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# Association between estrogen receptor alpha gene polymorphisms and bone mineral density in Polish female patients with Graves' disease

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**Graves' (GD) hyperthyroidism leads to reduced bone mineral density (BMD) accompanied by accelerated bone turnover. Ample studies have identified association between estrogen receptor *(ESR1)* gene polymorphism and decreased BMD and osteoporosis. In contrast, number of publications that link *ESR1*, BMD and Graves' disease is limited. The purpose of this study was to identify the association between *ESR1* polymorphisms and BMD in premenopausal women with GD and to determine whether *ESR1* polymorphic variants can predispose to GD. The study included 75 women aged 23–46 years with GD and 163 healthy controls. BMD was measured at lumbar spine and femoral neck. We investigated two SNPs in the *ESR1* gene and analyzed genetic variants in the form of haplotypes reconstructed by statistical method. Three out of four possible haplotypes of the *Pvu*II and *Xba*I restriction fragment length polymorphisms were found in GD patients: px (55.3%), PX (33.3%) and Px (11.4%). Women homozygous for xx of *Xba*I and for pp of *Pvu*II had the lowest BMD at lumbar spine. Moreover, the px haplotype predisposed to reduced lumbar BMD. No associations were observed for femoral neck BMD. No statistically significant relationship were found between *ESR1* polymorphisms or their haplotypes and GD. These results indicate that the *Pvu*II and the *Xba*I polymorphisms of *ESR1* gene are associated with bone mineral density in premenopausal women with GD and may help to estimate the risk of bone loss particularly at lumbar spine. However, none of the *ESR1* gene alleles predict the risk of GD in Polish female patients.**

**Keywords:** *ESR1* gene polymorphisms, bone mineral density, Graves' disease, premenopausal women

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## INTRODUCTION

Hyperthyroidism is diagnosed in 2% of adult population, predominantly in women. Approximately 60–80% of cases of hyperthyroidisms are due to Graves' disease (GD) that tends to affect women in their third and fourth decade of life. This autoimmune, chronic disease has recurrent nature and affects many tissues, including bone. The disease has a strong genetic background associated with polymorphisms in the *HLA* and *CTLA-4* (cytotoxic T lymphocyte associated 4 genes (Donner *et al.*, 1997).

Hyperthyroidism disturbs bone metabolism and is an important risk factor for secondary osteoporosis. The pathophysiology is multifactorial. An excess of thyroid hormones induces both acceleration of bone turnover due to an increased number of active osteoclasts, increased osteoblasts activity and shortening of the bone remodeling cycle (Eriksen *et al.*, 1995; Murphy & Wiliams, 2004). This kind of bone damage is usually reversible. The duration of exposure to an excess of thyroid hormones is a major factor determining bone condition. The longer the time of hyperthyroidism, the greater decrease of bone mass. Normalization of bone status usually takes 6–12 months after achieving euthyreosis. Biochemical markers of bone metabolism come to normal ranges sooner, usually within 6 months (Diamond *et al.*, 1994). A BMD (bone mineral density) reduction in the axial skeleton is observed irrespective of gender. Higher incidence of osteoporosis in women concerns both primary and hyperthyroidism-related osteoporosis. Here, age is the most important factor for bone quality, because estrogen status has a strong influence on bone turnover. Thus, hyperthyroid premenopausal females without an estrogen deficiency are an adequate group to estimate the influence of high level of thyroid hormones and autoimmune process on bone density (Diamond *et al.*, 1994).

Bone mineral density is determined by large number of genes. The influence of genetic factors on bone metabolism and fracture risk has already been investigated widely.

Considering the important role of estrogens in bone turnover and their protective effect on BMD, numerous studies have targeted estrogen receptor alpha (*ESR1*) gene, in particular its polymorphisms defined by the restriction enzymes *Xba*I and *Pvu*II, to evaluate its association with bone mineral density. However, the results are inconsistent and the effects of these two polymorphisms on BMD and fracture risk remain ambiguous (Ioannidis *et al.,* 2002; Yamada *et al.*, 2002; van Meurs *et al.*, 2003; Ioannidis *et al.*, 2004). Estrogen receptor also modulates the transcription of target genes in response to estrogens, which appears to play an important role in immune response and immune-mediated diseases (Cutolo *et al.*, 1995).

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**Abbreviations:** BMD, bone mineral density; ESR1, estrogen receptor alpha; FN, femoral neck; GD, Graves' disease; LS, lumbar spine

We have recently showed that some polymorphic variants of another candidate gene — *VDR* (vitamin D receptor) — may predispose women to Graves' disease, but do not predict the risk of reduced BMD in these patients (Horst-Sikorska *et al.*, 2008).

In view of the possible role of estrogens in the pathogenesis of GD as well as their effect on BMD, the aim of this study was to identify the relationship between the *ESR1* polymorphisms and bone mineral density in premenopausal women with Graves' and to determine whether *ESR1* polymorphic variants predispose females to the autoimmune disease of the thyroid gland.

## MATERIALS AND METHODS

Patients were recruited from the Outpatient Endocrinology Clinic of District Hospital in Poznań. The study group comprised 75 premenopausal women 26–46 years old (average age 37 years) previously diagnosed with Graves' disease. Patients were checked for past medical history, underwent physical examination, and a variety of laboratory parameters (see below) and bone mineral density were measured. They did not report relevant complaints regarding the central skeleton, fractures, or loss of height, and regularly menstruating at the time of the study. The study group was homogenous for physical activity (below 2 h weekly), smoking habits (non-smokers) and alcohol consumption (less than 1 unit daily). Family history of osteoporotic fractures was not analyzed. The presence of significant co-morbidities influencing bone metabolism (e.g., hyperparathyroidism, diabetes mellitus, renal failure, hematological diseases, severe gastroenterological disorders) was a reason for exclusion from the study.

Laboratory tests to assess the patients' endocrine status, recommended at the beginning of the study, included free thyroxine (fT4; normal range: 11.5–21 pmol/L), free triiodothyronine (fT3; normal range: 3.95–6.3 pmol/L), thyroid-stimulating hormone (TSH; normal range: 0.27–4.2 mU/L) and TSH-receptor antibodies (TSHRAb; normal range: <2 IU/L). The diagnosis of GD hyperthyroidism was established by the presence of clinical symptoms, elevated fT4 and/or fT3 with decreased TSH serum concentrations together with elevated level of TSHRAb.

Patients were divided into two groups on the basis of thyroid function. The first group comprised both women freshly diagnosed with overt hypertyroidism and patients with the TSH level normalized for less than 3 months. Women whose serum TSH level had remained within normal ranges for over 12 months formed the other group of patients. In those patients active hyperthyroidism, confirmed by lab tests, had lasted for 3–9 months.

Bone mineral density was assessed by dual energy X-ray absorptiometry (DEXA) with a Lunar DPX-Plus device calibrated with a spine phantom. The BMD measurements concerned lumbar spine (LS, vertebrae L1–L4) and femoral neck (FN). Osteoporosis was defined as a T-score below –2.5SD according to WHO criteria (Kanis, 2007). The results of BMD measurements were interpreted with emphasis on the differences between particular genotypes and haplotypes.

The control group consisted of 163 healthy unrelated females aged 47–89 years (average 65 years), with no personal or family history of autoimmune diseases. Older women were chosen as controls deliberately, due to generally lower incidence of autoimmune disease, particularly GD, in older population. Each subject was given written informed consent.

The study was approved by the local ethics committee.

**Genotyping.** The analysis of polymorphisms in the first intron of *ESR1* gene was performed for GD patients and the controls. Genomic DNA was isolated from peripheral blood by the standard method with guanidine izotiocyanate (GTC) and analyzed by the method reported by Yaich *et al.* (1992) and Kobayashi *et al.* (1996) with own modification. Genomic DNA (200 ng) was amplified in 20 μl of buffer solution (50 mM KCl, 10 mM Tris/HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.25 mM dNTP). *Taq* polymerase (0.5 unit; Sigma) and 7.5 pmol of each oligonucleotide primer. PCR reaction was performed for 30 cycles with the following steps: denaturation at 94 °C for 40 s, annealing at 57 °C for 40 s and extension at 72 °C for 100 s. PCR product was digested with *Pvu*II and *Xba*I restriction endonucleases (Fermentas) for restriction fragment length polymorphism (RFLP) analysis. Products of digestion were separated in 1.5% agarose gels and stained with ethidium bromide. The possible genotypes were: PP, Pp, pp for *Pvu*II and XX, Xx, xx for *Xba*I, where P and X denote the absence, while p and x the presence of the restriction sites.

**Statistical analysis.** Student's *t*-test was used to compare the means of two samples. To check whether data follow normal distribution Shapiro-Wilks test was used. The assumption of homogeneity of variances was analyzed by F test. Relationships between BMD and clinical parameters — age, body mass, weight and height — were analyzed by Spearman's rank correlation coefficient (Statistica v.6.0, StatSoft). Analysis of the compliance of distribution of genotypes and haplogenotypes with the distribution in the Hardy-Weinberg equilibrium was carried out by a program available at http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. Hardy-Weinberg equilibrium was found for all the polymorphisms and haplogenotypes analyzed. The analysis of the linkage disequilibrium (LD) of examined polymorphisms was performed with the assistance of the 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

**Table 1. Polymorphisms in the *ESR1* and methods of their genotyping**
methodical nomenclature recommended by Human Genome Variation Society (www.hgvs.org)

| Gene | Analyzed polymorphisms | | | Method used for genotyping |
|---|---|---|---|---|
| | SNP number (db SNP) | Common nomenclature used in paper (alleles) | Methological nomenclature | |
| *ESR1* | rs2234693 | *Pvu*II (p P) | c.453-397T>C | RFLP (restriction endonuclease *Pvu*II) |
| | rs9340799 | *Xba*I (x X) | c.453-351A>G | RFLP (restriction endonuclease *Xba*I) |

**Table 2. Mean BMD values in studied group**

| | mean value for FN BMD (g/cm$^2$) | mean value for LS BMD (g/cm$^2$) |
|---|---|---|
| hyperthyroidism or < 3-month euthyreosis; n = 39 | 0.971±0.054 | 1.154±0.125 |
| over 12-month euthyreosis; n = 36 | 1.004±0.025 | 1.198±0.109 |
| *P* value | 0.178 | 0.529 |

0 indicates no LD between different polymorphisms, and D' value of 1 indicates complete LD.

Association analysis was performed for three possible effects of the influence of a given polymorphism: allele dose effect, effect of recessiveness and effect of dominance. Bone mineral density was considered as non-adjusted and adjusted by: age, body mass and height of subjects. The significance was calculated using the analysis of variance (ANOVA). For post-hoc analysis Tukey test was used. The impact of allele or haplotype dose on BMD was analyzed by simple regression (non-adjusted BMD) or multiple regression (adjusted BMD). In addition, an analysis of associations (*case-control* type) of *ESR1* gene polymorphisms with Graves' disease development was done. It was carried out for the following three possible effects of action: the allele dose (c2 Armitage test for trend), recessive and dominant action (c2 Person's and odds ratio for a significant value of c2 test only). All tests were analysed at the significance level of a = 0.05, and the analyses were performed using STATISTICA v. 6.0 (StatSoft Inc., http://www.statsoft.com).

## RESULTS

Densitometric parameters at femoral neck (FN) and lumbar spine (LS) were analyzed in GD patients. Mean LS BMD was 1.172 g/cm$^2$ and mean FN BMD was 0.985 g/cm$^2$ for the whole group. Only 2% of patients fulfilled the densitometric criteria of osteoporosis with a T-score below –2.5SD. A comparison of BMD between women with overt hyperthyroidism (↓TSH, ↑fT4) or short-lasting euthyreosis (normalized TSH and fT4) and those euthyreotic for longer than 12 months showed a non-significant dependence of BMD values at both lumbar spine and femoral neck on the duration of hyperthyroidism regression.

The BMD values were lower in the group of patients with decreased TSH or TSH normalized for less than 3 months than in the group with normalized TSH for at least 12 months (see Table 2).

Spearman's rank correlation analysis showed correlations between body mass and height and both LS and FN BMD. The age gave a significant negative correlation only with FN BMD. Results are presented in Table 3.

**Table 3. Spearman's rank correlation analysis between anthropometric parameters and FN/LS BMD**

| | FN BMD | | LS BMD | |
|---|---|---|---|---|
| | Spearman's rank correlation coefficient | *P* | Spearman's rank correlation coefficient | *P* |
| Age | *r* = –0.235 | 0.043 | *r* = –0.124 | 0.29 |
| Body mass | *r* = 0.282 | 0.014 | *r* = 0.333 | 0.004 |
| Height | *r* = 0.308 | 0.007 | *r* = 0.262 | 0.023 |

### Association analysis of *ESR1* gene polymorphisms with BMD value

The *Pvu*II and *Xba*I polymorphisms were tested separately for their potential influence on bone mineral density. As regards *Xba*I on effect of allele x dose was observed for lumbar spine. In women homozygous for xx mean adjusted BMD had the lowest value ($P = 0.023$). An effect of recessiveness was found for the pp genotype of *Pvu*II polymorphism. Bone mineral density in pp homozygotes was significantly lower than in the GD women with the PP and the Pp genotypes ($P = 0.039$). The results are shown in Table 4.

### Association analysis of *ESR1* gene haplotypes and BMD value

A strong linkage disequilibrium (LD) was found between the two polymorphic variants of *ESR1*, *Pvu*II and *Xba*I, with D' = 1.0. These polymorphisms formed three haplotypes in GD women: the most frequent px (55.3%), PX (33.3%), and the rarest — Px (11.3%).

In the whole group no cases of pX haplotype were observed. Each haplotype was studied for its relation with low bone density in the femoral neck and lumbar spine.

Women homozygous for the px haplotype had the lowest LS BMD adjusted for age, height and body mass (1.113 g/cm$^2$). Bone mineral density of heterozygotes with only one copy of the px haplotype was 1.190 g/cm$^2$, while for non-carriers the value was the highest (1.199 g/cm$^2$).

However, these results were not statistically significant ($P = 0.070$). For this haplotype the effect of recessiveness was observed for lumbar BMD when adjusted for age, weight and height. No such effect was shown for the non-adjusted parameter. Women without the px haplotype or with only one copy of it had a higher adjusted lumbar bone mineral density (1.192 g/cm$^2$) than women with px haplotype on both chromosomes (1.113 g/cm$^2$) ($P = 0.039$). No such influence was found for the femoral neck BMD. Table 5 presents results of the analysis of association between LS and FN BMD in relation to haplotype px of *ESR1* gene.

A dose effect was observed for the PX haplotype. Women homozygous for PX had the highest adjusted LS BMD (1.248 g/cm$^2$) as opposed to non carriers of this haplotype who had the lowest non- and adjusted LS BMD (1.113 g/cm$^2$). The effect was staistically significant ($P = 0.023$). Also the effect of dominance was noted for this haplotype. Women without the PX haplotype had a lower adjusted LS BMD (1.130 g/cm$^2$) than women with the PX haplotype on one or both chromosomes (1.203 g/cm$^2$, $P = 0.049$). Table 6 contains the results of analysis of the association between LS and FN BMD with haplotype PX of *ESR1* gene. There was no influence for femoral neck BMD.

The third most frequent haplotype (Px) had no statistically sig-

**Table 4. Distribution of *ESR1* gene polymorphisms and their association analysis**

| | | 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 ($g/cm^2$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *ESR1 Xba*I | | | | | | | | | | | |
| Allele dose effect | | n=75 | | | | | | | | | |
| Allele dose | XX | 9 | 12.0% | 1.002 | ± 0.154 | 0.976 | ±0.133 | 1.265 | ± 0.205 | 1.248 | ± 0.143 |
| | Xx | 32 | 42.7% | 0.990 | ± 0.140 | 0.994 | ±0.138 | 1.171 | ± 0.138 | 1.190 | ± 0.148 |
| | xx | 34 | 45.3% | 0.967 | ± 0.151 | 0.970 | ±0.139 | 1.144 | ±0.163 | 1.113 | ±0.149 |
| Significance | | | | *P*=0.437 | | *P*=0.726 | | *P*=0.066 | | *P*=0.023 | |
| Effects of recessiveness and dominance | | | | | | | | | | | |
| Genotype | XX+Xx | 41 | 54.7% | 0.993 | ±0.141 | 0.990 | ±0.137 | 1.192 | ±0.157 | 1.203 | ±0.148 |
| | xx | 34 | 45.3% | 0.967 | ±0.151 | 0.970 | ±0.138 | 1.144 | ±0.163 | 1.130 | ±0.150 |
| Significance | | | | *P*=0.448 | | *P*=0.552 | | *P*=0.204 | | *P*=0.049 | |
| Genotype | XX | 9 | 12.0% | 1.002 | ± 0.154 | 0.975 | ±0.131 | 1.265 | ± 0.205 | 1.245 | ± 0.145 |
| | Xx+xx | 66 | 88.0% | 0.978 | ± 0.145 | 0.982 | ±0.134 | 1.157 | ± 0.151 | 1.160 | ± 0.142 |
| Significance | | | | *P*=0.644 | | *P*=0.887 | | *P*=0.058 | | *P*=0.104 | |
| *ESR1* PvuII | | | | | | | | | | | |
| Allele dose effect | | n=75 | | | | | | | | | |
| Allele dose | PP | 13 | 17.3% | 0.983 | ± 0.133 | 0.963 | ±0.133 | 1.206 | ± 0.199 | 1.199 | ± 0.144 |
| | Pp | 41 | 54.7% | 0.990 | ± 0.142 | 0.997 | ±0.131 | 1.182 | ± 0.151 | 1.190 | ± 0.142 |
| | pp | 21 | 28.0% | 0.962 | ± 0.162 | 0.961 | ±0.134 | 1.126 | ±0.149 | 1.113 | ±0.146 |
| Significance | | | | *P*= 0.615 | | *P*=0.833 | | *P*=0.133 | | *P*=0.070 | |
| Effects of recessiveness and dominance | | | | | | | | | | | |
| Genotype | PP+Pp | 54 | 72.0% | 0.989 | ±0.139 | 0.989 | ±0.131 | 1.187 | ±0.162 | 1.192 | ±0.142 |
| | pp | 21 | 28.0% | 0.962 | ±0.162 | 0.961 | ±0.134 | 1.126 | ±0.149 | 1.113 | ±0.145 |
| Significance | | | | *P*=0.479 | | *P*=0.424 | | *P*=0.137 | | *P*=0.039 | |
| Genotype | PP | 13 | 17.3% | 0.983 | ± 0.133 | 0.962 | ±0.133 | 1.206 | ± 0.199 | 1.197 | ± 0.147 |
| | Pp+pp | 62 | 82.7% | 0.981 | ± 0.148 | 0.985 | ±0.131 | 1.163 | ± 0.152 | 1.165 | ± 0.145 |
| Significance | | | | *P*= 0.965 | | *P*=0.570 | | *P*=0.384 | | *P*=0.474 | |

nificant association with bone mineral density neither for femoral neck or lumbar spine (see Table 7).

### A case-control study

A case-control analysis of association with predisposition to GD was performed for the *ESR1* polymorphisms (*Pvu*II, *Xba*I) and their haplotypes. The most frequent genotype was Pp of *Pvu*II for both GD patients and the controls. When the *Xba*I was tested, the Xx genotype was the most frequent in the controls while xx in women with Graves' (see Table 8). The distribution of the px, PX, and Px haplotypes among the Graves' patients and the control group is shown in Table 9.

No statistically significant associations with the autoimmune disease were found for *ESR1* polymorphisms studied or for their haplotypes. The lack of an association found may be caused by low power of performed tests (the power of particular comparisons varied from 12% to 87%) or may not occur at all.

## DISCUSSION

The main purpose of the present study was to look for a relationship between the *ESR1* gene polymorphisms and BMD as an indicator of osteoporosis in Graves' patients. The second aim was to find out whether the *ESR1* polymorphic variants predispose premenopausal women to this autoimmune disease of the thyroid gland.

Many studies have identified an association between the *ESR1* genotype and decreased BMD or osteoporosis. Bone loss in Graves' patients has been also shown in several studies suggesting an impact of autoimmunity on bone turnover. However, the number of publications that link *ESR1*, BMD and Graves' disease is small. Here, we tried to answer the question about possible association

**Table 5. Association analysis of haplotype px of *ESR1* gene with BMD for FN and LS**

| *ESR1* px | | 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 | | | | | | | | | |
| Haplotype dose | 0 | 13 | 17.3% | 0.983 | ±0.133 | 0.963 | ±0.133 | 1.206 | ±0.199 | 1.199 | ±0.144 |
| | 1 | 41 | 54.7% | 0.990 | ±0.142 | 0.997 | ±0.131 | 1.182 | ±0.151 | 1.190 | ±0.142 |
| | 2 | 21 | 28.0% | 0.962 | ±0.162 | 0.961 | ±0.134 | 1.126 | ±0.149 | 1.113 | ±0.146 |
| Significance | | | | *P*=0.615 | | *P*=0.833 | | *P*=0.133 | | *P*=0.070 | |
| Effects of recessiveness and dominance | | | | | | | | | | | |
| Genotype | 0+1 | 54 | 72.0% | 0.989 | ±0.139 | 0.989 | ±0.131 | 1.187 | ±0.162 | 1.192 | ±0.142 |
| | 2 | 21 | 28.0% | 0.962 | ±0.162 | 0.961 | ±0.134 | 1.126 | ±0.149 | 1.113 | ±0.145 |
| Significance | | | | *P*=0.479 | | *P*=0.424 | | *P*=0.137 | | *P*=0.039 | |
| Genotype | 0 | 13 | 17.3% | 0.983 | ±0.133 | 0.962 | ±0.133 | 1.206 | ±0.199 | 1.197 | ±0.147 |
| | 1+2 | 62 | 82.7% | 0.981 | ±0.148 | 0.985 | ±0.131 | 1.163 | ±0.152 | 1.165 | ±0.145 |
| Significance | | | | *P*=0.965 | | *P*=0.570 | | *P*=0.384 | | *P*=0.474 | |

between *ESR1* polymorphisms and bone mineral density in the context of autoimmune processes underlying GD. This is why two aspects were considered: the influence of hyperthyroidism on bone and impact of *ESR1* polymorphic variants on bone mineral density.

The present study investigated premenopausal, regularly menstruating women with a similar lifestyle without any other diseases affecting bone metabolism. This approach ensured that estrogen deficiency, one of the most important factors for osteoporosis, was eliminated. First, bone mineral density of 75 premenopausal GD women was correlated with age, body mass and height. The correlation between BMD and weight/height was positive, whereas between BMD and age — negative. Therefore, it was proven that the risk factors for osteoporosis established for the whole population are also valid for young women with hyperthyroidism due to GD. One of the accepted explanations of the positive relationship between weight and BMD is that body weight increases bone mass. The stronger the mechanical stress on bone, the more effective are the processes of osteosynthesis and mineralization and in consequence — higher peak bone mass (Walker-Bone *et al.*, 2002).

However, the influence of age on BMD remains unclear. We found a negative correlation between age and FN BMD, but not LS BMD.

In our study we paid special attention to the selection of the patients. In most studies on the discussed issue addressed here, the study groups comprised much older subjects. Our females comprising the study group were younger and menstruating. This gave us the opportunity to explore other determinants of BMD than the estrogen deficit. Changes in BMD in patients with GD in our

**Table 6. Association analysis of haplotype PX of *ESR1* gene with BMD for FN and LS**

| *ESR1* PX | | 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 | | | | | | | | | |
| Haplotype dose | 0 | 34 | 45.0% | 0.967 | ±0.151 | 0.970 | ±0.139 | 1.144 | ±0.163 | 1.113 | ±0.149 |
| | 1 | 32 | 42.7% | 0.990 | ±0.140 | 0.994 | ±0.138 | 1.171 | ±0.138 | 1.190 | ±0.148 |
| | 2 | 9 | 12.0% | 1.002 | ±0.154 | 0.976 | ±0.133 | 1.265 | ±0.205 | 1.248 | ±0.143 |
| Significance | | | | *P*=0.437 | | *P*=0.726 | | *P*=0.066 | | *P*=0.023 | |
| Effects of recessiveness and dominance | | | | | | | | | | | |
| Genotype | 0+1 | 66 | 88.0% | 0.978 | ±0.145 | 0.982 | ±0.131 | 1.157 | ±0.151 | 1.160 | ±0.142 |
| | 2 | 9 | 12.0% | 1.002 | ±0.154 | 0.975 | ±0.133 | 1.265 | ±0.205 | 1.245 | ±0.145 |
| Significance | | | | *P*=0.644 | | *P*=0.887 | | *P*=0.058 | | *P*=0.104 | |
| Genotype | 0 | 34 | 45.3% | 0.967 | ±0.151 | 0.970 | ±0.138 | 1.144 | ±0.163 | 1.130 | ±0.150 |
| | 1+2 | 41 | 54.7% | 0.993 | ±0.141 | 0.990 | ±0.137 | 1.192 | ±0.157 | 1.203 | ±0.148 |
| Significance | | | | *P*=0.448 | | *P*=0.552 | | *P*=0.204 | | *P*=0.049 | |

**Table 7. Association analysis of haplotype Px of *ESR1* gene with BMD for FN and LS.**

| *ESR1* Px | | 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 | | | | | | | | | |
| Haplotype dose | 0 | 58 | 77.3% | 0.985 | ±0.152 | 0.983 | ±1.131 | 1.176 | ±0.157 | 1.176 | ±0.146 |
| | 1 | 17 | 22.7% | 0.967 | ±0.123 | 0.975 | ±0.134 | 1.150 | ±0.173 | 1.151 | ±0.148 |
| | 2 | 0 | 0.0% | | | | | | | | |
| Significance | | | | *P* = 0.643 | | *P* = 0.831 | | *P* = 0.558 | | *P* = 0.546 | |
| Effects of recessiveness and dominance | | | | | | | | | | | |
| Genotype | 0+1 | 75 | 100.0% | | | | | | | | |
| | 2 | 0 | 0.0% | | | | | | | | |
| Significance | | | | | | | | | | | |
| Genotype | 0 | 58 | 77.3% | 0.985 | ±0.152 | 0.983 | ±0.131 | 1.176 | ±0.157 | 1.176 | ±0.146 |
| | 1+2 | 17 | 22.7% | 0.967 | ±0.123 | 0.975 | ±0.134 | 1.150 | ±0.173 | 1.151 | ±0.148 |
| Significance | | | | *P* = 0.643 | | *P* = 0.831 | | *P* = 0.558 | | *P* = 0.546 | |

study resulted from an excess of active thyroid hormones and *ESR1* polymorphisms, while the protective effect of estrogens on bone was maintained.

The lower BMD values in GD women is a consequence of hyperthyroidism leading to higher bone turnover and accelerated bone loss. Bone loss induced by hyperthyroidism is usually reversible, but achieving euthyreosis does not restore bone quality immediately. The recovery of balance between bone formation and resorption takes 7.5 to 12 months from the TSH normalization and this period is usually sufficient to increase BMD value (Jodar *et al.*, 1997; Horst-Sikorska *et al.*, 2005). Much earlier, just after 6 months, the biochemical markers of bone metabolism reach normal values (Diamond *et al.*, 1994). No significant correlation between the duration of hyperthyroidism remission and improvement of BMD was observed in the studied females with GD. However, higher BMD values in both lumbar spine and femoral neck were found in females euthyreotic for more than 1 year, which suggests sufficiency of bone repair processes after normalization of thyroid function.

Hyperthyroidism causes an increase of osteoclastic resorption particularly in the cortical bone, so loss of cortical bone is characteristic for hyperthyroidism-related osteoporosis (Hofbauer *et al.*, 1999; Greenspan *et al.*, 1999; Lakatos 2003). High prevalence of cortical bone loss was noticed by Majima *et al.* (2006a) in GD male patients. Those authors paid special attention to BMD measurements and its monitoring as crucial in Graves' patients, irrespectively of gender and age (Majima *et al.*, 2006a; 2006b).

We did not corroborate this. The low frequency of osteoporosis in the studied women defined as T-score $<-2.5$SD was probably related to the fact that the study involved regularly menstruating women in whom

**Table 8. Association analysis of *ESR1* polymorphisms (*Pvu*II, *Xba*I) with Graves' disease**

| *ESR1* polymorphisms | | Genotype frequency | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Controls | | GD | | | | |
| | | n | % | n | % | Effects of recessiveness and dominance | | |
| *ESR1* PvuII | | n = 163 | | n = 75 | | | d.f. = 1 | |
| Genotype | PP | 43 | 26.4 | 13 | 17.3 | PP *vs* Pp+pp | c2 = 2.34 | *P* = 0.126 |
| | Pp | 78 | 47.9 | 41 | 54.7 | | | |
| | pp | 42 | 25.8 | 21 | 28.0 | pp *vs* Pp+PP | c2 = 0.13 | *P* = 0.717 |
| Pearson's $\chi 2$ | | c2 = 2.36 | | d.f. = 2 | *P* = 0.307 | | | |
| Armitage's $\chi 2$ trend test | | c2 = 1.31 | | d.f.=1 | *P* = 0.253 | | | |
| *ESR1 Xba*I | | n = 163 | | n = 75 | | | d.f. = 1 | |
| Genotype | XX | 14 | 8.6 | 9 | 12.0 | XX *vs* Xx+xx | c2 = 0.68 | *P* = 0.408 |
| | Xx | 84 | 51.5 | 32 | 42.7 | | | |
| | xx | 65 | 39.9 | 34 | 45.3 | xx *vs* Xx+XX | c2 = 0.63 | *P* = 0.428 |
| Pearson's $\chi 2$ | | c2 = 1.82 | | d.f. = 2 | *P* = 0.404 | | | |
| Armitage's $\chi 2$ trend test | | c2 = 0.05 | | d.f. = 1 | *P* = 0.819 | | | |

**Table 9. Frequency of px, PX, Px haplotypes of *ESR1* gene in patients with Graves' disease and in controls**

| | | Haplotype frequency | | | |
|---|---|---|---|---|---|
| | | Controls | | GD | |
| | | n | % | n | % |
| Haplotype | px | 162 | 49.7 | 83 | 55.3 |
| | Remaining | 164 | 50.3 | 67 | 44.7 |
| Pearson's $\chi 2$ | | c2 = 1.3 | d.f. = 1 | *P* = 0.253 | |
| Odds ratio | | OR = 0.8 | 95%CI [ 0.54–1.18] | | |
| Haplotype | PX | 112 | 34.4 | 50 | 33.3 |
| | Remaining | 214 | 65.6 | 100 | 66.7 |
| Pearson's $\chi 2$ | | c2 = 0.05 | d.f. = 1 | *P* = 0.827 | |
| Odds ratio | | OR = 1.05 | 95%CI [ 0.70–1.58] | | |
| Haplotype | Px | 52 | 16.0 | 17 | 11.3 |
| | Remaining | 274 | 84.0 | 133 | 88.7 |
| Pearson's $\chi 2$ | | c2 = 1.77 | d.f. = 1 | *P* = 0.184 | |
| Odds ratio | | OR = 1.49 | 95%CI [0.83–2.67] | | |

estrogen secretion ensured efficient bone remodeling. It is interesting, as although cortical bone is more metabolically active, and thus more prone to factors decreasing bone mass, no significant changes — contrary to trabecular bone — were observed. Probably remodeling processes in cortical bone were more effective or duration of thyrotoxicosis was too short to disturb bone metabolism considerably. We hypothesize, that in our study group sufficient action of estrogens was a strong protective factor on bone and eliminated negative influence of thyroid hormones' excess.

Only a few studies have been published on the expression of TSHR in human bone cells or on the influence of serum TSH on bone parameters. Direct effect of TSH on BMD, independent of thyroid hormones' level, was found recently by Heemstra *et al.* (2008). Van der Deure *et al.* (2008) found a stronger effect of fT4 than TSH on BMD. They noticed that femoral neck BMD increased with serum TSH level (van der Deure *et al.*, 2008). A recent cross-sectional study by Baqi *et al.* (2010) confirmed the role of TSH in bone metabolism. A highly significant influence of TSH on BMD at FN and LS independent of age was found. Patients with normal TSH level had favorable bone status while women with low level of TSH had lower BMD. Those authors concluded that the impact of THS on BMD may be substantial. That study was conducted in 113 postmenopausal women, including GD patients, so the conclusions should be carefully compared with our results. The question what causes differences in the bone status among patients with GD was addressed in an analysis of polymorphic variants of selected genes, the function of their protein products and literature data on osteoporosis in GD (Walker-Bone *et al.*, 2002; Lakatos, 2003).

We confirmed the association between LS BMD and the allelic variants of *ESR1* polymorphisms (*Pvu*II, *Xba*I). A dose effect was observed for allele x of *Xba*I. Women homozygous for xx had the lowest mean BMD adjusted for age, body mass and height as compared to Xx heterozygotes and XX homozygotes. This was true for the lumbar bone, but not for femoral neck. In turn, the correlation between p allele dose of *Pvu*II and the BMD values was negative. Effect of recessiveness was found for the pp genotype. The lumbar BMD in women homozygous for the p allele was significantly decreased, while P allele carriers had higher values of LS BMD. A possible explanation for BMD reduction, despite a lack of estrogens deficit, is that p and x alleles of *ESR1* somewhow determine weaker biological impact of estrogens on bone. In these women an excess of thyroid hormones may lead to more intense bone resorption than in the P and X alleles carriers.

The association of BMD with *ESR1* genotypes has been identified in several studies (Kobayashi *et al.*, 1996; Mahonen *et al.*, 1997; Willing *et al.*, 1998). Authors of those reports emphasize that the genotype that predicts low or high bone mineral density may be population specific. Indeed, Japanese women with the PP genotype had lower BMD while in American and Finnish females decreased bone mineral density was associated with the pp genotype. A multicenter study by Ioannidis *et al.* (2004) performed on 18 917 patients from eight European centers did not confirm any relation of *Pvu*II polymorphism with BMD and risk fracture. In turn patients with XX genotype of *Xba*I had a slightly higher FN BMD and reduced fracture risk. The relationship between xx genotype and low BMD was documented also by Willing *et al.* (1998)

The present study also indicated a recessive effect for the px haplotype of the *ESR1* gene. Women with two copies of the px haplotype had the lowest lumbar BMD compared to patients without or with only one copy of this haplotype. This rule was observed for adjusted bone mineral density at LS. PX homozygotes had the highest adjusted LS BMD while non-carriers of this haplotype had the lowest non- and adjusted LS BMD. There was no influence of any haplotype of *ESR1* on BMD at femoral neck.

In studies on postmenopausal women with hyperthyroidism estrogen deficiency which definitely has a strong impact on bone turnover, was an important factor.

In our study premenopausal women presenting normal estrogen level were shown to have different BMD values depending on the *ESR1* haplotype. The *ESR1* px variant was shown to predispose to increased bone resorption and the lowest values of lumbar BMD. This observation could indicate yet another genetic risk factor for osteoporosis and help identify women with hyperthyroidism particularly predisposed to secondary osteoporosis. Our results are partly consistent with published data. There is some convincing evidence on the 19 association between *ESR1* px haplotype and lower bone mineral density (Colin *et al.*, 2003; van Meurs *et al.*, 2003; Zhang *et al.*, 2003). It should also be noticed that more publications report a relationship between the Px rather than the px haplotype with low BMD (Kobayashi *et al.*, 1996; Albagha *et al.*, 2001). An analysis of *Pvu*II and *Xba*I polymorphisms of *ESR1* in British subjects reported an association between Px haplotype and decreased BMD (Albagha *et al.*, 2001). A Chinese study revealed association between PX haplotype and low spine

mineral density while PX carriers had decreased hip BMD (Zhang *et al.*, 2003). However, other studies that showed no associations between the polymorphic variants of *ESR1* and BMD (Gennari *et al.*, 1998; Bechereni *et al.*, 2000; Yamada *et al.*, 2002). Yamada *et al.* (2002) found no differences in bone mineral density among Japanese women under 60 years regarding the *Pvu*II and *Xba*I genotypes. Bechereni *et al.* (2000) made the same observations for Italian women. This conflicting observations may result from a diverse distribution of the polymorphisms in populations, and the impact of environmental factors such as dietary habits, physical activity and exposure to sunlight.

Our results show that none of the polymorphic variants of the *ESR*1 gene was particularly responsible for the development of Graves' disease despite the well documented role of estrogens in human autoimmunity. There were no statistically significant differences in the frequency of particular polymorphic alleles of *ESR1* and their haplotypes between patients with GD and controls. These results are consistent with previous studies (Ban *et al.*, 2000; 2001). Also Japanese and Russian populations were studied for the predisposition to Graves' disease in relation to human estrogen receptor alpha and beta genes' microsatellite polymorhisms, with no associations between *ESR1* (alpha) or *ESR2* (beta) and autoimmune thyroid diseases being found (Ban *et al.*, 2001; Chistiakov *et al.*, 2002).

In view of the above inconsistencies between studies, our results should be interpreted with caution. Osteoporosis is a complex multigenic disease. Moreover, multiple factors and metabolic processes are involved in bone turnover and bone quality, that are in part independent of the genetic makeup of individuals. Beside BMD measured by DEXA, bone geometry, its architecture and various other parameters influence bone strength. These factors should also be considered in estimation of osteoporosis risk. Our study focused on the association between the *Pvu*II and *Xba*I polymorphisms of the *ESR1* gene and bone mineral density in premenopausal women with GD. The results suggest that estrogen receptor alpha gene may have important influence on bone mineral density in these patients, particularly at lumbar spine. The main limitation of the study was small sample size. The observed associations may represent a true trend that might be population-specific. However, further large-scale investigations are required to confirm the evidence of *ERS1* gene linkage to BMD and to clarify whether its polymorphisms may be applicable as genetic markers of osteoporosis prediction among women with Graves' disease.

## CONCLUSIONS

1. Analysis of *Pvu*II and *Xba*I polymorphisms of *ESR1* gene may be considered to estimate the risk of osteoporosis in premenopausal Polish women with GD because:
— homozygotes: xx of *Xba*I and pp of *Pvu*II were shown to have reduced bone mineral density at the lumbar spine
— px haplotype predisposed to lower lumbar spine BMD.
2. *ESR1* gene polymorphisms do not predict the risk of Graves' disease in Polish female patients.

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