The combinations of polymorphisms in vitamin D receptor, osteoprotegerin and tumour necrosis factor superfamily member 11 genes are associated with bone mineral density

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Abstract

1 α ,25-dihydroxyvitamin D₃ upregulates tumour necrosis factor superfamily member 11 (*TNFSF11*) that codes for the receptor activator of nuclear factor κ B ligand (RANKL), and downregulates osteoprotegerin (*OPG*) expression. We have analyzed the individual effects of polymorphisms in the vitamin D receptor gene (*VDR*), *OPG* and *TNFSF11*, and searched for interactions between them. Six hundred and forty one subjects were evaluated: 239 osteoporotic and 228 non-osteoporotic post-menopausal, 57 pre-menopausal women and 117 elderly men. The subjects were genotyped for *BsmI*, *FokI* and *Cdx2* in *VDR*, K3N in *OPG* and $-290C > T$, $-643C > T$ and $-693G > C$ in *TNFSF11* gene. Bone mineral density (BMD) and biochemical markers were measured. In the osteoporotic women, femoral neck BMD (BMD-fn) showed associations with *BsmI*(*VDR*) and *Cdx2*(*VDR*) ($P=0.015$ and 0.047 respectively), and lumbar spine BMD (BMD-ls) with K3N(*OPG*) and $-290C > T$ (*TNFSF11*) ($P=0.021$ and 0.017). No association with BMD was found in the non-osteoporotic women. In the pre-menopausal women, the *Cdx2*(*VDR*) polymorphism was associated with BMD-fn and total hip BMD ($P=0.011$ and 0.011). In elderly men, *FokI*(*VDR*) was associated with BMD-fn and BMD-ls ($P=0.040$ and 0.036). Interestingly, the $-290C > T$ (*TNFSF11*)-K3N(*OPG*) combination was associated with BMD-th ($P=0.041$) in the osteoporotic women. In the non-osteoporotic women, the combination K3N(*OPG*)-*Cdx2*(*VDR*) was associated with BMD-ls, BMD-th and BMD-fn ($P=0.032$, 0.049 and 0.022), and the combination $-290C > T$ (*TNFSF11*)-K3N(*OPG*) with BMD-fn ($P=0.029$). For the first time, the presence of gene–gene interactions between *VDR*, *OPG* and *TNFSF11* genes was studied. Our results strongly suggest further confirmation of their combined influence on larger cohorts.

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Introduction

Osteoporosis is a common multifactorial disease characterized by reduced bone mass and strength, changes in the microarchitecture of bone tissue and an increased risk of fracture. Fracture affects up to 30% of women and 12% of men at some point. Bone mineral density (BMD) is accepted as the primary clinical predictor of fracture risk and is genetically determined. Twin and family studies show heritability of 50–85% for peak bone mass (Ralston & de Crombrughe 2006).

1 α ,25-dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃) plays a central role in skeletal metabolism by binding to its nuclear steroid receptor, vitamin D receptor (VDR). 1 α ,25(OH)₂D₃ can stimulate bone formation and resorption, regulating bone turnover by acting on both osteoblastic and osteoclastic lineage cells (Baldock *et al.* 2006). Recent data show that 1 α ,25(OH)₂D₃ is also involved in the regulation of local factors such as

osteoprotegerin (OPG)/receptor activator of nuclear factor κ B ligand (RANKL)/RANK system. In the OPG/RANKL/RANK system, OPG acts as a decoy receptor for RANKL (Yasuda *et al.* 1999) and inhibits osteoclast differentiation, survival and function *in vitro* and bone resorption *in vivo* (Kondo *et al.* 2004) by preventing the interaction of RANKL with its receptor RANK. 1 α ,25(OH)₂D₃ affects OPG and RANKL in a reciprocal manner, promoting osteoclastogenesis by upregulating the expression of RANKL and downregulating the expression of OPG (Kondo *et al.* 2004). In mice, 1 α ,25(OH)₂D₃ transiently suppressed *OPG* gene expression by suppressing *OPG* promoter activity through the AP-1 binding site and by accelerating degradation of *OPG* mRNA (Kondo *et al.* 2004). In the tumour necrosis factor superfamily member 11 (*TNFSF11*) gene that codes for RANKL protein, vitamin D response elements (VDREs) have been identified in the mouse promoter region (Kitazawa *et al.* 1999).

In mice and in humans, several distal regulatory regions in *TNFSF11* gene promoter have been found to mediate the activity of $1\alpha,25(\text{OH})_2\text{D}_3$ through VDR and contain functional VDREs (Kim *et al.* 2006, Nerenz *et al.* 2008).

As osteoporosis is a disease with a strong genetic component, much effort is focused on the identification of single nucleotide polymorphisms (SNPs) associated with the osteoporotic phenotype (Ralston & de Crombrughe 2006). The outbreak of genetic association studies on osteoporosis started with the reported association between VDR alleles and BMD (Morrison *et al.* 1994). Numerous association studies have been performed on the VDR gene and BMD showing conflicting results (Thakkestian *et al.* 2004, Fang *et al.* 2005, Zintzaras *et al.* 2006). In a prospective multicentre large-scale association study involving 26 242 participants, only the Cdx2 polymorphism in the VDR gene was found to be associated with the risk of vertebral fractures (Uitterlinden *et al.* 2006). In the OPG gene, several association studies have consistently shown an association of the K3N polymorphism with the osteoporotic phenotype (Langdahl *et al.* 2002, Arko *et al.* 2005, Choi *et al.* 2005, Zhao *et al.* 2005, Kim *et al.* 2007, García-Unzueta *et al.* 2008). However, some studies revealed a non-significant association of K3N with the osteoporotic phenotype (Wynne *et al.* 2002, Ueland *et al.* 2007). Two recently published genome-wide association analyses have identified the OPG gene as playing a key role in the biological characteristics of bone (Richards *et al.* 2008, Styrkarsdottir *et al.* 2008). Association with the osteoporotic phenotype has not been widely studied in the *TNFSF11* gene (Hsu *et al.* 2006, Kim *et al.* 2007, Mencej *et al.* 2008). However, a functional role has been established for some SNPs in the *TNFSF11* promoter (Mencej *et al.* 2008).

Based on the new data on the crosstalk between RANKL/RANK/OPG and vitamin D pathways, we have examined the possibility of their combined influence on the risk of osteoporosis. The VDR, OPG and *TNFSF11* genes have been studied as candidate genes for the regulation of susceptibility to osteoporosis. As the combined influence of the risk alleles of the VDR, OPG and *TNFSF11* gene SNPs on BMD has not been elucidated, our aim was to examine the potential combined influence of risk genotypes on BMD at different sites in post-menopausal women.

Materials and methods

Subjects

We evaluated 641 subjects, who had been referred to the outpatient departments of the University Medical Centre, Ljubljana, the General and Teaching Hospital, Celje or the Teaching Hospital, Maribor, Slovenia for

BMD measurement. On the basis of gender and menopausal status, the subjects were divided into four subgroups: 239 osteoporotic and 228 non-osteoporotic post-menopausal women, 57 pre-menopausal women and 117 elderly men. Each patient 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 (WHO 1994). The study was approved by the Ethical Committee of the Republic of Slovenia and written informed consent was obtained from each subject participating in the study. The study was conducted in compliance with national legislation and the Declaration of Helsinki.

Genotyping

Genomic DNA was isolated from peripheral blood leukocytes using standard methodology. The TaqMan allelic discrimination method, using the TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) with slight modifications, was used for genotyping of the following SNPs: K3N (rs2073618) in the OPG gene, and $-290\text{C}>\text{T}$ (rs9525641) in the *TNFSF11* gene. Reactions were performed on an ABI Prism 7000 sequence detection system (Applied Biosystems).

PCR primers were designed for SNPs $-643\text{C}>\text{T}$ (rs9533156) and $-693\text{G}>\text{C}$ (rs9533155) in the *TNFSF11* gene, and *BsmI* (rs1544410), *FokI* (rs2228570) and Cdx2 (rs11568820) in the VDR gene, based on the gene sequences available in GenBank (accession numbers AF544022 for *TNFSF11* and AY342401 for VDR). The subjects were genotyped for the presence of $-643\text{C}>\text{T}$ (*TNFSF11*) and $-693\text{G}>\text{C}$ (*TNFSF11*) as described previously (Mencej *et al.* 2006).

For genotyping *BsmI* (VDR) and *FokI* (VDR) two pairs of primers were used for duplex PCR: the first pair of sense 5'-GGCAACCTGAAGGGAGACGTA-3' and antisense 5'-CTCTTTGGACCTCATCACCGAC-3' primers, and the second pair of sense 5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3' and antisense 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3' primers. Cycling conditions consisted of an initial 12 min at 95 °C followed by 40 cycles of 30 s at 94 °C, 45 s at 56 °C, 30 s at 72 °C, and finally, 8 min at 72 °C. For genotyping Cdx2 (VDR) the following PCR primers were used: sense 5'-ACTGCAGCCTTGACCTCCTA-3' and antisense 5'-AAAGCAAACCAAGGGGTCTT-3'. Cycling conditions consisted of an initial 10 min at 95 °C followed by 36 cycles of 45 s at 95 °C, 20 s at 58 °C, 45 s at 72 °C and finally, 7 min at 72 °C. The PCR mixture (20 µl) contained genomic DNA (100 ng), 1× PCR buffer, 2.0 mM MgCl₂, 0.3 mM each of the four deoxyribonucleotides, 0.125–0.375 µM each of

oligonucleotide primers and 1·0 U AmpliTaq Gold polymerase (Applied Biosystems, Roche Molecular Systems, Inc). For analysis of the *VDR* gene SNPs, the following restriction enzymes were used as outlined by the manufacturer (New England Biolabs, Beverly, MA, USA): *BsaI* for *BsaI*, *FokI* for *FokI* and *HypCHIII* for *Cdx2* polymorphism.

BMD measurement

BMD measurements at the lumbar (L1–L4) spine (BMD-ls), total hip (BMD-th) and femoral neck (BMD-fn) were performed by dual-energy X-ray absorptiometry (QDR-4500, Hologic Inc., Waltham, MA, USA) in Ljubljana, Celje and Maribor. Cross-calibration study of all three facilities was performed by having one technologist do ten phantom scans with repositioning, as recommended by the ISCD for the systems with the same technology (manufacturer and model; Shepherd *et al.* 2006). We found a negligible difference in mean BMD between facilities, so a correction factor was not considered necessary.

Biochemical markers of bone turnover

Biochemical markers of bone turnover were measured in a subset of 152 post-menopausal women. Blood samples were collected between 0800 and 1000 h after an overnight fast. The plasma and serum samples were analysed in a routine laboratory using standard procedures as outlined by the manufacturers. Osteocalcin (OC) in heparinized plasma was measured by a solid-phase, two-site chemiluminescent enzyme-labelled immunometric assay (Immulite Osteocalcin, Diagnostic Product Corporation, Los Angeles, CA, USA). Serum bone alkaline phosphatase (BALP) was measured by RIA (Tandem-R Ostase, Beckman Coulter, Fullerton, CA, USA). Serum C-terminal crosslinking telopeptides of type I collagen (CTX) were measured by enzyme immunoassay (Serum CrossLaps ELISA, Nordic Bioscience Diagnostics A/S, Herlev, Denmark). Serum-free soluble RANKL (sRANKL) was measured by an enzyme immunoassay (sRANKL ELISA, Biomedica, Vienna, Austria) and OPG by enzyme immunoassay (Human Osteoprotegerin ELISA, BioVendor Laboratory Medicine Inc., Modrice, Czech Republic).

Statistical analysis

Hardy–Weinberg equilibrium was tested for each SNP and group of participants using the χ^2 test. Frequency distributions of genotypes in the osteoporotic and non-osteoporotic post-menopausal women were compared for each studied SNP using the χ^2 test. The standardized

measures of linkage disequilibrium (LD), denoted as D' and r^2 , were assessed using the EMLD software that calculates pair-wise LD based on SNP genotype data from unrelated individuals (<https://cge.mdanderson.org/~quang/software/pub.htm>). 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 (Mueller 2004). Kolmogorov–Smirnov normality test was conducted before association analysis and data transformation was performed where appropriate. The differences in age, weight, height, body mass index (BMI), age at menopause and years since menopause between genotype subgroups were tested using ANOVA. No differences were observed. In the present study, each subgroup of subjects was analyzed separately. Therefore, no comparison of basic characteristics was made between groups of subjects, as no influence on the results of the association analysis would be expected. Differences in BMD and biochemical markers between the genotype subgroups were tested using ANOVA or non-parametric Kruskal–Wallis test. To analyze the interaction between different pairs of SNPs, the differences in BMD were tested with two-way ANOVA. Statistical analyses were carried out using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) and p values less than 0·05 were considered statistically significant.

Results

In the present study, the impact of the individual SNPs on the *VDR*, *OPG* and *TNFSF11* genes was established to identify the risk polymorphisms for assessing their combined influence on the BMD. The genotype frequencies of all studied SNPs were determined by screening DNA samples from 641 subjects. The genotype frequencies of all the subgroups are summarized in Table 1. The genotype frequency distributions of all seven polymorphic SNPs were in Hardy–Weinberg equilibrium. Comparison of genotype frequencies between osteoporotic and non-osteoporotic post-menopausal women revealed statistically significant differences only in *FokI*(*VDR*) and *Cdx2*(*VDR*) ($P=0\cdot041$ and $0\cdot037$).

In the *TNFSF11* gene promoter SNPs, evidence of strong LD was shown within each of the three possible pairs: $-290C>T$ and $-643C>T$, $D'=0\cdot96$, $r^2=0\cdot87$; $-290C>T$ and $-693G>C$, $D'=0\cdot93$, $r^2=0\cdot84$; $-643C>T$ and $-693G>C$, $D'=0\cdot92$, $r^2=0\cdot78$. In the *VDR* gene SNPs, no evidence of LD was obtained

Table 1 Genotype frequency distributions in osteoporotic and non-osteoporotic post-menopausal women, pre-menopausal women and in elderly men

	Osteoporotic post-menopausal women	Non-osteoporotic post-menopausal women	Pre-menopausal women	Men
<i>N</i>	239	228	57	117
<i>BsmI</i> (rs1544410)				
GG	103 (42.9)	88 (38.6)	21 (36.8)	32 (27.4)
AG	110 (45.8)	100 (43.9)	27 (47.4)	58 (49.6)
AA	27 (11.2)	40 (17.5)	9 (15.8)	27 (23.1)
<i>FokI</i> (rs2228570)				
CC	88 (36.7)	105 (46.1)	20 (35.1)	42 (35.9)
CT	108 (45.0)	97 (42.5)	25 (43.9)	59 (50.4)
TT	44 (18.3)	26 (11.4)	12 (21.1)	16 (13.7)
<i>Cdx2</i> (rs11568820)				
GG	155 (64.6)	172 (75.4)	43 (75.4)	80 (68.4)
GA	75 (31.2)	48 (21.1)	11 (19.3)	32 (27.4)
AA	9 (3.8)	8 (3.5)	3 (5.3)	5 (4.3)
<i>K3N</i> (rs2073618)				
GG	59 (24.6)	39 (17.1)	17 (29.8)	31 (26.5)
GC	117 (48.8)	112 (49.1)	24 (42.1)	54 (46.2)
CC	63 (26.6)	77 (33.8)	16 (28.1)	32 (27.4)
–290C>T (rs9525641)				
CC	48 (20.0)	55 (24.1)	18 (31.6)	21 (17.9)
CT	118 (49.2)	106 (46.5)	28 (49.1)	51 (43.6)
TT	74 (30.8)	67 (29.4)	11 (19.3)	45 (38.5)
–643C>T (rs9533156)				
CC	42 (17.5)	54 (23.7)	16 (28.1)	22 (18.8)
CT	126 (52.5)	106 (46.5)	30 (52.6)	51 (43.6)
TT	72 (30.0)	68 (29.8)	11 (19.3)	44 (37.6)
–693G>C (rs9533155)				
GG	42 (17.5)	53 (23.2)	15 (26.3)	20 (17.1)
GC	126 (52.5)	110 (48.2)	34 (59.6)	51 (43.6)
CC	72 (30.0)	65 (28.5)	8 (14.0)	46 (39.3)

OPG, osteoprotegerin; TNFSF11, tumour necrosis factor superfamily, member 11 and VDR, vitamin D receptor. Values are numbers (%).

between any of the three possible pairs: *BsmI* and *FokI*, $D' = 0.04$, $r^2 = 0.002$; *BsmI* and *Cdx2*, $D' = 0.16$, $r^2 = 0.008$; *FokI* and *Cdx2*, $D' = 0.18$, $r^2 = 0.004$.

Subject characteristics are presented in Table 2. For each genotype, the differences in age, weight, height and BMI between genotype subgroups were compared in the subgroups of non-osteoporotic and osteoporotic post-menopausal women, pre-menopausal women and in men. In the non-osteoporotic and osteoporotic post-menopausal women, the difference in age at menopause and years since menopause were also tested. No statistically significant differences were identified.

In the osteoporotic post-menopausal women, statistical analysis showed an association with BMD for the following SNPs: *BsmI* (VDR) and *Cdx2* (VDR), *K3N* (OPG) and –290C>T (TNFSF11) (Table 3). In the non-osteoporotic women, no association with BMD was found in any of the SNPs under study. In the pre-menopausal women, association of BMD-fn and BMD-th with the *Cdx2* (VDR) polymorphism was identified ($P = 0.011$ and 0.011). In elderly men, association of BMD-fn and BMD-ls with the *FokI* (VDR) polymorphism was identified ($P = 0.040$ and 0.036).

The concentrations of OC, BALP, CTX, RANKL and OPG were measured in 152 post-menopausal women. In the non-osteoporotic women, association was found between –693G>C (TNFSF11) and OC and BALP concentrations ($P = 0.048$ and 0.005). In the other studied SNPs, no significant association with biochemical markers of bone turnover was identified in any of the groups.

Multiple SNPs were associated with BMD only in the osteoporotic and non-osteoporotic post-menopausal women. No significant differences were found between the frequencies of combined genotypes in the osteoporotic and non-osteoporotic post-menopausal women. The results of the combined influences of different pairs of SNPs are summarized in Table 4.

Discussion

In our study, a total of 7 SNPs were studied in the VDR, OPG and TNFSF11 gene. In the osteoporotic post-menopausal women, BMD-fn was associated with *BsmI* (VDR) and *Cdx2* (VDR) and BMD-ls with *K3N* (OPG) and –290C>T (TNFSF11). In the

Table 2 Characteristics of the post-menopausal women, non-osteoporotic and osteoporotic, pre-menopausal women and men

	Osteoporotic post-menopausal women	Non-osteoporotic post-menopausal women	Pre-menopausal women	Men
Number of subjects	239	228	57	117
Age (years)	64.5±8.2	61.5±8.3	45.4±4.6	67.6±6.1
Weight (kg)	66.0±10.0	73.6±13.0	65.4±11.8	81.7±12.8
Height (m)	158.6±6.2	161.2±5.8	163.8±6.2	171.1±5.9
BMI (kg/m ²)	26.2±3.9	28.4±5.0	24.3±4.1	27.9±3.8
Age at menopause (years)	48.9±4.4	49.8±4.1	/	/
Years since menopause (years)	15.3±9.4	10.4±8.6	/	/
Femoral neck BMD (g/cm ²)	0.625±0.083	0.765±0.121	0.782±0.077	0.813±0.159
Femoral neck T-score	-2.3±0.8	-1.0±1.0	-0.7±0.7	-1.2±1.2
Total hip BMD (g/cm ²)	0.755±0.099	0.916±0.137	0.921±0.092	1.024±0.165
Total hip T-score	-1.5±0.8	-0.3±1.1	-0.3±0.7	-0.2±1.1
Lumbar spine BMD (g/cm ²)	0.742±0.088	0.974±0.139	1.001±0.115	1.057±0.176
Lumbar spine T-score	-2.8±0.9	-0.8±1.2	-0.6±1.0	-0.4±1.5
Osteocalcin (µg/l)	28.3±13.8	15.2±5.6	/	/
Bone alkaline phosphatase (µg/l)	12.4±5.6	11.8±4.0	/	/
CTX (pmol/l)	4343±2333	3940±1918	/	/
sRANKL (pmol/l)	0.3±0.6	0.4±0.5	/	/
OPG (pmol/l)	64.3±188.7	145.7±170.6	/	/

BMI, body mass index; BMD, bone mineral density; CTX, C-terminal crosslinking telopeptides of type I collagen (CTX); sRANKL, soluble receptor activator of nuclear factor κ B ligand and OPG, osteoprotegerin.

non-osteoporotic post-menopausal women, only OC and BALP concentrations were associated with -693G>C(*TNFSF11*), but no association with BMD was found in any of the studied SNPs. In the pre-menopausal women, the Cdx2(*VDR*) polymorphism was associated with BMD-fn and BMD-th. In elderly men, *FokI*(*VDR*) was associated with BMD-fn and BMD-ls. The combination of -290C>T(*TNFSF11*) and K3N(*OPG*) showed the association with BMD-th in the osteoporotic and with BMD-fn in the non-osteoporotic post-menopausal women. In the non-osteoporotic post-menopausal women, the combined influence of K3N(*OPG*) and Cdx2(*VDR*) on BMD was shown at all sites.

The analyzed genes were selected on the basis of the recently published data showing that $1\alpha,25(\text{OH})_2\text{D}_3$ suppresses OPG expression and enhances RANKL expression, indicating crosstalk of these two bone regulatory systems (Kondo *et al.* 2004). Therefore, seven SNPs were selected from the *VDR*, *OPG* and *TNFSF11* genes. The SNPs from the *VDR* (*BsmI*, *FokI* and Cdx2) and *OPG* (K3N) genes were selected, since they have often been associated with osteoporotic phenotype. For *BsmI*(*VDR*) and K3N(*OPG*), the association with BMD has also been shown in a smaller group of post-menopausal women from our population (Marc *et al.* 2000, Arko *et al.* 2005). In the *TNFSF11* gene, SNPs -290C>T, -643C>T and -693G>C were selected on the basis of their previously proposed functional role in the regulation of the *TNFSF11* expression (Mencej *et al.* 2008).

In the comparison of the osteoporotic and non-osteoporotic post-menopausal women, a significant difference in the genotype frequencies was observed for *FokI*(*VDR*) and Cdx2(*VDR*). However, these differences were not considered to be relevant since, in *FokI*(*VDR*), no influence on BMD was found and, in Cdx2(*VDR*), the genotypes GG and GA had different frequency distribution but comparable BMD values.

In post-menopausal osteoporosis, *BsmI*(*VDR*) and Cdx2(*VDR*), but not *FokI*(*VDR*), were found to be associated with BMD-fn. The *VDR* gene is one of the most widely studied osteoporosis-related genes and numerous genetic association studies have yielded conflicting results (Thakkestian *et al.* 2004, Fang *et al.* 2005, Zintzaras *et al.* 2006). For *BsmI*(*VDR*), the meta-analysis has shown contradictory results in BMD-fn (Cooper & Umbach 1996, Gong *et al.* 1999, Thakkestian *et al.* 2004). In the GENOMOS study, no association of *BsmI*-*Apal*-*TaqI*(*VDR*) haplotype with any osteoporotic phenotype was found, whereas Cdx2(*VDR*) was associated with risk of vertebral fracture (Uitterlinden *et al.* 2006). In *FokI*(*VDR*), no association with BMD has been found (Uitterlinden *et al.* 2006, Zintzaras *et al.* 2006).

In SNPs of the RANKL/RANK/OPG pathway genes, association between BMD-ls and K3N(*OPG*) and -290C>T(*TNFSF11*) was established for post-menopausal osteoporosis in the present study. In K3N(*OPG*), women with GG genotype had significantly lower BMD-ls than those with GC or CC genotype, which is in accordance with several earlier studies (Langdahl *et al.* 2002, Arko *et al.* 2005, Choi *et al.* 2005, Zhao *et al.* 2005,

Table 3 Bone mineral density in osteoporotic post-menopausal women in different genotype subgroups

<i>BsmI</i> in <i>VDR</i>	GG	GA	AA	<i>P</i> values*
BMD-ls (g/cm ²)	0.742 ± 0.097	0.747 ± 0.079	0.725 ± 0.087	0.514
BMD-th (g/cm ²)	0.751 ± 0.102	0.768 ± 0.092	0.720 ± 0.104	0.065
BMD-fn (g/cm ²)	0.616 ± 0.079	0.640 ± 0.086	0.595 ± 0.079	0.015
<i>FokI</i> in <i>VDR</i>	CC	CT	TT	<i>P</i> values*
BMD-ls (g/cm ²)	0.738 ± 0.089	0.746 ± 0.081	0.744 ± 0.102	0.814
BMD-th (g/cm ²)	0.763 ± 0.098	0.749 ± 0.103	0.754 ± 0.086	0.635
BMD-fn (g/cm ²)	0.626 ± 0.084	0.626 ± 0.085	0.620 ± 0.080	0.903
<i>Cdx2</i> in <i>VDR</i>	GG	GA	AA	<i>P</i> values*
BMD-ls (g/cm ²)	0.750 ± 0.093	0.727 ± 0.079	0.731 ± 0.070	0.161
BMD-th (g/cm ²)	0.751 ± 0.095	0.753 ± 0.100	0.811 ± 0.097	0.194
BMD-fn (g/cm ²)	0.620 ± 0.077	0.623 ± 0.085	0.690 ± 0.112	0.047
<i>K3N</i> in <i>OPG</i>	GG	GC	CC	<i>P</i> values*
BMD-ls (g/cm ²)	0.715 ± 0.094	0.753 ± 0.087	0.747 ± 0.080	0.021
BMD-th (g/cm ²)	0.738 ± 0.095	0.761 ± 0.098	0.756 ± 0.096	0.343
BMD-fn (g/cm ²)	0.615 ± 0.097	0.633 ± 0.070	0.616 ± 0.087	0.242
−290C>T in <i>TNFSF11</i>	CC	CT	TT	<i>P</i> values*
BMD-ls (g/cm ²)	0.712 ± 0.097	0.745 ± 0.081	0.758 ± 0.089	0.017
BMD-th (g/cm ²)	0.749 ± 0.111	0.755 ± 0.096	0.760 ± 0.092	0.853
BMD-fn (g/cm ²)	0.605 ± 0.098	0.629 ± 0.080	0.631 ± 0.078	0.199
−643C>T in <i>TNFSF11</i>	CC	CT	TT	<i>P</i> values*
BMD-ls (g/cm ²)	0.718 ± 0.095	0.741 ± 0.083	0.759 ± 0.089	0.053
BMD-th (g/cm ²)	0.753 ± 0.103	0.753 ± 0.102	0.760 ± 0.091	0.879
BMD-fn (g/cm ²)	0.609 ± 0.096	0.627 ± 0.082	0.631 ± 0.077	0.368
−693G>C in <i>TNFSF11</i>	GG	GC	CC	<i>P</i> values*
BMD-ls (g/cm ²)	0.718 ± 0.095	0.743 ± 0.083	0.756 ± 0.091	0.093
BMD-th (g/cm ²)	0.747 ± 0.099	0.755 ± 0.101	0.760 ± 0.094	0.778
BMD-fn (g/cm ²)	0.607 ± 0.095	0.627 ± 0.082	0.631 ± 0.078	0.280

OPG, osteoprotegerin; *TNFSF11*, tumour necrosis factor superfamily, member 11; *VDR*, vitamin D receptor; BMD-ls, lumbar spine bone mineral density; BMD-th, total hip bone mineral density and BMD-fn, femoral neck bone mineral density. Values are means ± s.d.s for bone mineral densities. **P* values were obtained in comparison of mean values with ANOVA testing. *P* values <0.05 were considered statistically significant and are written in bold.

Kim *et al.* 2007, García-Unzueta *et al.* 2008). Our previous study on the *TNFSF11* gene promoter SNPs, involving 404 post-menopausal women, showed association with BMD-ls for −290C>T, −643C>T and −693G>C (Mencej *et al.* 2008). In the present study, the association was only shown for −290C>T(*TNFSF11*), whereas in −643C>T(*TNFSF11*) and −693G>C(*TNFSF11*) the association was of borderline significance (*P*=0.053 and 0.093 respectively).

In the pre-menopausal women, only *Cdx2*(*VDR*) was shown to be associated with BMD-fn and BMD-th. On the contrary, the GENOMOS study, involving pre- and post-menopausal women, revealed no association between the *VDR* gene SNPs and BMD in women (Uitterlinden *et al.* 2006). So far, the *TNFSF11* gene promoter SNPs −290C>T, −643C>T and −693G>C have not been studied in pre-menopausal women, but the present results did not reveal their possible

role in pre-menopausal BMD regulation. It seems that the *VDR* gene could play a role in the BMD regulation before menopause, but the data need to be further confirmed.

In elderly men, *FokI*(*VDR*) was associated with BMD-fn and BMD-ls. However, the GENOMOS study found no association of *FokI*(*VDR*) and *BsmI*(*VDR*) with osteoporotic phenotype in men, although *Cdx2*(*VDR*) was associated with the risk of vertebral fracture (Uitterlinden *et al.* 2006). Our results have also shown that *K3N*(*OPG*) has not been associated with osteoporotic phenotype in men. The *TNFSF11* gene promoter SNPs −290C>T, −643C>T and −693G>C have not been studied in elderly men and no association with BMD was shown in the present study. The data indicate the possible importance of the *VDR* gene, but not of the *OPG* and *TNFSF11* genes, in bone mass regulation in elderly men.

Table 4 The analysis of interactions between polymorphisms in vitamin D receptor (*VDR*), osteoprotegerin (*OPG*) and tumour necrosis factor superfamily member 11 (*TNFSF11*) genes in osteoporotic and non-osteoporotic post-menopausal women

	Osteoporotic post-menopausal women	Non-osteoporotic post-menopausal women
K3N – Cdx2		
BMD-ls	0.723	0.032
BMD-th	0.704	0.049
BMD-fn	0.329	0.022
K3N – BsmI		
BMD-ls	0.143	0.335
BMD-th	0.525	0.291
BMD-fn	0.061	0.278
–290C>T – Cdx2		
BMD-ls	0.632	0.517
BMD-th	0.584	0.331
BMD-fn	0.926	0.263
–290C>T – BsmI		
BMD-ls	0.457	0.645
BMD-th	0.439	0.447
BMD-fn	0.440	0.654
–290C>T – K3N		
BMD-ls	0.177	0.477
BMD-th	0.041	0.097
BMD-fn	0.057	0.029

BMD-ls, lumbar spine bone mineral density; BMD-th, total hip bone mineral density and BMD-fn, femoral neck bone mineral density. Numbers are *P* values. *P* values were obtained with two-way ANOVA testing. *P* values <0.05 were considered statistically significant and are written in bold.

The main aim of our study was to investigate any interactions between different SNPs in the *VDR*, *OPG* and *TNFSF11* genes. So far, the interaction had been studied only between *OPG* and *TNFSF11* gene polymorphisms. The K3N(*OPG*) polymorphism, alone and in combination with the rs2277438 polymorphism in the *TNFSF11* gene, was identified to influence BMD-ls (Kim *et al.* 2007). Studying individual polymorphisms in the *VDR*, *OPG* and *TNFSF11* genes, an association with BMD was found with *BsmI*(*VDR*) and *Cdx2*(*VDR*), K3N(*OPG*) and –290C>T(*TNFSF11*), but only in osteoporotic post-menopausal women. These significantly associated SNP were selected for further interaction analysis and the following combinations were prepared: K3N(*OPG*)-*Cdx2*(*VDR*), K3N(*OPG*)-*BsmI*(*VDR*), –290C>T(*TNFSF11*)-*Cdx2*(*VDR*), –290C>T(*TNFSF11*)-*BsmI*(*VDR*) and –290C>T(*TNFSF11*)-K3N(*OPG*). Since the pre-menopausal women and elderly men subgroups were rather small, the combined influence of different pairs of SNPs was only studied in the osteoporotic and non-osteoporotic post-menopausal women. Although no association of individual SNPs was observed in the non-osteoporotic women, the interactions were also studied in this subgroup. The combined influence can sometimes be observed even with the lack of individual effects. Comparison of

the frequencies of combined genotypes between the osteoporotic and non-osteoporotic post-menopausal women revealed no significant differences. In the osteoporotic post-menopausal women, the combination of –290C>T(*TNFSF11*) and K3N(*OPG*) was associated significantly with BMD-th (*P*=0.041). Interestingly, the individual effects of –290C>T(*TNFSF11*) and K3N(*OPG*) on BMD-th were not significant, suggesting the importance of the interaction between the studied polymorphisms. Association of borderline significance with BMD-fn was observed for the K3N(*OPG*)-*BsmI*(*VDR*) and –290C>T(*TNFSF11*)-K3N(*OPG*) genetic combinations (*P*=0.061 and 0.057). In the former, it seems that the *BsmI*(*VDR*) polymorphism is running the association, since the individual effect of the *BsmI*(*VDR*) on BMD-fn was also observed. In the latter, the CT and TT genotypes of the –290C>T(*TNFSF11*) polymorphism seem to additionally increase BMD-fn in the K3N(*OPG*) genotypes.

In the non-osteoporotic post-menopausal women, the K3N(*OPG*)-*Cdx2*(*VDR*) combination was associated with BMD at all skeletal sites. The individual effects of K3N(*OPG*) and *Cdx2*(*VDR*) were not significant at any skeletal site, suggesting the importance of the studied interaction. In non-osteoporotic women, the C allele of the K3N(*OPG*) was observed to markedly reduce BMDs in combination with the A allele of the *Cdx2*(*VDR*). In the –290C>T(*TNFSF11*)-K3N(*OPG*) combination, the association was observed with BMD-fn and the association of borderline significance with BMD-th. As the individual effect of the –290C>T(*TNFSF11*) was also observed, it seems that the –290C>T(*TNFSF11*) is running the association.

Interestingly, different genes were shown to be associated with BMD in the studied subgroups. These findings suggest that various polymorphisms might influence BMD in different periods of life. The genetic influences differ between osteoporotic and non-osteoporotic post-menopausal women, suggesting that some polymorphisms might not cause osteoporosis, but when present can modify the degree of osteoporosis. These differences could have a great clinical impact, since even small changes in BMD may result in large changes of fracture risk. Diverse genetic influences were also observed in the pre- and post-menopausal women. The differences could be a result of numerous genetic interactions that were partly also addressed in our study. The association may also be modified by the influence of other osteoporosis-related polymorphisms and several environmental factors, such as dietary calcium and vitamin D intake, physical activity, etc.

Regarding the limitations of the study, firstly it must be remembered that BMD is only one of several parameters related to bone quality and risk of fracture. No data on fragility fractures and the quality of bone were available for the analysis. Secondly, the sample size

is not optimal for the identification of genotype–phenotype association. A relatively small sample size allowed only a limited power to detect individual effects and interactions. By expanding the sample size, we could further investigate genetic influence of the studied genes on the development of osteoporosis. Thirdly, the osteoporotic phenotype is usually affected by environmental factors. In our study, no data on 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 variations in the *VDR*, *OPG* and *TNFSF11* genes, since only the most common variants were studied. Additionally, biochemical markers of bone turnover were only measured in 152 post-menopausal women, thus not reflecting the intensity of bone turnover in pre-menopausal women and in men.

In conclusion, the results of our study support the involvement of gene–gene interactions between the *VDR*, *OPG* and *TNFSF11* genes in the BMD regulation, particularly in osteoporotic and non-osteoporotic postmenopausal women. Under the combined influence of *VDR*, *OPG* and *TNFSF11* genes, the association with BMD was markedly increased compared with the individual genetic effects of the genes under study. In osteoporotic women, a combination of $-290C>T$ (*TNFSF11*) and $K3N$ (*OPG*) was particularly important in the association with osteoporotic phenotype. In non-osteoporotic women, the interaction of $K3N$ (*OPG*) and $Cdx2$ (*VDR*) influences bone phenotype at all skeletal sites. To further investigate the genetic interactions between the *VDR*, *OPG* and *TNFSF11* genes, the number of the studied SNPs should be increased and confirmed in the larger cohorts.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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