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Opposite effects of *GSTM1* – and *GSTT1* – gene deletion variants on bone mineral density

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Abstract. Oxidative stress is associated with osteoporosis. The glutathione S-transferases form the major detoxifying group of enzymes responsible for eliminating products of oxidative stress. We have therefore proposed *GSTM1* and *GSTT1* genes as candidates for studying the genetics of osteoporosis.
The aim of the present study was to examine possible association of *GSTM1* and *GSTT1* gene deletion polymorphisms, alone or in combination, with bone mineral density at femoral neck (BMD_fn), lumbar spine (BMD_ls) and total hip (BMD_th) in Slovenian elderly women and men.
GSTM1 and *GSTT1* gene deletion polymorphisms in 712 elderly people were analyzed using the triplex PCR method for the presence of *GSTM1* and *GSTT1* gene segments. BMD_fn, BMD_ls and BMD_th were measured by the dual-energy X-ray absorptiometry (DEXA) method. Results were analyzed using univariate statistic model adjusted for sex, body mass index (BMI) and age.
Our results showed the significant differences in BMD_th, BMD_ls and BMD_fn values ($p = 0.031$, 0.017 and 0.023, respectively) in subgroups of *GSTT1* gene deletion polymorphism. For *GSTM1* gene deletion polymorphism borderline significant association was found with BMD_ls ($p = 0.100$). Furthermore, subjects with homozygous deletion of *GSTT1* gene showed higher BMD values on all measured skeletal sites and, in contrast, subjects with homozygous deletion of *GSTM1* gene showed lower BMD values. Moreover, a gene-gene interaction study showed significant association of GSTM1-null and GSTT1-null polymorphisms with BMD_ls values ($p = 0.044$). Carriers with a combination of the presence of *GSTT1* gene and the homozygous absence of *GSTM1* gene fragment were associated with the lower BMD values at all skeletal sites.
The significant association of combination of *GSTT1* gene presence and homozygous absence of *GSTM1* gene with BMD was demonstrated, suggesting that it could be used, if validated in other studies, as genetic marker for low BMD.

Keywords: Osteoporosis, oxidative stress, antioxidant defence system, bone mineral density, triplex method, glutathione s-transferase

1. Introduction

Osteoporosis is a systemic skeletal age-related disease characterized by low bone mass and microarchitectural deterioration of bone tissue resulting in increased bone fragility and increased fracture risk (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis and Therapy 2001*). The incidence of osteoporosis in Slovenia reflects the aging of the population. The percentage of Slovenians being diagnosed with osteoporosis increases from 12% at age 50 to 50% at 90 [1].

Oxidative stress, increased in elderly people, is associated with the development of osteoporosis [2–4] by modulating osteoblast differentiation and survival [5] and by stimulating bone resorption [6,7]. Significantly increased levels of lipid peroxidation and hydrogen peroxide and decreased levels of antioxidant enzymes

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have been found in ovariectomized rats [8–10] and postmenopausal women [11,12]. Further, microarray analysis on primary human osteoblast cell lines from osteoporotic patients showed significant differential expression profile of genes involved in the antioxidative defence system [13].

The glutathione S-transferases (GSTs) form a supergene phase II detoxifying antioxidant enzyme family, highly expressed in liver and also in bone cells [14]. Two GSTs, GSTM1 and GSTT1, play an important role in cellular protection by conjugating several environmental carcinogens, endogenous compounds such as peroxidized lipids and inactive products formed as secondary metabolites during oxidative stress, with endogenous ligands to facilitate their excretion [15–18].

Twin and family studies have shown that genetic factors account for 50–85% of the variance in bone mineral density (BMD) [19]. Numerous studies and meta-analyses of SNPs in osteoporosis candidate genes have shown association with BMD, however very few studies were done on polymorphisms of genes coding for enzymes involved in the antioxidative defense system, such as catalase (*CAT*) and glutathione peroxidase 1(*GPX1*) [20,21]. No associations have been identified with the GST gene family.

Two variants have been identified in the *GSTM1* gene: the GSTM1*0 allele, caused by a gene deletion and thus associated with lack of enzyme activity, and the C allele of Lys173Asp polymorphism (rs74837985) which does not appear to affect enzyme activity [22].

Two functionally important variants have been identified in *GSTT1* gene: the GSTT1*0 allele, caused by a gene deletion, and the A allele of Thr104Pro polymorphism (rs11550605) [23]. The A allele and the GSTT1*0 alleles are associated with non-functional phenotypes.

While there are data on their potentiation of risk of cytogenetic damage in many diseases, only one osteoporosis-association study on deletion polymorphisms of *GSTT1* and *GSTM1* genes has been reported; it showed no significant association with bone density values in an elderly Japanese population [24].

However, the linkage study by Ioannidis et al. [25] showed the greatest significance of quantitative trait locus (QTL) on chromosome 1p13.3-q23.3, on which the *GSTM1* gene is located, for lumbar spine BMD. Moreover, linkage studies by Zhang et al. [26] and Streeten et al. [27] showed the presence in 1p36 of QTLs affecting BMD at multiple skeletal sites. This region is near that of *GSTM1*.

The aim of the present study was to identify whether there are any associations of the GSTM1 and GSTT1 null polymorphisms, alone or in combination with BMD, measured at lumbar spine, femoral neck and total hip.

Table 1
Characteristics of the whole study group

	Mean ± S.D. ($N = 712$)
Age (years)	62.04 ± 9.79
Height (cm)	162.28 ± 7.47
Weight (kg)	71.56 ± 13.32
BMI (kg/m^2)	27.25 ± 4.46
Years since menopause (only women)	11.91 ± 9.61
Sex (N (%) men/women)	119 (16.7) / 593 (83.3)
BMD_fn (g/cm^2)	0.72 ± 0.14
BMD_tot (g/cm^2)	0.88 ± 0.16
BMD_ls (g/cm^2)	0.91 ± 0.18

Abbreviations: BMI, body mass index; BMD, bone mineral density; fn, femoral neck; tot, total hip; ls, lumbar spine; N, number of samples.

2. Materials and methods

2.1. Subjects

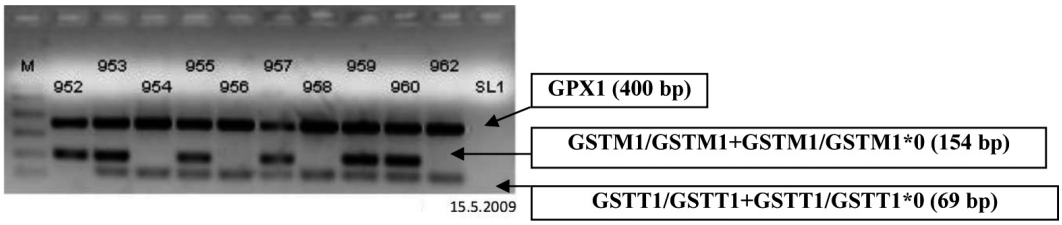
The study included 712 elderly Slovenian people (593 women and 119 men); 292 (266 women and 26 men) had diagnosis of osteoporosis according to ISCD criteria (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis and Therapy 2001*). Subjects were referred to the outpatient departments of the University Medical Centre, Ljubljana, the General and Teaching Hospital, Celje, and the University Clinical 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. 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. Characteristics of the study group are listed in Table 1.

2.2. Blood sampling and genotyping

Genomic DNA was isolated from peripheral blood leukocytes using the QIAmp DNA Blood Midi isolation kit (Qiagen, Germany). Three pairs of oligonucleotide primers (Table 2) were designed based on the sequences of *GSTT1, GSTM1* and *GPX1* genes available in GenBank (accession no. NM_000637.2). Fragments for the all three genes were amplified simultane-

Table 2
Primer sequences and reaction conditions used for multiplex-PCR for detection of GST-null polymorphisms

Gene/variant	Amplicon size	Sense (F) and antisense (R) primer	Cycling conditions
GSTT1/null	69bp	F: 5-ATGTGACCCTGCAGTTGC-3	95°C, 10 min
		R: 5-AGATGTGAGGACCAGTAAGG-3	95°C, 60 s
GSTM1/null	154bp	F: 5-GCTTCACGTGTTATGGAGGTT-3	57°C, 60 s (35 cycles)
		R: 5-CGGGAGATGAAGTCCTTCAGA-3	72°C, 60 s
GPX1/positive control	400bp	F: 5-AGCCCAACTTCATGCTCTTC-3	72°C, 8 min
		R: 5-AGATGTGAGGACCAGTAAGG-3	[8°C, ∞]



M, marker with lengths of 50, 150, 300, 500, 700bp ; No. 952-962, ID of samples; SL1, negative control

Fig. 1. Multiplex PCR products on 2% agarose gel (90 V, 25 min).

ously by the triplex-PCR method, with the following cycling conditions for all primer pairs together in the same reaction (Table 2): an initial 10 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 57°C, 1 min at 72°C, and finally 8 min at 72°C. The PCR mixture (20 μL) contained genomic DNA (100 ng), 1x PCR buffer, 0.4 mM of each of the four deoxyribonucleotides, 1.5 mM $MgCl_2$, 0.20 μmol/L of each primer and 3 U of AmpliTaq Gold polymerase (Applied Biosystems, Roche Molecular Systems Inc., Brandenburg, New Jersey, USA). Aliquots of PCR products were electrophoresed on 2% agarose gel to check their quality and quantity. GPX1 was used as positive control in each reaction. The absence of an amplification product, together with the presence of a positive control band, indicated the null (variant) type for both polymorphisms (Fig. 1).

The samples with null polymorphisms were repeated in PCR reaction with only one related pair of primers of each gene, to confirm its deletion polymorphism.

2.3. BMD measurement

BMD was measured at the lumbar (L1-L4) spine (BMD_ls), total hip (BMD_th) and femoral neck (BMD_fn) by dual-energy X-ray absorptiometry (DEXA) (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. Statistical analysis

Genotype frequencies in patients and control subgroups were compared using a likelihood ratio-χ^2 test.

Kolmogorov-Smirnov normality test was conducted before association analysis and data transformation was performed where appropriate. Univariate general linear model (ANCOVA), with adjustment for sex, BMI and age (in a logarithmic scale for some tests), and followed by post hoc test (LSD), was used to assess the statistical differences between BMD measurements on subjects with different deletion genotypes of GSTM1 and GSTT1 polymorphisms. Kruskal-Wallis non-parametric test was used to evaluate the association of not normally distributed parameters with different genotypes. P values less than 0.05 were considered statistically significant. All statistical analyses were performed using the statistical software package SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

The power calculations were done by using G*Power version 3.0.3. The whole study group had a 97.9% statistical power to detect low effect sizes (0.15) with an alpha level of 0.05.

3. Results

3.1. Subject characteristics and genotype distribution

Characteristics of the whole elderly study group are presented in Table 1. No statistically significant differences in age, weight, height, BMI and years

since menopause among women between genotype subgroups were observed.

As the PCR method was not suitable for distinguishing between homozygotes (+/+, wild type) and heterozygotes (+/−), these two groups were considered together (non-null genotypes, GSTT1/GSTT1+GSTT1/GSTT1*0, GSTM1/GSTM1+GSTM1/GSTM1*0, respectively) and compared with the homozygous variant group (null genotypes, GSTT1*0/GSTT1*0, GSTM1*0/GSTM1*0, respectively). Hardy Weinberg Equilibrium testing was thus not performed.

The frequencies of GSTT1 and GSTM1 deletion polymorphisms in the whole study group are presented in Table 3. Comparison of genotype frequencies between osteoporotic and non-osteoporotic elderly people did not reveal any significant difference for either deletion polymorphism.

3.2. *Association of individual deletion polymorphisms with BMD values*

In the whole study group of elderly people, significant differences of BMD_fn ($p = 0.023$), BMD_th ($p = 0.031$) and BMD_ls ($p = 0.017$) between different genotype subgroups of GSTT1 deletion polymorphism were observed (Figs 2 and 3). Borderline significant differences of BMD_ls values between GSTM1 deletion genotype subgroups have been shown ($p = 0.100$). Statistical analysis was performed using ANCOVA adjusted for sex, BMI and age.

3.3. *Association of combined GSTM1 and GSTT1 gene deletion variants with BMD values*

Significant differences of BMD_ls and borderline significant differences of BMD_fn between subgroups of combined presence of *GSTT1* and absence of *GSTM1* genes adjusted for sex, BMI and age were found ($p = 0.044$ and 0.119, respectively). Post hoc analysis revealed significant differences of BMD_fn between GSTT1+GSTM1*0 and GSTT1*0+GSTM1*0 (p value of 0.007) and between GSTT1+GSTM1 and GSTT1*0+GSTM1*0 combination subgroups (p value of 0.030). Moreover, borderline significant differences of BMD_ls between GSTT1+GSTM1*0 and GSTT1*0+GSTM1 combination subgroups were observed ($p = 0.074$). All results of the combined influences of the two deletion gene variants are summarized in Fig. 4.

4. Discussion

Homozygous deletion of the *GSTT1* gene is shown to be significantly associated with higher BMDs measured at femoral neck, lumbar spine and total hip. In contrast, association of homozygous deletion of *GSTM1* gene with BMD values at lumbar spine in elderly Slovenians is of borderline significance. Moreover, the combination of the presence/absence of the *GSTM1* and *GSTT1* genes showed significant effects on BMD_ls values. So variations in genes coding for GST antioxidative enzymes are associated with BMD.

The GST family is involved in the conjugation and thus excretion of toxic oxidant products, which are increased in osteoporotic people [15–18]. Polymorphisms or deletions within these genes alter the catalytic activity of the GSTs to varying extents. The study by Miranda-Vilela et al. [28] showed that, under oxidative stress induced by H_2O_2, the highest and lowest extents of DNA damage depended on the interaction between GSTM1-T1 polymorphisms.

In our study, the significant associations of GSTT1-gene deletion variant, alone or in combination with GSTM1- gene deletion variant, add to the list of antioxidative enzyme gene polymorphisms associated with BMD values. Contrary to the results presented here, it has been reported that GSTM1 and GSTT1 gene deletion genotypes do not significantly affect susceptibility to osteoporosis in Japanese osteoporotic patients assessed using ultrasound bone tissue measurement [24]. According to published data, the genetic diversity in the Japanese population shows slightly higher GSTM1*0 genotype (55.8%) and significantly higher GSTT1*0 genotype (40.0%) frequencies than in the Slovenian population (49.0% and 21.3%, respectively). The Slovenian population is more homogenous having smaller range of years of tested individuals.

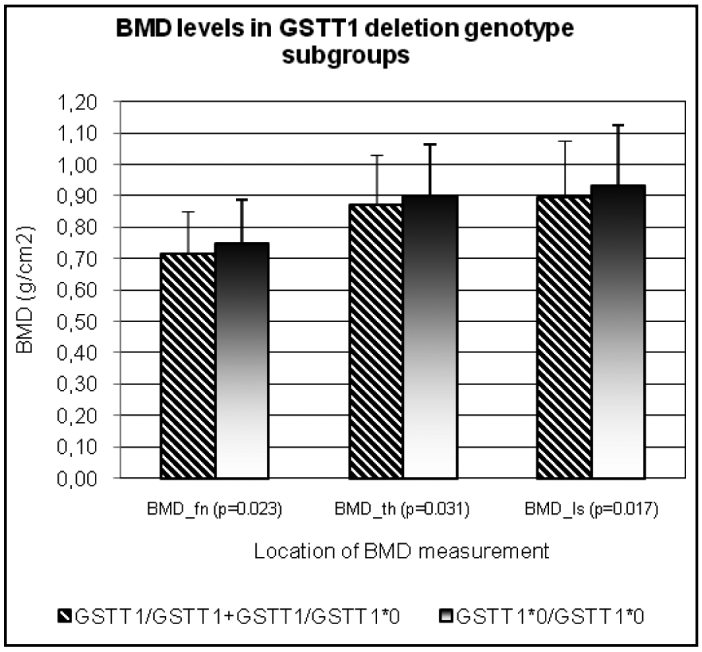
The GSTM1*0 and GSTT1*0 genotype frequencies of the control population in our study were in accord with other reported frequencies of the control 12.525 Caucasians from the meta-analysis [29]. These alleles are common, with the GSTT1- and GSTM1- null genotypes occuring in 10-20% and 40-65% of the Caucasian population, respectively [30]. Moreover, a much lower frequency of GSTM1*0 and a much higher frequency of GSTT1*0 carriers was detected in the osteoporotic subgroup than in the control group in our study, although the differences were not significant.

It is interesting that two polymorphisms studied here have opposite effects on BMD values. The absence of *GSTT1* gene is significantly associated with the higher

Table 3
Genotype and combination frequencies of the GSTT1 (presence: GSTT1/GSTT1+ GSTT1/ GSTT1*0 and absence: GSTT1*0/GSTT1*0) and the GSTM1 (presence: GSTM1/GSTM1+ GSTM1/GSTM1*0 and absence: GSTM1*0/ GSTM1*0) polymorphisms in whole study group

	Genotype frequencies ($N_{\text{total}} = 712$) % (N) (whole group/ osteoporotic-/ non-osteoporotic- subgroups)	P value Hi-square (osteoporotic vs. nonosteoporotic)
GSTT1 and GSTM1 genotypes		
GSTM1/GSTM1+ GSTM1/GSTM1*0	48.9 (348) / 47.3 (131) / 50.6 (204)	0.641
GSTM1*0/GSTM1*0	51.1 (364) / 52.7 (146) / 49.4 (199)	
GSTT1/GSTT1+ GSTT1/GSTT1*0	81.1 (578) / 84.5 (234) / 78.7 (317)	0.289
GSTT1*0/GSTT1*0	18.8 (134) / 15.5 (43) / 21.3 (86)	
GSTT1 and GSTM1 polymorphism combinations		
Combination 1: GSTT1/GSTT1+GSTM1/GSTM1	39.3 (280) / 38.6 (107) / 40.2 (163)	0.624
Combination 2: GSTT1/GSTT1+GSTM1*0/GSTM1*0	41.8 (298) / 45.8 (127) / 38.5 (156)	
Combination 3: GSTT1*0/GSTT1*0+ GSTM1/GSTM1	9.4 (66) / 8.7 (24) / 10.1 (41)	
Combination 4: GSTT1*0/GSTT1*0+ GSTM1*0/GSTM1*0	9.5 (68) / 6.9 (19) / 11.1 (45)	

N, number of samples.



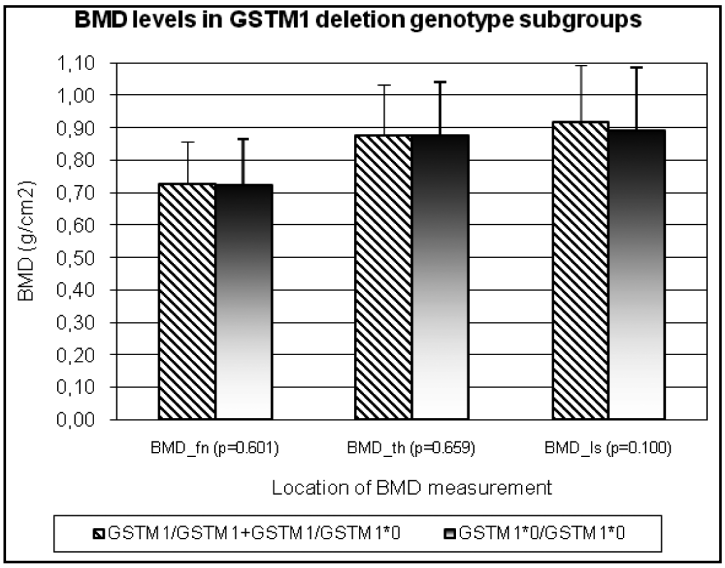
*ANCOVA, adjusted for sex, age and body mass index

Fig. 2. BMD values in GSTT1 deletion genotype subgroups.

BMD values at all skeletal sites, with the greatest significance on BMD_ls, whereas the absence of *GSTM1* gene is borderline associated with lower BMD values, again with the greatest significance on BMD_ls.

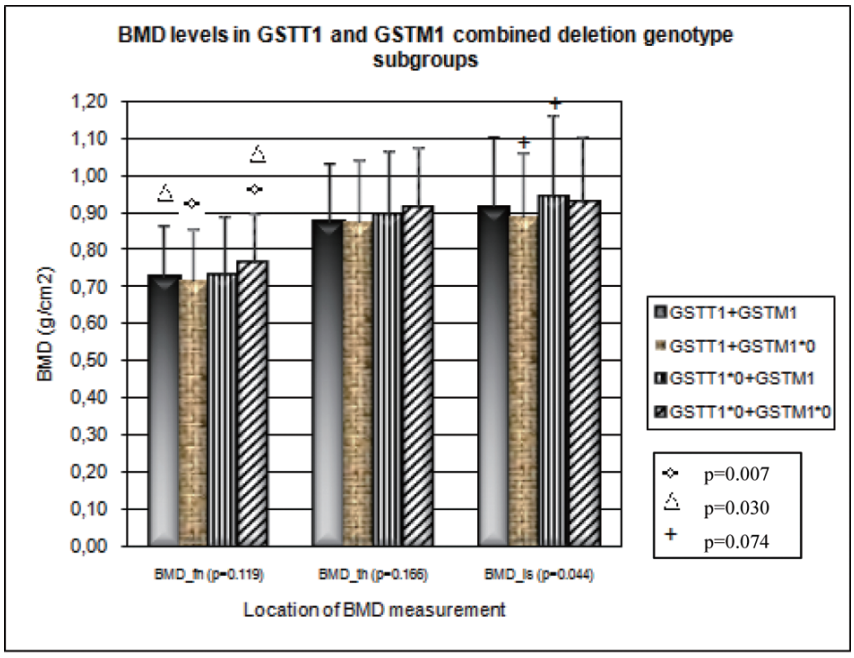
The significant role of *GSTM1* gene region in BMD_ls has been reported earlier in the linkage study of Ioannidis et al. [25], where the QTLs with the greatest effect were shown on chromosome 1p13.3-q23.3, also the *GSTM1* gene specific region for lumbar spine BMD. However, to date, no linkage study has shown any significant, for *GSTT1* gene specific, chromosome region associated with BMD values.

Although *GSTM1* expression is concentrated in liver, it is involved in the conjugation (and thus transport and excretion) of a broad range of endobiotics and xenobiotics from different tissues [31] possibly also influence gene expression in different tissues. In human osteoblasts, *GSTM1* is 2-times more expressed than a *GSTT1* gene, consequently resulting in 2-times more effective detoxification in bone cells [[21], 'un-



*ANCOVA, adjusted for sex, age and body mass index

Fig. 3. BMD values in GSTM1 deletion genotype subgroups.



*ANCOVA, adjusted for sex, age and body mass index

Fig. 4. BMD values in combined GSTT1/GSTM1-gene deletion genotype subgroups.

published results']. When the *GSTM1* gene is deleted, it can lead to a greater adverse effect on bone osteoblast cells, resulting in lower BMD, which is in accordance with our results. Moreover, the deletion of *GSTT1* gene is associated with the higher BMD values in our study and, due to lower expression of *GSTT1* gene than that of *GSTM1* gene in osteoblasts, the influence of the *GSTT1* gene on bone is less pronounced than that of the *GSTM1* gene. However the loss of GSTT1 enzyme activity by deletion polymorphism can affect other metabolic pathways that could be strongly activated, for example other genes in the GST family, thus causing the pre-

ventive effect on osteoblast cells. This fact could be supported, however indirectly, by the study of Fuciarelli et al. [32] who hypothesized that there is probably a regulative compensatory mechanism that involves the increased activity of GSTT1 enzyme when the GSTM1 enzyme is lacking. Further studies are needed on the activities of GSTT1 and GSTM1 and their relation to deletion genotypes.

In our study, the subjects with a combination of present *GSTM1* and absent *GSTT1* genes showed the higher BMD_ls values, while the lowest BMDs at all skeletal sites were shown in subjects with the inverse – presence of *GSTT1* and absence of *GSTM1* genes. Moreover, it appears that carriers with homozygous deletion of *GSTT1* gene show the preventive effect on BMD values via the upregulation of other enzymes in the GST family due to lack of GSTT1 enzyme activity. Another explanation of this effect could be that GSTT1, but not GSTM1, is also known to have Phase I activity and the ability to activate oxidative stress related molecules rather than their inactivation by Phase II detoxification step [33,34]. Moreover, the presence of *GSTT1* gene and homozygous absence of *GSTM1* gene combination is much more frequent in osteoporotic people than in controls and exhibits the lowest BMD values on all skeletal sites. This combination could be used as a marker for susceptibility to low BMD values.

The present study has some limitations. First, the specific mechanisms of the studied GSTT1 and GSTM1 enzymes in the pathogenesis of osteoporosis are far from clear. Both genes have been considered to encode carcinogen-metabolising enzymes implying they mediate the detoxification of potential mutagens such as polycyclic aromatic hydrocarbons, which are found in many common exposures such as cigarette smoke, diesel fuel and grilled meats [35]. GST substrates also include ROS, products of endogenous lipid peroxidation and inactivating organic hydroperoxides including thymine hydroperoxide and arachidonic acid hydroperoxide, which may arise from inflammation. Oxidative stress causes alterations in redox balance by affecting changes in the GSH:GSSG ratio which in turn influences expression of immunologically and bone relevant transcription factors including NF-κB. This factor induces expression of TNF-α, which activates the expression of RANKL in osteoblasts [36,37], and further increases osteoclastogenesis. This possibility appears attractive as *GSTM1* deleted gene is associated with lower values of BMD.

Secondly, no data on fragility fractures and bone quality were available for the analysis.

Furthermore, no other SNPs in both genes were investigated. However, only the gene deletion polymorphisms were studied where the complete loss of GSTT1 and GSTM1 enzyme activities occurs.

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, lowering the possibility of false positive associations.

Our findings need to be confirmed in new patient cohorts. Clearly, associations between both GST-null polymorphisms and clinical phenotypes will be mediated by interactions with other polymorphic loci of other genes. The Xu et al. [38] reported that *GSTM1* is tightly clustered with 3 other *GST*-mu genes which are spaced approximately 20 kb apart and arranged in the following order: 5-prime–*GSTM4*–*GSTM2*–*GSTM1*–*GSTM5*–3-prime. Thus all the *GSTM1* neighbouring genes, clustered in the same GST family, could show synergistic effects on BMD. It is necessary to examine large number of candidate genes GSTs family in order to identify the genotype combinations that determine risk for osteoporosis. It is also possible that *GSTT1* is in linkage disequilibrium with the true candidate gene nearby on chromosome 22 and it is worth to study furtherly on these regions.

In conclusion, in our study, we have demonstrated that homozygous deletion of the *GSTT1* gene is significantly associated with higher BMDs measured at femoral neck, lumbar spine and total hip. In contrast, homozygous deletion of *GSTM1* gene is borderline significantly associated with lower BMD values only at lumbar spine in elderly Slovenians. Deletion of *GSTT1* gene showed a "protective" effect, probably by compensatory increase of other GST enzymes. Moreover, the combination of lacking *GSTM1* and present *GSTT1* genes was significantly associated with lower BMD-ls values.

The results of our study provide evidence for the hypothesis that oxidative stress in osteoporosis can be accelerated, not only by increased production of ROS but also by reduced ability to conjugate oxidant products and thus eliminate them. This is caused at least partly by deletion of gene segments of *GSTM1*. Through an understanding of the molecular pathways responsible for bone remodeling in osteoporosis patients we will be able to produce novel agents targeting these pathways and thus improve the management of osteoporosis.

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