329
VDR TaqI						
Allele dose effect		n = 75				
Allele dose	TT	30 (40.0%)	0.998 (± 0.114)	0.991 (± 0.135)	1.187 (± 0.147)	1.176 (± 0.149)
	Tt	39 (52.0%)	0.972 (± 0.159)	0.976 (± 0.134)	1.156 (± 0.150)	1.159 (± 0.148)
	tt	6 (8.0%)	0.959 (± 0.198)	0.966 (± 0.137)	1.181 (± 0.281)	**1.217** (± 0.152)
Significance			p = 0.416	p = 0.586	p = 0.616	p = 0.892
Effects of recessiveness and dominance						
Genotype	TT+Tt	69 (92.0%)	0.983 (± 0.141)	0.983 (± 0.131)	1.169 (± 0.149)	1.166 (± 0.145)
	tt	6 (8.0%)	0.959 (± 0.198)	0.966 (± 0.137)	1.181 (± 0.281)	1.217 (± 0.151)
Significance			p = 0.695	p = 0.772	p = 0.860	p = 0.433
Genotype	TT	30 (40.0%)	0.998 (± 0.114)	0.991 (± 0.134)	1.187 (± 0.147)	1.176 (± 0.149)
	Tt+tt	45 (60.0%)	0.970 (± 0.162)	0.974 (± 0.133)	1.159 (± 0.169)	1.166 (± 0.148)
Significance			p = 0.422	p = 0.276	p = 0.467	p = 0.793
VDR FokI						
Allele dose effect		n = 75				

	FF	21 (28.0%)	0.953 (± 0.127)	0.949 (± 0.133)	1.154 (± 0.130)	1.156 (± 0.149)
Allele dose	Ff	41 (54.7%)	0.992 (± 0.156)	0.998 (± 0.130)	1.176 (± 0.181)	1.182 (± 0.146)
	ff	13 (17.3%)	0.991 (± 0.139)	0.981 (± 0.134)	1.180 (± 0.144)	1.155 (± 0.150)
Significance			p = 0.391	p = 0.384	p = 0.617	p = 0.911
Effects of recessiveness and dominance						
Genotype	FF+Ff	62 (82.7%)	0.979 (± 0.14)	0.981 (± 0.131)	1.168 (± 0.165)	1.174 (± 0.145)
	ff	13 (17.3%)	0.991 (± 0.139)	0.980 (± 0.134)	1.180 (± 0.144)	1.154 (± 0.149)
Significance			p = 0.787	p = 0.969	p = 0.817	p = 0.665
Genotype	FF	21 (28.0%)	0.953 (± 0.127)	0.949 (± 0.132)	1.154 (± 0.130)	1.156 (± 0.148)
	Ff+ff	54 (72.0%)	0.992 (± 0.151)	0.994 (± 0.130)	1.177 (± 0.171)	1.176 (± 0.146)
Significance			p = 0.297	p = 0.191	p = 0.586	p = 0.598

Association analysis of *VDR* gene polymorphisms with BMD

Each polymorphism in Graves' patients was analyzed in respect of its association with bone mineral density within the femoral neck (FN BMD) and lumbar spine (LS BMD). The analysis showed no significant allele-dose effect on BMD values for FN or LS. These results concerned all polymorphisms and BMD values, both non-adjusted and adjusted by age, body mass, or height. The analyzed effects of recessiveness and dominance revealed also no influence of *VDR* polymorphisms on the adjusted and non-adjusted BMD values in GD patients. The results are shown in Table 4.

Association analysis of *VDR* gene haplotypes with BMD

Strong linkage disequilibrium (LD) was revealed for three polymorphisms: *Bsm*I, *Apa*I and *Taq*I localized in 3' end of the *VDR* gene. The strongest LD (D' = 1.0) was observed for *Bsm*I, and *Taq*I and weaker for *Apa*I and *Bsm*I (D' = 0.98). The three most frequent haplotypes among the studied women were: baT, BAt and bAT (99.2%). Haplotype baT was present in 47.9%, BAt in 34.9% and bAT in 16.4% of the women. The most frequent haplotypes were studied for their association with bone density both for FN and LS. There was no association of baT, BAt, and bAT haplotypes with BMD. No dose effect was observed, nor the effects of recessiveness or dominance. We may only suggest that the lowest BMD (adjusted and non-adjusted) were observed in FN of GD women homozygous for the BAt haplotype. In turn, the lowest BMD for LS concerned heterozygotic women with one copy of the bAT haplotype. The results were not statistically significant and are shown in Table 5.

Association analysis of SNPs and haplotypes with Graves' disease — case-control study

The analysis of association with predisposition to the disease was performed for all *VDR* gene polymorphisms as well as for the most frequent haplotypes (baT, BAt, bAT). Only the presence of the dominant allele F of the *Fok*I polymorphism was found to have a weak tendency to be associated with Graves' disease (p = 0.058). The risk of GD development, estimated by odds ratio, was almost two times higher for individuals carrying allele F (see Table 6). For the other polymorphisms there were no statistically significant associations, so the allele dose effect or effects of recessiveness and dominance were not observed. No association was also found for the most frequent haplotypes (Table 7). The distribution of alleles between the patients and controls was compared as shown in Table 6.

DISCUSSION

The presented study was concerned for 75 young premenopausal, regularly menstruating women with diagnosed Graves' disease who had similar lifestyles and did not suffer from other diseases affecting bone metabolism. In this way estrogens deficit — one of the most important factors for bone loss — was likely eliminated and an excess of thyroid hormones was the most probable reason for BMD decrease. Association of *VDR* gene polymorphisms (*Bsm*I, *Apa*I, *Taq*I, *Fok*I) with bone mineral density as well as with predisposition to Graves' disease was studied.

The bone mineral density in the 75 women with Graves' disease was significantly correlated with the body mass and height. Higher weight is related to a higher bone mass. It means that the stronger the mechanical stress on the bone is the

Table 5. Association analysis of the 3′ end polymorphism haplotypes of *VDR* gene (baT, BAt, bAT) with BMD values for FN and LS

VDR baT		Number of samples (n)	Non-adjusted BMD value for FN (g/cm^2)	Adjusted BMD value for FN (g/cm^2)	Non-adjusted BMD value for LS (g/cm^2)	Adjusted BMD value for LS (g/cm^2)
Dose effect of haplotype		n = 75				
	0	18 (24.7%)	0.979 (± 0.163)	0.989 (± 0.134)	1.184 (± 0.185)	1.199 (± 0.146)
Haplotype dose	1	40 (54.8%)	0.974 (± 0.152)	0.978 (± 0.134)	1.139 (± 0.151)	1.142 (± 0.146)
	2	15 (20.5%)	0.998 (± 0.110)	0.975 (± 0.138)	1.230 (± 0.153)	1.206 (± 0.150)
Significance			p = 0.741	p = 0.754	p = 0.495	p = 0.973
Effects of recessiveness and dominance						
Genotype	0+1	58 (79.5%)	0.976 (± 0.154)	0.982 (± 0.133)	1.153 (± 0.162)	1.159 (± 0.147)
	2	15 (20.5%)	0.998 (± 0.110)	0.975 (± 0.137)	1.230 (± 0.153)	1.207 (± 0.151)
Significance			p = 0.602	p = 0.864	p = 0.103	p = 0.289
Genotype	0	18 (24.7%)	0.979 (± 0.163)	0.989 (± 0.133)	1.184 (± 0.185)	1.200 (± 0.147)
	1+2	55 (75.3%)	0.981 (± 0.141)	0.977 (± 0.132)	1.164 (± 0.156)	1.159 (± 0.146)
Significance			p = 0.978	p= 0.748	p = 0.652	p = 0.312
VDR BAt						
Dose effect of haplotype		n = 75				
	0	28 (38.4%)	0.997 (± 0.115)	0.990 (± 0.136)	1.185 (± 0.152)	1.173 (± 0.151)
Haplotype dose	1	39 (53.4%)	0.972 (± 0.159)	0.975 (± 0.135)	1.156 (± 0.150)	1.159 (± 0.150)
	2	6 (8.2%)	**0.959** (± 0.198)	**0.968** (± 0.139)	1.181 (± 0.281)	1.200 (± 0.154)
Significance			p = 0.445	p = 0.637	p = 0.666	p = 0.834
Effects of recessiveness and dominance						
Genotype	0+1	67 (91.8%)	0.982 (± 0.142)	0.981 (± 0.132)	1.168 (± 0.151)	1.165 (± 0.146)
	2	6 (8.2%)	0.959 (± 0.198)	0.968 (± 0.138)	1.181 (± 0.281)	1.216 (± 0.153)
Significance			p = 0.708	p = 0.818	p = 0.847	p = 0.432
Genotype	0	28 (38.4%)	0.997 (± 0.115)	0.990 (± 0.135)	1.185 (± 0.152)	1.173 (± 0.150)
	1+2	45 (61.6%)	0.970 (± 0.162)	0.974 (± 0.133)	1.159 (± 0.169)	1.166 (± 0.149)
Significance			p = 0.453	p = 0.643	p = 0.514	p = 0.861
*VDR b*AT						
Dose effect of haplotype		n = 75				
	0	52 (71.2%)	0.978 (± 0.148)	0.976 (± 0.131)	1.180 (± 0.171)	1.178 (± 0.145)
Haplotype dose	1	18 (24.7%)	0.971 (± 0.152)	0.976 (± 0.133)	**1.116** (± 0.131)	**1.126** (± 0.147)
	2	3 (4.1%)	1.066 (± 0.036)	1.080 (± 0.136)	1.292 (± 0.018)	1.274 (± 0.150)
Significance			p = 0.611	p = 0.409	p = 0.786	p = 0.833
Effects of recessiveness and dominance						
Genotype	0+1	70 (95.9%)	0.977 (± 0.148)	0.976 (± 0.130)	1.164 (± 0.164)	1.165 (± 0.145)
	2	3 (4.1%)	1.066 (± 0.036)	1.080 (± 0.135)	1.292 (± 0.018)	1.272 (± 0.150)
Significance			p = 0.300	p = 0.195	p = 0.182	p = 0.231
Genotype	0	52 (71.2%)	0.978 (± 0.148)	0.976 (± 0.132)	1.180 (± 0.171)	1.177 (± 0.147)
	1+2	21 (28.8%)	0.985 (± 0.145)	0.991 (± 0.133)	1.141 (± 0.137)	1.148 (± 0.148)
Significance			p = 0.869	p = 0.655	p = 0.360	p = 0.443

more effective are the processes of osteosynthesis and mineralization, which leads to a higher peak bone mass (Walker-Bone *et al*., 2002). The impact of weight may also be explained by additional estrogen (estron) synthesis in adipose tissue, which prevents bone loss, as was previously reported for primary

Table 6. Association analysis of *VDR* polymorphisms (*Taq*I, *Apa*I, *Bsm*I, *Fok*I) with Graves' disease

Polymorphisms in ***VDR*** gene		Genotype frequency						
		Controls		GD				
		n		n	(%)	Effect of dominance and recessiveness		
*VDR Fok*I		n=163		n=75			d.f.=1	
	FF	40	(24.5%)	21	(28.0%)	FF *vs*. Ff+ff	$\chi2 = 0.32$	p = 0.570
Genotype	Ff	76	(46.6%)	41	(54.7%)			
	ff	47	(28.8%)	13	(17.3%)	ff *vs*. Ff+FF	$\chi2 = 3.60$	***p = 0.058***
Pearson's $\chi2$		$\chi2 = 3.61$		d.f.=2	*p = 0.164*	**OR = 1.93**		[0.97 – 3.84]
Armitage's $\chi2$ trend test		$\chi2 = 2.26$		d.f.=1	*p = 0.133*			
*VDR Taq*I		n=163		n=75			d.f.=1	
	TT	65	(39.9%)	30	(40.0%)	TT *vs*. Tt+tt	$\chi2 = 0.00$	p = 0.986
Genotype	Tt	75	(46.0%)	39	(52.0%)			
	tt	23	(14.1%)	6	(8.0%)	tt *vs*. Tt+TT	$\chi2 = 1.79$	p = 0.181
Pearson's $\chi2$		$\chi2 = 1.96$		d.f.=2	*p = 0.376*			
Armitage's $\chi2$ trend test		$\chi2 = 0.45$		d.f.=1	*p = 0.503*			
*VDR Apa*I		n=163		n=75			d.f.=1	
	AA	36	(22.1%)	18	(24.0%)	AA *vs*. Aa+aa	$\chi2 = 0.11$	p = 0.743
Genotype	Aa	90	(55.2%)	42	(56.0%)			
	aa	37	(22.7%)	15	(20.0%)	aa *vs*. Aa+AA	$\chi2 = 0.22$	p = 0.640
C		$\chi2 = 0.26$		d.f.=2	*p = 0.878*			
Armitage's $\chi2$ trend test		$\chi2 = 0.25$		d.f.=1	*p = 0.620*			
*VDR Bsm*I		n=163		n=75			d.f.=1	
	BB	23	(14.1%)	7	(9.3%)	BB *vs*. Bb+bb	$\chi2 = 1.06$	p = 0.302
Genotype	Bb	77	(47.2%)	40	(53.3%)			
	bb	63	(38.7%)	28	(37.3%)	bb *vs*. Bb+BB	$\chi2 = 0.04$	p = 0.846
Pearson's $\chi2$		$\chi2 = 1.34$		d.f.=2	*p = 0.511*			
Armitage's $\chi2$ trend test		$\chi2 = 0.14$		d.f.=1	*p = 0.709*			

Table 7. Analysis of association between baT, BAt, bAT haplotypes and Graves' disease

Haplotypes for 3′ end of VDR gene polymorphisms		Number of patients with two copies, one copy or without respective haplotype						
		Controls		GD				
		n		n	(%)	Effect of dominance and recessivness		
baT		n=161		n=73			d.f.=1	
	0	36	(22.4%)	18	(24.7%)	0 *vs*. 1+2	$\chi2 = 0.15$	p = 0.699
Haplotype dose	1	88	(54.7%)	40	(54.8%)			
	2	37	(23.0%)	15	(20.5%)	2 *vs*. 1+0	$\chi2 = 0.17$	p = 0.678
Pearson's $\chi2$		$\chi2 = 0.25$		d.f.=2	*p = 0.883*			
Armitage's $\chi2$ trend test		$\chi2 = 0.25$		d.f.=1	*p = 0.618*			
BAt		n=161		n=73			d.f.=1	
	0	63	(39.1%)	28	(38.4%)	0 *vs*. 1+2	$\chi2 = 0.01$	p = 0.910
Haplotype dose	1	75	(46.6%)	39	(53.4%)			
	2	23	(14.3%)	6	(8.2%)	2 *vs*. 1+0	$\chi2 = 1.70$	p = 0.192
Pearson's $\chi2$				d.f.=2	*p = 0.371*			
Armitage's $\chi2$ trend test				d.f.=1	*p = 0.573*			
bAT		n=161		n=73			d.f.=1	
	0	124	(77.0%)	52	(71.2%)	0 *vs*. 1+2	$\chi2 = 0.90$	p = 0.342
Haplotype dose	1	35	(21.7%)	18	(24.7%)			
	2	2	(1.2%)	3	(4.1%)	2 *vs*. 1+0	$\chi2 = 1.98$	p = 0.160
Pearson's $\chi2$				d.f.=2	*p = 0.310*			
Armitage's $\chi2$ trend test				d.f.=1	*p = 0.210*			

osteoporosis. A positive correlation was also shown for BMD and age in the femoral neck but not in the lumbar spine. The reason for the significant correlation between age and FN BMD value is not so obvious. It may result from the strong influence of estrogens on bone turnover in young women particularly in this region of the skeleton. Because age, body mass and height were strongly correlated with bone mineral density, the association analyses were performed both for adjusted and non-adjusted BMD to avoid imprecise results.

Hyperthyroidism results in accelerated bone loss, by leading to a higher bone turnover. Osteoporosis induced by hyperthyroidism is usually reversible as long as there are no osteoporotic fractures. Trabecular bone is more metabolically active and more sensitive to unfavorable factors, so BMD changes in lumbar spine appear rapidly after hyperthyreoidism manifestation. However, the bone density reduction confirmed in DPX measurements concerned both trabecular (LS) and cortical (FN) bone (Diamond *et al*., 1991). In our study lower BMD values (related to peak bone mass) were found in both locations in patients with hyperthyroidism and those with normalized TSH level lasting less than 12 months. Bone mass was comparably improved both in the trabecular bone and in the compact one when comparing both subgroups of patients. Mean BMD values were higher both in LS and FN in women with euthyreosis lasting over 12 months, but no statistically significant influence of the duration of hyperthyroidism remission on BMD value was found. The increase of bone mineral density after TSH normalization confirms the hypothesis that the rate of bone repair processes after elimination of an unfavorable factor is high; after 1 year the BMD reached values corresponding to peak bone mass. We hypothesize that young women with sufficient action of estrogens, a strong protective factor, promptly eliminate the consequence of excess of thyroid hormones for both spongy and trabecular bones. On the other hand, treatment of GD hyperthyroidism can additionally sufficiently improve bone mineral density. Achieving euthyreosis does not restore bone quality immediately. According to previous studies the recovery of balance between bone formation and resorption takes from 7.5 to 12 months after TSH normalization and this period is usually sufficient for the BMD value to increase (Jodar *et al*., 1997; Horst-Sikorska *et al*., 2005). Much earlier, after just 6 months, the biochemical markers of bone metabolism reach normal values (Diamond *et al*., 1994).

The answer to the question what causes the differences in bone conditions among the analyzed patients with GD has been searched through the analysis of polymorphic variants of several genes, the function of their protein products, and literature data about their function in osteoporosis. Research on polymorphic alleles of genes that control bone mineral metabolism makes it possible to select patients with susceptibility to bone mass reduction. Several vitamin D receptor gene polymorphisms are among the most important and the most frequently analyzed genetic risk factors for primary osteoporosis (Ferrari *et al*., 1998; Braga *et al*., 2002; Morita *et al*., 2004). Morrison *et al*. (1994; 1997) observed an association of allele B of *Bsm*I polymorphism with lower BMD in the lumbar spine and femoral neck. According to Langdahl *et al*. (2000) BB and Bb genotypes were also more frequent in patients with osteoporotic fractures. Investigation of the *VDR* polymorphisms in relation to osteoporosis induced by hyperthyroidism was less common. Our study did not demonstrate a statistically significant association between the analyzed polymorphisms of *VDR* gene and mineral density in young women with Graves' disease. Results of similar studies for different populations are discrepant (Obermayer-Pietsch *et al*., 2000; Ban *et al*., 2000a; 2000b). Obermayer-Pietsch showed an association between the BB genotype of *VDR-Bsm*I and low bone mass in Austrian patients with diagnosed hyperthyroidism. There are also a few studies not confirming any associations that are related to our results. No significant correlation of *VDR* gene allelic variations with the BMD in Japanese GD women was found (Ban *et al*., 2000b). However, those authors noticed that Japanese female homozygous for allele F of *VDR-Fok*I polymorphism had higher risk of osteoporosis when remission lasted less than 5 years. In turn, a reports on premenopausal women (Dutch, Canadian) revealed an association of allele b of *VDR-Bsm*I with lower bone mineral density (Hansen *et al*., 1998; Rubin *et al*., 1999). Similar conclusions concerned *Fok*I polymorphic variants and bone metabolism. Among different populations this polymorphism in premenopausal women was investigated. The authors proved that the ff genotype predisposed to decreased mineral density in the lumbar spine (Kubota *et al*., 2001).

The localization of polymorphisms very close to each other within a single gene gives a chance for linkage disequilibrium and formation of haplotypes. A particular allele of one polymorphism present on a chromosome together with another polymorphism allele make an arrangement of alleles inherited together and called a haplotype. Analysis of haplotypes rather than of single polymorphisms increases the chance for a more precise detection of any association between gene polymorphisms and the studied feature, e.g. BMD value. The *Fok*I polymorphism is localized over 30 kbp away from the *Bsm*I, *Apa*I, and *Taq*I polymorphisms and that is why it may not

show linkage disequilibrium. Here we found strong linkage disequilibrium for the *VDR* 3' end polymorphisms: *Bsm*I, *Apa*I and *Taq*I, which formed three the most frequent haplotypes in Graves' women: baT, Bat and bAT, with the prevalence of baT. These results are consistent with previous studies for both Polish and other European populations (Kalak *et al*., 2000; Ramoz-Lopez *et al*., 2005). The most frequent haplotypes were studied for their association with bone mineral density of femur or lumbar spine. Among the studied patients, women homozygous for the BAt haplotype had the lowest BMD values (both adjusted and non-adjusted) in the femoral neck. In turn, the lowest BMD for LS concerned heterozygotic women with one copy of the bAT haplotype. Unfortunately our results were not statistically significant and require further studies on a larger group.

The *VDR* gene was also investigated for a role in genetic susceptibility to Graves' disease. The distribution of genotype frequencies differed between patients and controls. A comparison between healthy women and GD cases showed that the f allele of *Fok*I polymorphism was more common in controls. In contrast, the presence of F allele was more frequent in Graves' patients, which may suggest predisposition to the disease in F allele carriers. The odds ratio indicated a 2-fold higher risk of disease in women with the F allele, however, with low level of significance ($p = 0.058$). Japanese authors (Ban *et al*., 2000b) found overrepresentation of the FF genotype in women with Graves' disease with statistical significance, which suggests that the F allele may predispose to GD. Other polymorphic variants, *Bsm*I and *Apa*I, were also studied in the Japanese (Ban *et al*., 2000a). Authors found that carriers of B and A alleles were predisposed to Graves' disease. Previous Polish studies revealed that the BB and FF genotypes are also more frequent among GD patients, thus increasing susceptibility to the disease. In turn, the distribution of *Apa*I and *Tag*I *VDR* polymorphisms was comparable to that in control subjects (Kuryłowicz *et al*., 2005). Unfortunately, recent English report concerning 768 cases did not find any associations between Graves' disease and *VDR* gene polymorphisms (Collins *et al*, 2004).

In summary, the four studied polymorphisms (*Bsm*I, *Apa*I, *Taq*I, *Fok*I) of the *VDR* gene do not predict the risk of decreased BMD in young women with Graves'. This is why the relationship between *VDR* polymorphisms and the risk of bone mineral density decrease induced by an excess of thyroid hormones in Polish GD patients is not certain. However, it may be speculated that F allele carriers of the *VDR-Fok*I polymorphism may be predisposed to Graves' disease.

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