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The antioxidant enzyme *GPX1* gene polymorphisms are associated with low BMD and increased bone turnover markers

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Abstract. Recently, oxidative stress has been suggested as participating in the development of osteoporosis. Glutathione peroxidase 1 (GPX1) is one of antioxidant enzymes responsible for the defence of cells against oxidative damage and thus for protection against age related diseases such as osteoporosis.
The aim of present study was to associate genetic variances of GPX1 enzyme with bone mineral density (BMD) and biochemical bone turnover markers and to show the influence of antioxidative defence system in genetics of osteoporosis.
We evaluated 682 Slovenian subjects: 571 elderly women and 111 elderly men. All subjects were genotyped for the presence of *GPX1* gene polymorphisms Pro198Leu and polyAla region. BMD and biochemical markers were also measured. General linear model analysis, adjusted to height, and (one-way) analysis of variance were used to assess differences between the genotype.and haplotype subgroups, respectively.
The significant or borderline significant associations were found between the polyAla or the Pro198Leu polymorphisms and total hip BMD (0.018; 0.023, respectively), femoral neck BMD (0.117; 0.026, respectively) and lumbar spine BMD (0.032; 0.086, respectively), and with biochemical bone turnover markers such as plasma osteocalcin (0.027; 0.025, respectively) and serum C-terminal telopeptide of type I collagen concentrations (0.114; 0.012, respectively) in whole group. Haplotype analysis revealed that the 6-T haplotype is associated significantly with low BMD values ($p < 0.025$) at all measured locations of the skeleton, and with high plasma osteocalcin concentrations ($p = 0.008$).
This study shows for the first time that the polymorphisms polyAla and Pro198Leu of the *GPX1* gene, individually and in combination, are associated with BMD and therefore may be useful as genetic markers for bone disease. Moreover, it implies the important contribution of the oxidative stress to pathogenesis of osteoporosis.

Keywords: Osteoporosis, oxidative stress, glutathione peroxidase 1, bone mineral density, osteocalcin

1. Introduction

Osteoporosis is a complex, polygenic disorder, characterized by low bone mineral density (BMD), of which genetic predisposition accounts for between 50 and 85% of the variance [1–5]. The contributions of specific gene polymorphisms are likely to be relatively small; however they may still be important in the pathogenesis of osteoporosis [3,5].

Recently, oxidative stress has been reported as participating in the development of osteoporosis [2,6–11]. Osteoporosis belongs to the age-related diseases, in which increased risk of oxidative stress, excessive production of reactive oxygen species (ROS), and decreased effectiveness of the antioxidant defence system occur [1,2,6–8,12–14]. In tissue homogenates from the

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femora of ovariectomized rats, a popular model of postmenopausal osteoporosis, significantly increased levels of lipid peroxidation and hydrogen peroxide and decreased levels of enzymatic antioxidants like superoxide dismutase (SOD), glutathione peroxide (GPX) and glutathione transferases (GSTs) were found, compared to control group [10,15–19]. Moreover increased production of ROS in the form of superoxide assessed by increased levels of lipid peroxidation end-product malondialdehyde was detected in postmenopausal women, aged 40–65 years. [7,20].

Osteoblasts produce enzymatic antioxidants such as GPX1, which is a major intracellular enzyme that catalyzes the degradation of peroxides by oxidizing glutathione with the formation of its conjugates, thereby preventing cellular injury [22–24]. Furthermore, osteoclast-generated superoxides contribute to bone degradation and, interestingly, H_2O_2 may stimulate osteoclast differentiation, RANKL expression in human osteoblast-like MG63 cell line, and osteoclast formation, and enhance activity of mature osteoclasts in mouse calvariae [10,15,19,21].

In addition, in comparative protein expression profiling study of circulating monocytes (CMCs) in Chinese premenopausal females with extremely discordant BMD a SOD2 and GPX1 showed differential protein expressions, which might affect CMCs' transendothelium differentiation, and/or downstream osteoclast functions, thus contribute to differential osteoclastogenesis and finally lead to BMD variation [22].

Moreover, the gene expression profile of 2T3 preosteoblasts, exposed to microgravity to stimulate the bone loss via inhibition of differentiation of preosteoblasts into mature osteoblasts, showed downregulation of GPX1 [23]. Additionally, microarray analysis of primary human osteoblast cell lines of osteoporotic and non-osteoporotic women and men, performed in our laboratory, showed significant differential expression profile of genes, involved in antioxidative defence system [24].

Another evidence for possible involvement of antioxidative defence gene *GPX1*, located at 3p21.3, comes from meta-analysis of the genome-wide linkage study and bone mineral density performed on 12,685 individuals. This study has shown the linkage of osteoporosis to chromosome 3p22.2-3p14.1 position, where *GPX1* is located [25]. This result was independently confirmed two years after by Huang et al. showing that the susceptibility loci for osteoporosis are at 3p14-25 [26].

Individuals with reduced GPX1 activity exhibit an increased incidence of oxidative stress-related diseases such as breast cancer, colon, prostate, and lung cancer [27–31]. Peroxidase overexpression, supported by selenium, was shown to suppress tumour cell growth in nude mice [32,33].

On the DNA level, a list of genes and polymorphisms has been found associated with BMD or other phenotypes of osteoporosis. However, little effort has been made to establish whether there is an association between osteoporosis in humans and functional polymorphisms of antioxidative stress-related genes. So far the significant link has already been obtained between genetic polymorphisms of catalase and BMD values [1]. Furthermore, the significantly lower antioxidant activities of the GPX1 protein has been observed in the groups of subjects with osteoporosis [6,21], however no correlation was established so far on the DNA level.

GPX1 gene contains many polymorphic sites. A single substitution of C to T at codon 198, which changes Pro to Leu, reduces the activity by 10% in comparison to wild type enzyme [28]. Furthermore, *in vitro* functional analysis indicated that 198Leu, in combination with 6Ala repeats of PolyAla polymorphism, decreases the GPX1 protein activity by 40% [34]. This suggests that Ala repeats only together with Pro198Leu influence the GPX1 activity [35]. The importance of this polymorphism was shown by Winter and colleagues. Presence of one or two 6Ala alleles appeared to be a modest risk of coronary artery disease [36]. Haplotype combinations of -602A/G, $+2$C/T, 5Ala/6Ala and Pro198Leu in *GPX1* are significantly associated with the intimae-media thickness of carotid arteries and risk of cardiovascular diseases in type 2 diabetes patients [34]. Pro198Leu polymorphism also affects altered personality traits in healthy persons [37]. Association between the activity of GPX1 enzyme and the development of osteoporosis has been established in mouse and postmenopausal women [7,17,20], however as far as our knowledge, no evidence associating genetic variance of *GPX1* with osteoporosis has been reported.

According to evidences presented above and our microarray data [24] on expression of GPX1 we hypothesized that BMD and biochemical bone turnover markers could be associated with two known functional polymorphisms in *GPX1* gene – a polyAla polymorphism and the Pro198Leu polymorphism – individually and in combination, and that the genetic component of the antioxidative stress response is involved in bone metabolism.

2. Materials and methods

2.1. Subjects

The study included 682 Slovenian subjects, consisted of 571 elderly women and 111 elderly men. The subjects were referred to the outpatient departments of the University Medical Centre, Ljubljana, the General and Teaching Hospital, Celje, and the University Medical Centre, Maribor, Slovenia for BMD measurement. Each subject was examined clinically and routine biochemical tests were performed to exclude systemic and metabolic bone diseases other than primary osteoporosis. None had previously taken any drug known to influence bone metabolism. Osteoporosis was defined according to the World Health Organization criteria [38]. All of the participants gave their written informed consent before enrolment in the study. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia.

2.2. Genotyping

2.2.1. Blood sampling

Peripheral venous blood was drawn into Vacutainer tubes containing EDTA (Becton Dickinson, Rutherford, USA) and stored at $-20°C$ for a maximum of 4 days. Genomic DNA was isolated from peripheral blood leukocytes using standard methodology [39].

2.2.2. Polymerase chain reaction (PCR)

Because of *GPX1* pseudogene, we designed new pair of primers for amplification of DNA fragment. The sequences of primers were as follows:

PolyAla-forward: 5‘-GCCGCCGGCCAGTTAAAAGG-3‘,

PolyAla-reverse: 5‘-AAGTAGTACCTTGCCCCGCAGG-3‘,

Pro198Leu-forward: 5‘-GTTCTAGCTGCCCTTCTCTC-3‘,

Pro198Leu-reverse 5‘-ACAGGACATACACACAGTTCTG-3‘.

Fragments for the PolyAla region and the Pro198Leu polymorphism of the gene *GPX1* were amplified by PCR. Following cycling conditions for each primer pair were used: an initial 10 min at 95°C followed by 38 cycles of 1 min at 95°C, 30 s at 62.5°C, 30 s at 72°C, and finally 8 min at 72°C; and an initial 10 min at 95°C followed by 35 cycles of 1 min at 95°C, 30 s at 58°C, 30 s at 72°C, and finally 8 min at 72°C, respectively.

The PCR mixture (20 μL) contained genomic DNA (100ng), 1xPCR buffer, 0,2 mM of each of the four deoxyribonucleotides, 1.5 mM $MgCl_2$, 0.25 μmol/L μM each of oligonucleotide primers and 0.5U of AmpliTaq Gold polymerase (Applied Biosystems, Roche Molecular Systems Inc., Brandenburg, New Jersey, USA).

2.2.3. Restriction fragment length polymorphism (RFLP) analysis

An ApaI-RFLP assay for Pro198Leu polymorphism was constructed using NEBCutter V2.0 [40]. Before the restriction, PCR products were synthesized successfully for 682 samples. In RFLP analysis the restriction of the polymorphic region and the constitutive region with ApaI enzyme, obtained from New England Biolabs (Beverly, MA, USA), was used as outlined by the manufacturer. The products of the restriction were analysed by 3% agarose gel electrophoresis.

2.2.4. Denaturing high performance liquid chromatography (DHPLC)

12 μl of the PCR products were injected into a preheated reverse-phase column (DNASep Column, Transgenomic) of a WAVE MD DHPLC system (Transgenomic) equilibrated by an ion pairing agent TEAA 0.1 M (Triethylammonium acetate). PolyAla polymorphism was determined using a sizing method by a linear acetonitrile gradient, achieved by mixing a buffer A (TEAA 0.1 M) with a buffer B (TEAA 0.1 M and acetonitrile 25%) with 1.4% per minute gradient increase from the start gradient of 40% up to stop gradient of 60% of the buffer B at constant temperature 54°C and constant flow rate of 0.9 ml/min. The eluted DNA was detected at 260 nm (Fig. 1). Genotypes of 682 individual PCRs were determined using the sizing calling routine (Transgenomic Navigator Software 1.6.2.). All of six different genotype combinations were also detected and confirmed by automated sequencing.

Genotyping of both polymorphisms was repeated on a random 5% of samples and results were identical to the original run.

2.2.5. Direct sequencing of the GPX1 amplicons

PCR products with different genotypes of Pro198Leu and PolyAla polymorphic regions were purified using GenElute PCR Clean Up Kit (Sigma-Aldrich Co., St.Louis, MO, USA) and confirmed by sequencing. Samples were sequenced by MWG Biotech AG (Ebersberg, Germany) with the Value Read Service, using the fluorescence-based dideoxy chain terminator method.

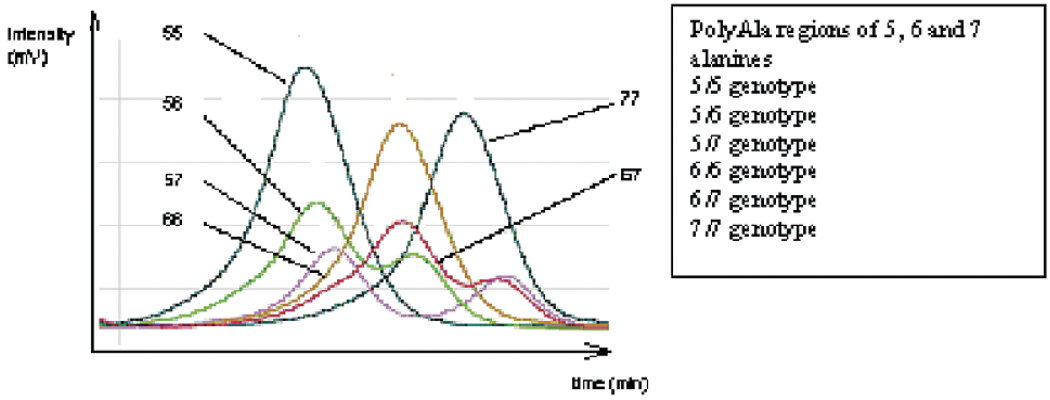

Fig. 1. Results of denaturing high performance liquid cromatography related to different genotypes of PolyAla polymorphism.

2.3. BMD measurement

BMD was measured at the lumbar (L2–L4) spine (BMDls), total hip (BMDtot) and femoral neck (BMDfn) by dual-energy X-ray absorptiometry (DXA) (QDR-4500, Hologic Inc., Waltham, MA, USA) in Ljubljana, Celje and Maribor. A cross-calibration study of the precision of measurements between the centres had previously been performed. A correction factor was not considered necessary.

2.4. Measurement of biochemical bone turnover markers

The plasma and serum samples were analysed in a routine laboratory using standard procedures as outlined by the manufacturers. Blood samples were collected between 8:00 a.m. and 10:00 a.m. after an overnight fast.

Osteocalcin (pOC) in heparinized plasma was determined in a subgroup of 289 subjects by a solid phase, two-site chemiluminescent enzyme-labelled immunometric assay (Immulite Osteocalcin Diagnostic Product Corporation, Los Angeles, CA, USA). The intra- and inter-assay variations were 3.9 and 5.2%. Concentrations of serum C-terminal crosslinking telopeptides of type I collagen (sCTX) were measured in a subgroup of 247 subjects by the enzyme-linked immunosorbent assay (Serum CrossLaps ELISA, Nordic Bioscience Diagnostics A/S, Herlev, Denmark).

2.5. Statistical analysis

Hardy-Weinberg equilibrium was tested for each polymorphism using the χ^2 test. Kolmogorov-Smirnov normality test was conducted before association analysis and data transformation was performed where appropriate. The standardized measures of LD, denoted as D' and r^2, were assessed using the EMLD software [41], which calculates pair-wise LD based on SNP genotype data from unrelated individuals. For two independent loci, the difference (D) is that between the actual and expected gametic frequencies and is usually expressed as a standardized difference (D'). D measures the statistical association of alleles in forming gametes and is related to the Pearson correlation coefficient (r). D' is used to assess the probability of historical recombination, whereas r^2 is the most relevant measure in the association studies [41]. SNPAlyze V7.0 Standard software program was used to estimate haplotypes from genotype data for individual participants. For the purpose of statistical analysis, we used only haplotype data in which the probability of correct haplotype in an individual participant assignment was estimated to be 80% or greater. In haplotype analysis, subjects were coded according to whether they had two copies, one copy, or no copies of the haplotype under study.

For each genotype, the differences in age, weight, height and body mass index (BMI) between genotype subgroups were compared in elderly women and in elderly men.

Differences in BMD, pOC and sCTX between the genotype and haplotype subgroups were tested using analysis of variance (one-way ANOVA) followed by Scheffe's post hoc test with significance level of 95% and general linear model analysis, using height as a covariate. P values less than 0.05 were considered statistically significant. All statistical analyses were performed using the statistical software package SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Table 1
Characteristics of the whole study group

	Mean ± S.D. ($N = 682$)
Age (years)	62.04 ± 9.79
Height (cm)	162.28 ± 7.40
Weight (kg)	71.37 ± 13.13
BMI (kg/m2)	27.12 ± 4.46
Years since menopause (only women)	11,91 ± 9.61
Sex (N (%) men/women)	111 (16.28) / 571 (83.72)
Femoral neck BMD (g/cm2)	0.72 ± 0.14
Total hip BMD (g/cm2)	0.88 ± 0.16
Lumbar spine BMD (g/cm2)	0.91 ± 0.18
sCTX (pmol/L)	3564.01 ± 2008.03
osteocalcin (μg/L)	18.59 ± 11.45

Abbreviations: BMI, body mass index; BMD, bone mineral density; sCTX, serum C-terminal crosslinking telopeptides of type I collagen; N, number of samples.

3. Results

3.1. Design of new primers for PCR reactions

For the genotyping of Pro198Leu polymorphism we used two different pairs of the primers. Firstly, the PCR fragments had been prepared by the primers as follows: forward: AGC CCA ACT TCA TGC TCT TC; reverse: CAG GTG TTC CTC CCT CGT AG. The data of genotypes of all samples had been obtained by using 10% PAGE after the restriction by the enzyme ApaI. Next we checked specific genotype such as CT, CC and TT with the sequencing and BLAST tool was used to search for similar sequences. We found the presence of pseudogene homologous to this region, therefore new primers, located outside of homologous regions, were designed and the specificity of PCR products were confirmed by the sequencing of all three different genotypes PCR fragments. Interestingly genotyping results obtained by new set of primers showed 4,3% lower frequencies of CT genotypes and 4,4% higher frequencies of TT genotypes of using specific PCR product in comparison to genotyping from PCR products obtained by initial pairs of primers.

3.2. Hardy-Weinberg equilibrium and genotype distribution

682 DNA samples were screened to determine the genotypes frequencies in the Slovenian population. Pro198Leu polymorphism in exon 2 was analysed by PCR-RFLP and PolyAla repeat polymorphism in exon 1 was analysed using DHPLC. Characteristics of the study group are listed in Table 1. The frequencies of all genotypes are summarized in Table 2.

The observed genotype frequency distributions for both polymorphisms were not significantly different from the Hardy-Weinberg distribution at the 5% level. The genotype distributions of the Pro198Leu polymorphism and the PolyAla repeat in the whole group ($N = 682$) were determined by Fisher exact test, with P values of 0.9747 and 0.336 respectively.

The subjects were divided according to their genotype. They did not differ in average age, height, weight, BMI or sex, except that the height in subgroups correlated with Pro198Leu polymorphism with p value of 0.034.

3.3. Association of Pro198Leu polymorphism with BMD and biochemical bone turnover markers

In whole group, ANCOVA analyses (adjusted to covariate of height) of BMD and bone turnover markers (in logarithmic scale where appropriate, to achieve normality of the distribution) showed significant associations of Pro198Leu polymorphism with BMD levels of femoral neck and total hip, concentrations of pOC and of sCTX with p values of 0.026, 0.023, 0.025 and 0.012, respectively (Fig. 2 (a)).

3.4. Association of PolyAla region genotypes with BMD and biochemical bone turnover markers

Significant associations were found in the whole group between the PolyAla region genotypes and the BMD of total hip and lumbar spine, and pOC concentration, with significances of 0.018, 0.032 and 0.027. The association with BMD of femoral neck was of borderline significance ($p = 0.117$) (Fig. 2 (b)).

3.5. Association of haplotypes 5-C, 6-T, 7-C with BMD and biochemical bone turnover markers

No evidence of strong LD was shown within two possible *GPX1* gene polymorphisms: Pro198Leu and PolyAla (D‘ $= 0.8745$, $r^2 = 0.2723$).

The interactions between different genotypes were studied by constructing 5-C, 5-T, 6-C, 6-T, 7-C and 7-T haplotypes using PolyAla repeat polymorphism with 5Ala, 6Ala and 7Ala alleles and Pro198Leu polymorphism with C and T alleles. Three haplotypes were selected with haplotype frequency > 0.05 (Table 2) and analysed further with respect to their association with BMD and biochemical markers. Significant associations were found between 6-T haplotype and BMD values of femoral neck, total hip and lumbar spine, with p

Table 2
Genotype and haplotype frequencies of the PolyAla region (5Ala/6Ala/7Ala) and the Pro198Leu (C/T) polymorphism in whole study group

PolyAla genotypes	Genotype frequencies ($N = 682$) % (N)	Pro198Leu genotypes	Genotype frequencies ($N = 682$) % (N)	Haplotype combinations	Haplotypes	Haplotype frequencies ($N = 673$)
5/5	25,8 (176)	C/C	48,1 (328)/**48,4* (*330*)	Haplotype 1: 5Ala-Pro	5-C$^{\#}$	0.4518 ($N = 304$)
6/6	10,6 (72)	C/T	43,5 (297)/**47,8* (*326*)	Haplotype 2: 6Ala-Leu	6-T$^{\#}$	0.2814 ($N = 189$)
7/7	5,6 (38)	T/T	8,4 (57)/*3, 8(26)	Haplotype 3: 7Ala-Pro	7-C$^{\#}$	0.2089 ($N = 141$)
5/6	26,7 (182)			Haplotype 4: 6Ala-Pro	6-C	0.0342 ($N = 23$)
5/7	16,1 (110)			Haplotype 5: 5Ala-Leu	5-T	0.0186 ($N = 13$)
6/7	15,2 (104)			Haplotype 6: 7Ala-Leu	7-T	0.0052 ($N = 3$)

N, number of samples; Ala, alanine; Pro, proline; $^{\#}$The selected haplotypes for further statistical analysis. *Results of genotyping on unspecific PCR fragments because of the pseudogene.

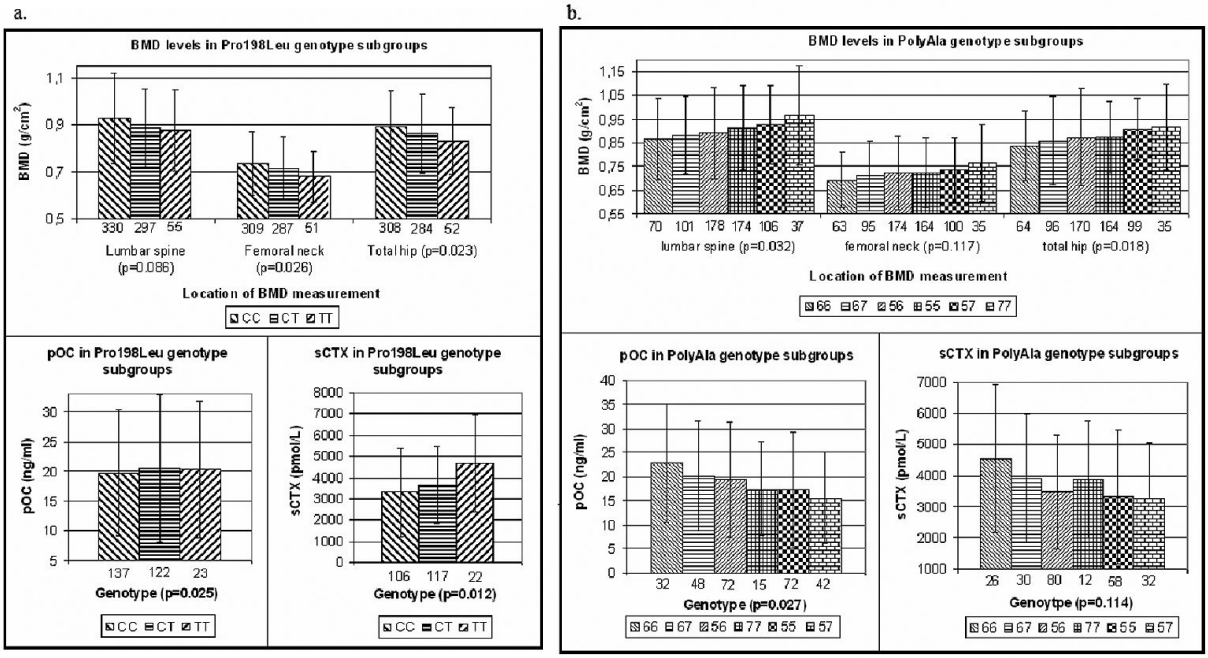

Fig. 2. BMD values and biochemical markers in relation to Pro198Leu genotypes (C/C, C/T and T/T (a)) and PolyAla repeat genotypes (5/5, 5/6, 5/7, 6/6, 6/7, 7/7 (b)). Numbers under each graph indicate the number of study samples included for statistical analysis.

values of 0.025, 0.007, 0.011, and pOC concentrations with a p value of 0.008 obtained by One-way ANOVA statistic analysis (Fig. 3). No association with sCTX was found in any of the haplotype combination under the study.

4. Discussion

In the present study we have shown for the first time that the genetic variability of the antioxidant enzyme GPX1 influences BMD on all measured skeletal sites and bone tissue markers as osteocalcin and the C-terminal telopeptide of collagen type I.

Since osteoporosis is an age related disease, in which excess production of reactive oxygen species and decrease of non-enzymatic and enzymatic defences to oxidative stress occur [1,42], we hypothesised that genetic variability in these genes has an important influence on the development of osteoporosis.

For this purpose we studied *GPX1* gene that codes for an antioxidant enzyme, which is involved in the degradation of peroxides and hydroperoxides and therefore contributes significantly to oxidative stress in normal cells [42]. Individuals with reduced GPX1 activity have a significantly increased incidence of oxidative stress-related diseases, such as breast, colon, bladder and lung cancer and coronary artery disease [27–29]. Association between the activity of GPX1 enzyme and osteoporosis has been established in mouse and postmenopausal women [7,17,20], however no evidence as-

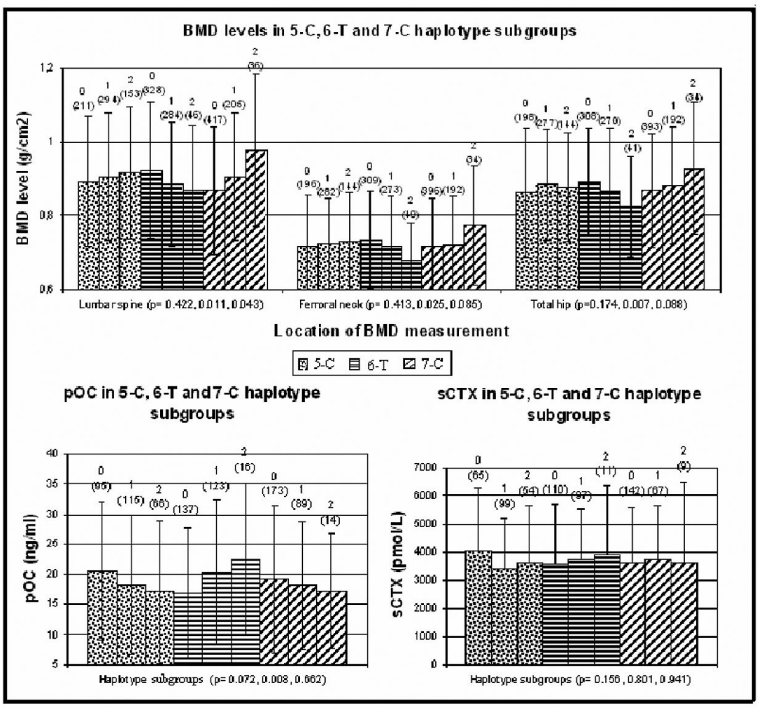

Fig. 3. BMD values and concentrations of biochemical markers (pOC and sCTX) in relation to different combinations of haplotypes in the whole study group. 0, 1, 2 indicate the number of haplotypes in each study subject, with selected haplotypes such as: 5-C (dotted), 6-T (horizontal lines), 7-C (dashed lines). P values below each graph are calculated by ANOVA statistical analysis for all three haplotype combinations (5-C, 6-T, 7-C) in line. Numbers in brackets above each graph indicate the number of included study samples for statistical analysis.

sociating genetic variance of *GPX1* with osteoporosis has been reported. *GPX1* contains multiple polymorphic sites. We selected two evidently functional variants, Pro198Leu and PolyAla of *GPX1* gene, associated with decreased activity of the GPX1 enzyme [28, 34], to conduct an association study with BMD and biochemical markers of osteoporosis.

In present study, the significant associations have been established between both Pro198Leu and PolyAla polymorphisms, individually or in combination, and BMD values of lumbar spine, femoral neck and total hip, and biochemical bone turnover markers, such as pOC level and sCTX concentration in whole group. This demonstrates an involvement of genetic variability in *GPX1* in the development of osteoporosis. Our results show that significantly ($p < 0.03$) higher BMD values of femoral neck and total hip were detected in individuals carrying the C/C genotype of Pro198Leu polymorphism and the lowest values of BMD in individuals carrying a minor homozygous genotype T/T. Results on polyAla polymorphism showed the lowest BMD values in the group carrying 6/6 genotype, followed by individuals carrying 5/5 Ala and then 7/7 Ala, which exhibited the highest BMD values. Differences between 6/6 and 7/7 genotype were significant ($p < 0.03$), showing that the 7/7 Ala variant protects against osteoporosis. These results were corroborated by observation of differences between heterozygotic individuals in whom those with variant 6/7 Ala had higher BMD than 6/6, and 5/7 higher than 5/5 but lower than 7/7.

Moreover, the haplotype analysis revealed significant association of haplotype combination 6-T with low BMD values at all locations of measurement ($p < 0.025$). Subjects carrying 7-C or 5-C haplotype combinations have higher BMD values, suggesting a lower risk of early development of osteoporosis.

The highest levels of serum CTx in homozygous genotype T/T in Pro198Leu polymorphism and in 6/6 genotype in PolyAla polymorphism are in accordance with the lowest BMD observed in these two genotypes. High bone turnover, as reflected by increased levels of serum CTx is being associated with low bone mass and is a major determinant of osteoporosis [43]. The mechanisms by which *GPX1* polymorphisms might induce increased bone resorption are not known. In this context it is interesting that plasma total antioxidant status and oxidative stress were shown to be higher in

postmenopausal women with osteoporosis than in controls [44]. Furthermore, reactive oxygen species seem to act as an intracellular signal mediator for osteoclast differentiation and consequently for osteoclast activity [45].

Differences in plasma osteocalcin concentrations were less pronounced than those in serum CTx concentrations. This finding is in line with osteocalcin high biological and diurnal variability as well as with presence of osteocalcin fragments in the serum, which makes it less accurate in predicting BMD [46,47].

An interesting observation was the association between genotypes of Pro198Leu polymorphism and height ($p = 0.034$) in the whole group. This finding might be explained by the fact that people with homozygotic Pro198 have stronger bones, given the higher BMD values, again supporting results presented in this study. However we do not have any data on subjects‘ height loss through time, which would corroborate our finding even further.

Finally, when reviewing the human genome we noted pseudogene of *GPX1* at the site 3q11-q12. The fragment of pseudogene has the same size as the amplified PCR product, prepared for Pro198Leu genotyping of the *GPX1* gene at first, based on the sequence available in GenBank (accession no. NC_000003.10). Moreover using standard 2% agarose gel and 10% polyacrylamide electrophoresis we were unable to separate the two products. Therefore we confirmed amplification of the pseudogene with automated sequencing. Novel primers listed in Materials and methods were chosen outside homologous regions and the specificity of the PCR product was checked using automated sequencing. This finding has deeper implications since the review of many studies [48–50] showed that the presence of pseudogene was not taken into consideration. This might be a due to the relatively late discovery [51] of pseudogene, however it still raises doubts about quality of their results. Importantly, both interrogated polymorphisms were in Hardy-Weinberg equilibrium showing the $p > 0.3$.

However, the present study has some limitations. Firstly, the specific action mechanism of GPX1 enzyme in the pathogenesis of osteoporosis is far from clear. Secondly, no data on fragility fractures and the bone quality of bone were available for the analysis. Thirdly, current sample size allowed only a limited power to detect interactions. By expanding the sample size, we could further investigate genetic influence of the studied gene on the development of osteoporosis. Moreover in our study, no data on external factors that influence BMD such as dietary calcium and vitamin D intake, smoking habits, alcohol consumption and physical activity were collected and analysed. However, our group was ethnically homogenous and the participants originate from the same environment and have similar lifestyles. In our study, we did not fully cover all genetic variation in the *GPX1* gene, since only variants that affect the activity of the enzyme were studied.

In summary, we conclude that the Pro198Leu and PolyAla polymorphisms are associated, individually or in combination, with BMD and bone turnover markers in Slovenian patients. Moreover, the 6-T haplotype was associated significantly with low levels of BMD and high concentrations of osteocalcin. These results support the role of the *GPX1* gene and of oxidative stress in the development of osteoporosis and indicate that genetic variance of the other proteins involved in the antioxidative response, such as catalase, glutathione reductase, glutathione S-transferase, could also be associated with osteoporosis. By further investigation of the genetic interactions between these genes, the number of the studied SNPs should be increased and confirmed in the larger cohorts. Further biological and/or functional evidence would be needed to confirm the suggested influence of *GPX1* variants on the BMD regulation. However, these results further emphasize and contribute to our understanding of the genetic component in osteoporosis.

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