

ASSOCIATION BETWEEN BIOCHEMICAL MARKERS OF BONE TURNOVER AND MARKERS IN THE ESTROGEN RECEPTOR (ER) GENE

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Summary. Bone metabolism is characterized by two opposite activities: the formation of new bone by osteoblasts and the degradation of old bone by the osteoclasts. Osteoporosis is a disease in which subtle modifications of bone remodeling can result in a substantial loss of bone over a prolonged period of time. The needs for non-invasive techniques that can be applied more widely and repeated several times in a single patient explain the development of markers of bone turnover that are measured in blood and urine. The prognostic value of parameters as risk indexes for osteoporosis is not enough. This makes it possible to use genetic markers, which together with already existing risk indexes will improve the identification of individuals with higher osteoporosis risk. The future development of genetic studies in osteoporosis will proceed in two major directions: identification of further candidate genes, their interaction with each other and their application to defined diseases of bone metabolism.

Key words: *osteocalcin, deoxypyridinoline, estrogen receptor (ER), bone mineral density (BMD)*

Abbreviations: *(BMD) bone mineral density; (ER) estrogen receptor; (Pyr) pyridinoline; D-Pyr) deoxypyridinoline; (Cr) creatinine*

INTRODUCTION

The formation of new bone by osteoblasts and the resorption of old bone by osteoclasts are two activities coupled in time and space. Bone mass depends on the balance between formation and resorption within a remodeling unit and on the number of remodeling units that are active within a given period of time in a defined area of bone. The necessity of development of markers giving the

opportunity of differential evaluation of the two processes of bone turnover – formation and resorption, explains the development of biochemical markers measured in blood and urine. They are easy to perform, atraumatic, making possible the early discovery of patients at risk, following of a patient's treatment and the effects of it.

Osteocalcin. Osteocalcin (Gla protein) is a small non-collagenous protein specific for bone tissue. It is a 49-residue polypeptide expressed by osteoblasts under the 1.25-dihydroxyvitamin D₃ control. Dentin and calcified cartilage also contain osteocalcin. It forms about 1% of the organic matrix of bone where it exists in association with the surface of the mineral crystallites. Osteocalcin is incorporated into the extracellular matrix of bone and a fraction is released into the circulation where it can be measured. Osteocalcin mRNA has also been detected in bone marrow, megakaryocytes and peripheral blood platelets but the protein itself is undetectable in human platelets [1]. This suggests that platelet osteocalcin does not contribute to serum levels. Circulating osteocalcin has short half-life and is rapidly cleared by the kidneys [2]. Serum levels correlate with skeletal growth at the time of puberty and are increased in conditions of increased bone turnover such as primary and secondary hyperparathyroidism, Paget's disease and acromegaly. It is decreased in hypothyroidism, hypoparathyroidism, glucocorticoid-treated patients and some with multiple myeloma and malignant hypercalcaemia [3, 4, 5, 6, 7, 8].

The assays for determining human osteocalcin utilize bovine osteocalcin as a tracer, standards and immunogen for the production of antibodies because human and bovine osteocalcin differ by only 5 aminoacids. Most antisera recognize the C-terminal region of the molecule which is identical in both human and bovine osteocalcin [9]. Fragments detected in serum of patients with high bone turnover have been released into the circulation during osteoclastic resorption of the bone matrix. When a series of monoclonal antibodies against various epitops of the human osteocalcin molecule is used, the intact molecule represents about a third of the immunoreactivity in serum of an adult. A third is represented by several small fragments and another third by a large N-terminal mid-molecule fragment (N-mid fragment) [10]. This large fragment (~43 aminoacids) is not released from degradation of bone matrix, because its circulating level does not change after acute treatment with bisphosphonate – a potent inhibitor of bone resorption. After a few hours at room temperature a significant fraction of intact osteocalcin is rapidly converted into the large N-mid-fragment resulting in a significant loss of immunoreactivity with most polyclonal antibodies. Measuring the intact molecule and the N-mid-fragment with antibodies results in a more robust and sensitive assay. Serum osteocalcin represents only a fraction of the synthetic pool, which escapes incorporation into the newly formed bone matrix and the amount of this fraction may change under different conditions of osteoblast physiology and pathology [11].

Pyridinium cross-links. Pyridinoline (Pyr) and deoxypyridinoline (D-Pyr) also called hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are two cross-links in collagen structure. These covalent cross-links are generated post-translational from lysine (Lys) and hydroxylysine (OH-Lys) residues and are unique to collagen and elastin. They create interchain bonds that stabilize the molecule within the extracel-

lular matrix (Fig. 1). The highest concentration of Pyr is found in articular cartilage where D-Pyr is present in minimal amounts [12, 13]. Pyr and D-Pyr are present in tendons and the aorta but absent from the skin [12]. Since the bone is the most abundant source of collagen and its rate of turnover is higher than other connective tissue, it is assumed that Pyr and D-Pyr in biological fluids are predominantly derived from bone. This makes these markers specific for bone resorption [14]. In human bone the ratio Pyr:D-Pyr is 3:1. Pyr and D-Pyr are released from bone matrix during its degradation by the osteoclasts. They are not reutilized for collagen synthesis. They are excreted in urine in a free (about 40%) and peptide-bound form (60%).

Total urinary Pyr and D-Pyr are markedly higher in children than in adults [13]. They increase by 50-100% at the time of menopause and go down to premenopausal levels under estrogen therapy [15]. In patients with vertebral osteoporosis, the D-Pyr levels are correlated with bone turnover measured by calcium kinetics [16] and bone histomorphometry [17, 18] but have poor correlations with urinary hydroxyproline. Pyr and D-Pyr contained in gelatin are not absorbed by the gut, which allows the collection of urine without any food restriction [19]. Immunoassays have been developed using antibodies against free Pyr, D-Pyr [20, 21] and against peptides in the cross-linking domains of type I collagen. Assays have been developed with antibodies against the N-telopeptide of helix domain [22], C-telopeptide of helix [23] and against a sequence of the C-telopeptide of a $\alpha 1$ chain [24] (**Fig. 1**). Urinary free Pyr correlates with the total Pyr excretion measured by HPLC and increases after the menopause and in patients with Paget's disease [25]. The enzyme-linked immunosorbent assay (ELISA) of free D-Pyr with monoclonal antibodies correlates with total D-Pyr measured by HPLC. It is increased in patients with osteoporosis and in diseases characterized by increased bone turnover such as primary and secondary hyperparathyroidism, Paget's disease and bone metastases from breast cancer [21]. The urinary ELISA developed against the N-telopeptide to helix (NTX) and against break-down products of type I collagen – C-telopeptide (CTX) shows a circadian rhythm similar to that of total pyridinoline excretion with peak in the early morning and a minimum in the afternoon.

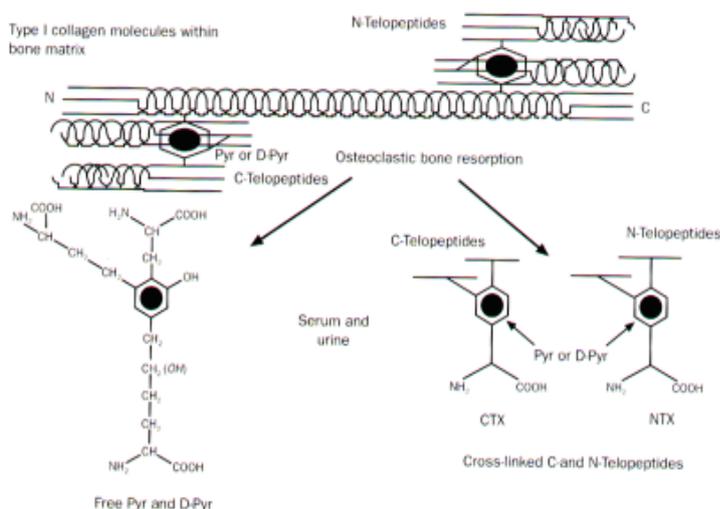


Fig. 1. Type I collagen breakdown products as markers of bone resorption. Type I collagen molecules in bone matrix are linked by pyridinoline cross-links in the region of N- and C-telopeptides. Pyr differs from D-Pyr by the presence of a hydroxyl residue. During osteoclastic bone resorption Pyr cross-links are released into the circulation and excreted in urine as a free form or linked to C- (CTX) or N-telopeptides (NTX) of collagen type I. Free Pyr, D-Pyr, CTX and NTX are measured in urine with specific immunoassays.

Estrogen receptor gene (ER). The effect of estrogen is related to maintenance of bone mineral density (BMD) after menopause and to protection from fractures [26]. Men with full estrogen resistance due to ER mutation [27] or aromatase deficiency [28] have significantly decreased BMD [29]. The decreased bone mass in a man with aromatase deficiency increased under the influence of estrogen therapy [30]. Many studies succeeded in discovering a relation between BMD and ER variants [31, 32, 33, 34, 35, 36, 37]. Willing et al [38] suggested in a population of 372 American women in pre- and peri-menopause that the ER locus genetic variability influences the achievement and maintenance of peak bone mass in younger women, which makes some individuals more susceptible to osteoporosis than others. The authors studied the relation between BMD levels with their variation during 3 years period and polymorphisms of ER gene (identified by PvuII and XbaI) and osteocalcin. ER genotypes were indicative of lumbar spine BMD and total body BMD. They identified significant effect of gene-gene interaction for vitamin D receptor (VDR) locus and PvuII and XbaI polymorphisms that influenced BMD in 171 women with genotype defined for both polymorphisms. Women with (-/-) PvuII ER and bb VDR genotype combination had very high total BMD while individuals with (-/-) PvuII ER and BB VDR genotype had significantly lower BMD. The ER genotype which predicts lower or higher BMD could be specific for different populations. In Japanese and Italian women, ER PvuII (-/-) genotype is related to low BMD [31, 35], while in American [37] and Finnish [36] groups PvuII (+/+) genotype is related to low BMD. The ER gene polymorphisms are related also to increased risk of osteoarthritis [39]. The gene-gene interaction is probably specific for populations [31, 38] and BMD like other complex traits is determined by several genes that act together. There is a potential physiological relation between ER and VDR – vitamin D is among substances that regulate P450-aromatase [40] and this enzyme drives the estrogen to its receptor and is expressed in osteoblasts [41].

MATERIALS AND METHODS

Subjects. The study includes 400 unrelated women that ranged between 36 and 77 years of age. 220 participants had low bone density (cases) and 180 had normal BMD (controls). All studied women are in menopause (natural or surgical). All of them have no other diseases causing secondary osteoporosis (gastrointestinal, liver diseases, kidney diseases or genetic). None of them have received inhibitors of bone resorption. Medications known to affect bone metabolism were used as exclusion criteria (glucocorticoids, heparin, antiepileptic drugs and immune suppressors).

Bone densitometry. All studied individuals are divided into two groups – cases and controls. Individuals composing the group of cases with low BMD phenotype are characterized with bone densitometry in the Endocrinology Clinic of Alexandrovska Hospital. BMD was measured at the lumbar spine (L1-L4) by dual-

energy X-ray absorptiometry (DXA) on a Hologic QDR 4500. The control group is also characterized with bone densitometry and has normal BMD.

Enzyme-linked immunosorbent assay (ELISA). Data used for the assessment of bone turnover of all studied individuals are from the routine analyzed in Central Clinical Laboratory of Alexandrovska hospital biochemical markers of bone formation (osteocalcin) and bone resorption (deoxypyridinoline). For the osteocalcin assessment, concurrent ELISA is applied with monoclonal antibodies against the intact molecule of human osteocalcin. The standards and controls are from purified human osteocalcin. For the deoxypyridinoline assessment, concurrent ELISA is applied with monoclonal antibodies. The reaction is utilized with p-nitrophenylphosphate and the values are given in relation to urine creatinine (Cr).

Genotyping. DNA was isolated from whole blood. Primers and PCR conditions for amplifying intron 1 of the ER gene were designed accordingly to Lorentzon et al [42]. 100 ng of the DNA were used as template in the PCR reaction. Digestion of the PCR product with PvuII or XbaI generates two fragments. Individuals homozygous for the PP and XX genotype have a single uncut fragment, while homozygous for the pp and xx genotype have two fragments. The heterozygotes Pp and Xx have all three bands. The products were electrophoresed through a 2% agarose gel. Individuals were scored according to the digestion pattern.

Statistical analysis. Data are evaluated by χ^2 -test and presented as means \pm SD. The body mass index (BMI) was calculated as: BMI = weight (kg) / [height (cm) / 100]². The statistical significance between cases and controls is analyzed by Student's t-test. The value p=0.05 is taken as limit for statistical significance. The coefficient of correlation is applied to measure the relation between two variants.

RESULTS AND DISCUSSION

The mechanism by which estrogen and ER exert their action upon bone mass is still not studied in detail. It is known that there are androgen and estrogen receptors on human osteoblasts while there are only estrogen receptors on osteoclasts. Estrogen can decrease osteoclastic bone resorption through influence on production and activation of transforming growth factor- β (TGF- β). Estrogen inhibits osteoclastic genesis by decreasing the production of IL-6 in bone marrow cells. The polymorphic sites defined by restriction enzymes PvuII and XbaI are localized in 1 intron of ER gene. Intron sequences contain transcription and splicing regulatory elements which deliver different levels of mRNA hence different isoforms of mature mRNA [43]. The polymorphisms are located in non-functional area of the gene and are in linkage disequilibrium with TA polymorphism in the promoter of ER gene. Probably the TA repeat influences the gene transcription.

Table 1 shows the characteristics of the studied groups by PvuII genotype. The three studied genotype groups among cases (PP, Pp, pp) do not differ significantly by number of pregnancies, vitamin D and calcium supplement with

food. The pp group has higher mean age and more years since menopause. A gene-dose effect on height expressed as BMI is observed. In the group of cases, the mean BMI value for Pp is 2.3 higher than the mean BMI value for pp ($p=0.013$). The mean BMI value for PP is 4.5 higher than the mean BMI for pp ($p=0.01$). This shows lower height (higher BMI value) for Pp group in comparison to pp and lowest height for PP group that has the highest BMI among cases. The height is lowest for PP group when compared with Pp and pp. The difference is well expressed in cases as well as in controls. In the control group, the mean BMI for Pp is 3.1 higher than mean BMI for pp ($p=0.46$). The mean BMI for PP is 8.4 higher than mean BMI for pp ($p<0.01$). These data show a lower height in Pp group compared to PP and the lowest height in PP that have the highest BMI among controls.

Table 1. Characteristic of the studied group by PvuII polymorphism

GENOTYPE	CASES			CONTROLS		
	PP	Pp	pp	PP	Pp	pp
N	58	110	52	21	106	53
age	51,5±8,4	53,5±11,7	57,3±11,4	51,2±3,6	52,2±1,9	50,3±5,6
Years since menopause	4,2±5,1	5,4±7,6	9,5±7,9	2,1±2,0	2,2±1,9	2,6±3,2
BMI	25,6±5,8	23,7±3,1	23,4±4,0	29,7±1,6	24,4±3,4	21,3±2,3
Pregnancies	0,5	2,0	1,6	2,5	1,1	2,0
Coffee consumers	86 %	94 %	92 %	67 %	65 %	100 %
Smokers	86 %	37 %	34 %	67 %	21 %	60 %
Alcohol consumers	50 %	25 %	71 %	34 %	14 %	0 %
Vitamin D supplement by fish and fish products	50 %	50 %	87 %	33 %	92 %	80 %
Ca supplement with dairy products	50 %	83 %	96 %	100 %	92 %	80 %
Family fractures	60 %	50 %	50 %	0 %	60 %	20 %
Osteoporotic fractures	50 %	33 %	13 %	0 %	0 %	0 %
Osteocalcin	14,9±3,6	13,5±3,3	11,5±1,2	8,1±1,3	8,6±1,2	7,3±1,9
Deoxypyridinoline	11,3±2,2	10,4±2,4	9,1±1,7	6,7±2,1	5,3±1,7	5,8±2,3
BMD g/cm ² (L1-L4)	0,701 ± 0,089	0,719 ± 0,098	0,768 ± 0,055	0,956 ± 0,010	0,983 ± 0,009	0,996 ± 0,019

BMD is the highest among pp and the lowest among PP group. Figure 2 shows the values of biochemical markers of bone turnover in cases and controls. Pp group has lower mean value of osteocalcin than PP and pp have the lowest value. The pp group has 2.0 ng/ml lower mean value of osteocalcin than Pp ($p=0.05$) and 3.4 ng/ml lower than PP ($p<0.01$). The correlation coefficient between osteocalcin and BMD for Pvull marker is 0.05. PP have the highest mean values of D-Pyr and the values for Pp are higher than those of pp group. The group of pp has 1.3 nmol/mmol Cr lower mean value of D-Pyr than Pp ($p>0.05$) and 2.2 nmol/mmol Cr lower than PP ($p=0.01$). The correlation coefficient between D-Pyr and BMD for Pvull marker is 0.09.

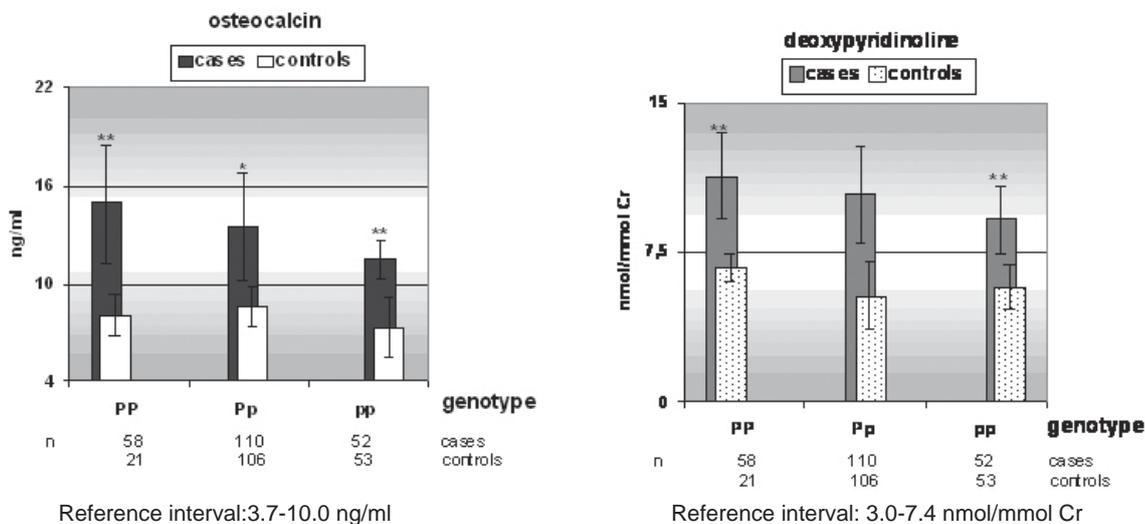


Fig. 2. Comparison between osteocalcin and deoxypyridinoline values in cases and controls for Pvull marker. Data are shown as means \pm 1SD; Student's t-test: * $p=0.05$ for pp/Pp and ** $p<0.01$ for pp/PP for osteocalcin $p=0.20$ for pp/Pp and ** $p=0.01$ for pp/PP for deoxypyridinoline

Table 2 shows the characteristics of the studied groups by Xbal genotype. The three studied genotype groups among cases (XX, Xx, xx) do not differ significantly in age, years since menopause, number of pregnancies, vitamin D and calcium supplement with food. Gene-dose effect on BMI is not observed. In the group of cases, the mean BMI for Xx is the highest and is 1.3 higher than mean BMI for xx ($p>0.05$). The mean value for XX is 0.5 lower than mean value of xx ($p>0.05$). These data show lower height (higher value of BMI) in Xx group compared to both other groups among cases. There is no considerable difference among individuals of the control group. For the controls the mean BMI in XX is 4.0 higher than mean value for xx ($p>0.05$). The xx mean value is 2.9 higher than that for Xx ($p>0.05$). It reveals a lower height (a higher value of BMI) for XX in comparison to both other groups in controls. There is no association of carrying allele X and BMI.

Table 2. Characteristic of the studied group by Xbal polymorphism

GENOTYPE	CASES			CONTROLS		
	XX	Xx	xx	XX	Xx	xx
N	73	110	37	25	113	42
age	51,5±7,9	50,2±10,7	50,0±2,6	51,1±4,0	50,0±1,6	52,0±2,2
Years since menopause	3,0±5,1	4,4±5,7	3,0±4,2	2,4±2,1	1,0±0,7	1,8±2,4
BMI	21,4±3,5	23,2±2,6	21,9±4,1	28,5±1,5	21,6±1,7	24,5±5,6
Pregnancies	3,0	1,3	2,0	3,0	1,0	2,0
Coffee consumers	79 %	67 %	92 %	100 %	40 %	100 %
Smokers	70 %	67 %	92 %	100 %	20 %	50 %
Alcohol consumers	82%	34 %	81 %	100 %	0 %	0 %
Vitamin D supplement by fish and fish products	80 %	91 %	95 %	100 %	60 %	50 %
Ca supplement with dairy products	97 %	88 %	86 %	100 %	80 %	100 %
Family fractures	100 %	66 %	0 %	0 %	40 %	0 %
Osteoporotic fractures	40 %	33 %	5 %	0 %	0 %	0 %
Osteocalcin	12,8±1,6	11,8±1,0	11,1±0,8	6,5±1,8	9,6±0,6	8,8±0,8
Deoxypyridinoline	13,2±2,6	10,1±1,2	8,8±1,1	5,4±1,0	4,8±1,3	4,5±1,3
BMD g/cm ² (L1-L4)	0,692 ± 0,174	0,800 ± 0,056	0,856 ± 0,073	0,967 ± 0,015	0,984 ± 0,008	1,009 ± 0,009

BMD is the highest in xx and the lowest in XX group. **Figure 3** displays the values of biochemical markers of bone turnover in cases and controls. Xx have lower mean osteocalcin value than XX and xx have the lowest value. The xx group has 0.7 ng/ml lower osteocalcin than Xx (p=0.13) and 1.7 ng/ml lower than XX (p<0.01). The correlation coefficient between osteocalcin and BMD for Xbal marker is 0.15. The highest mean values of D-Pyr are among XX and the values for Xx are higher than those for xx. The xx group has 1.3 nmol/mmol Cr lower D-Pyr than Xx (p=0.04) and 4.4 nmol/mmol Cr lower than XX (p<0.001). The correlation coefficient between D-Pyr and BMD for Xbal marker is 0.09.

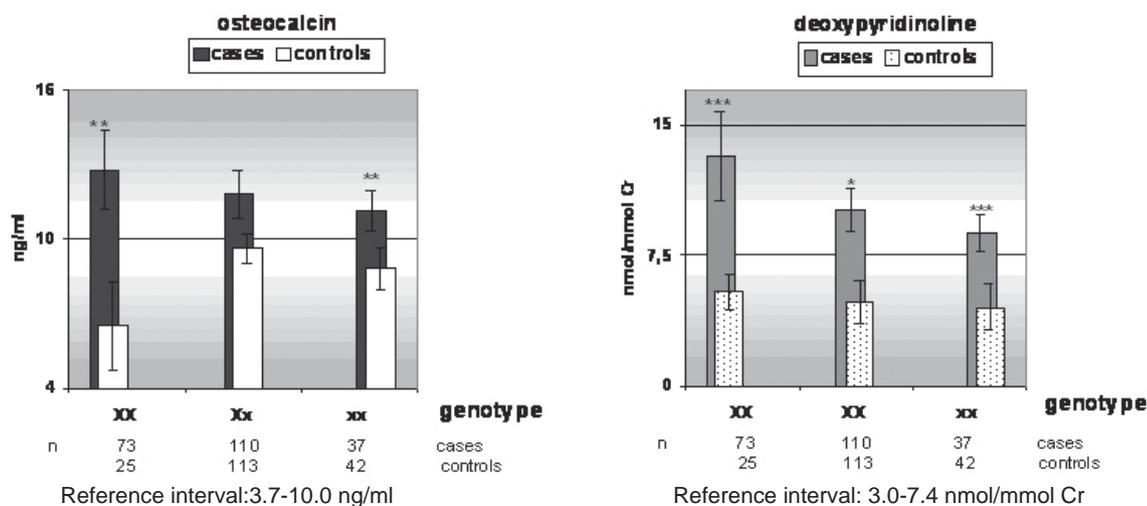


Fig. 3. Comparison between osteocalcin and deoxypyridinoline values in cases and controls for XbaI marker. Data are shown as means±1SD; Student's t-test: p=0.13 for xx/Xx and **p<0.01 for xx/XX for osteocalcin *p=0.04 for xx/Xx and ***p<0.001 for xx/XX for deoxypyridinoline

When examining the PvuII marker, PP genotype is more common among cases and is associated with lower BMD. The pp genotype is more common among controls and is associated with higher BMD. Heterozygotes Pp have mean value of BMD. There is gene-dose effect on height expressed in BMI for the PvuII marker. When comparing the three genotypes by PvuII, an association of PP genotype and lower height (higher BMD) is observed. In the studied group, the osteoporotic fractures are associated with P allele and the differences in risk are large enough (50%, 33% and 13% for PP, Pp and pp respectively). They are more common in the families of PP group (60%) compared to 50% for Pp and pp. There are statistically significant differences in the values of both biochemical markers of bone turnover for the three genotypes (PP, Pp, pp). In genotype PP, the D-Pyr values are the highest and bone loss is the most significant. The values of both markers in PP are strongly increased. The genetic marker PvuII correlates stronger with BMD (CC=0.33) than biochemical markers with BMD – osteocalcin (CC=0.05) and D-Pyr (CC=0.09).

In studying the XbaI marker, XX genotype is more common among cases and is associated to lower BMD. The xx genotype is more common among controls and is associated with higher BMD. Heterozygotes Xx have mean BMD value. There is no gene-dose effect on height or weight for XbaI. In the studied group, the osteoporotic fractures are associated with X allele and the differences in risk are not large (40%, 33% and 5% for XX, Xx and xx respectively). They are more common in the families of XX (100%) compared to 66% for Xx and 0% for xx group. There are also statistically significant differences in the values of both biochemical markers for the three genotypes (XX, Xx, xx). In genotype XX, the D-Pyr values are the highest and bone loss is the biggest. The osteocalcin values are moderately

higher and the D-Pyr – significantly higher in XX. The genetic marker Xbal correlates stronger with BMD (CC=0.51) when compared with the correlation between biochemical markers and BMD – for osteocalcin (CC=0.05) and D-Pyr (CC=0.09). The Xbal marker shows better correlation with BMD than Pvull marker that has correlation coefficient CC=0.33.

In conclusion we could suggest that a difference is found between BMD of ER homozygotes – Pvull and Xbal (PP and pp; XX and xx). The Pvull alleles are related to and the Xbal alleles are not related to body height and weight. The genetic variability of ER locus influences the achieving and maintaining of peak bone mass, that makes some individuals more susceptible to bone loss and osteoporosis. Both polymorphisms represent genetic variants that predispose to osteoporotic fractures through mechanisms which include decrease in quality and quantity of bone and influence the rate of bone loss.

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