

Associations of polymorphisms in the vitamin D receptor gene (BsmI and FokI) with bone mineral density in postmenopausal women in Malta

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Abstract Previous studies have suggested that variations in the vitamin D receptor (VDR) gene are related to bone mineral density (BMD). In this study, the T → C transition in the start codon and the G → A polymorphism at the 3' end of the VDR gene, identified by endonucleases FokI and BsmI, respectively, were analysed and correlated with BMD in postmenopausal Maltese women ($n = 104$). Genotype frequencies observed for the VDR start codon polymorphism (SCP) were CC: 60.4%; CT: 30.7% and TT: 8.9%, while those observed for the 3' in this study were GG: 16.4%; GA: 51.9%; AA: 31.7%. In postmenopausal women, both lumbar and femoral BMD were observed to be highest in CC homozygotes for the FokI genotype and in GG homozygotes for the BsmI genotype, although in both groups the difference between the genotypes was not statistically significant, even after adjusting BMD for age, BMI and years since menopause. No evidence of linkage disequilibrium between the two alleles was observed.

Keywords Bone mineral density · Maltese · Osteoporosis · Postmenopausal women · VDR gene polymorphisms

Introduction

Osteoporosis is a complex metabolic bone disease characterised by a low bone mass and deterioration of

the microarchitecture of bone, leading to increased bone fragility and fractures [1]. As many factors are involved in the control of bone formation and resorption, the heritability of osteoporosis is likely to be multifactorial, involving the interactions of both environmental and genetic influences. Various studies have shown that bone mineral density (BMD), the main determinant of osteoporosis, is under strong genetic control [2]. Among the candidate genes associated with BMD can be included those coding for collagen, COLIA1 [3], oestrogen receptor [4], tumour necrosis factor receptor 2 [5], osteoprotegerin [6], interleukin-6 [7], insulin growth factor-1 [8], transforming growth factor beta-1 [9], and the vitamin D receptor [2]. Also, Duncan and co-workers [10] showed suggestive linkage of the parathyroid hormone receptor type 1 gene to osteoporosis in a study carried out in families of probands with the disease, while Devoto et al. [11] showed evidence of linkage of various chromosomal regions to osteoporosis. Other chromosomal regions were associated with BMD by various types of analysis as sib pair linkage studies [12], transmission disequilibrium tests [13] and other genome scans [14].

So far, a number of polymorphisms of the VDR gene have been identified and correlated with BMD and with the development of other human diseases [15]. Three polymorphisms located at the 3' end of the VDR gene, which are identified using endonucleases, BsmI, ApaI and TaqI, have been associated with BMD and the increased risk for osteoporosis [15,16,17]. In 1994, Morrison and colleagues [2] correlated the A (previously denoted as b) allele of the VDR gene with higher bone density when compared to the G (previously denoted as B) allele and concluded that allelic variations of the VDR gene accounts for 75% of the genetic effects on bone density in a population of Australian women. These results were later partly withdrawn, when less correlation was found [18]. However, other studies have strongly supported this correlation in some populations [19,20,21], although this is not universally accepted [22,23].

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In 1996, Gross and colleagues [24] studied a novel polymorphism at the translation initiation site in exon 2 of the VDR gene, which can be detected by restriction fragment length polymorphism (RFLP) using endonuclease FokI. This polymorphism, resulting from a T → C transition, introduces codon 4 as the start codon and results in a shorter isoform of the VDR. In this study, it was observed that postmenopausal Mexican-American women having the TT (previously denoted as ff) genotype had 12.8% lower spinal BMD when compared to the CC (previously denoted as FF) genotype. These findings were also supported by other studies carried out in different populations of different age groups [25,26], but contrasting results were obtained by other workers [21,27].

In our study, we have examined the association of the VDR start codon polymorphism (SCP) identified by FokI and the BsmI polymorphism found in the intron between exons 8 and 9 with BMD, in a group of normal, osteopenic and osteoporotic postmenopausal Maltese women.

This study is of particular interest because of the ethnic origin of the Maltese population (circa 380,000), which is typical of a mixed Mediterranean population with admixture of Northern Europeans due to Malta being a British colony for 180 years (1799–1979). Prior to that occupancy, under different rulers, Malta had witnessed an influx of Greeks and others from the Eastern Mediterranean, and from Northern Africa [28].

Materials and methods

Subjects

A total of 104 Maltese postmenopausal women were recruited from new subjects referred by medical practitioners to the Bone Density Unit at the Department of Obstetrics and Gynaecology, St Luke's Hospital, Malta for an osteoporosis risk evaluation. Informed consent was obtained from all participants in the study that had been approved by the Research Ethics Committee of the University of Malta. All participants answered a questionnaire concerning medical conditions and the use of medications, family history of osteoporosis and dietary habits. Individuals who had conditions, or were on medications, known to affect bone metabolism were excluded from the study. Respondents who had undergone a surgical procedure such as total abdominal hysterectomy, bilateral surgical oophorectomy, together with those on steroid therapy, thyroxine, and oral contraceptives, hormone replacement therapy and on any medication for psychiatric disorders, were not included in this study. A small group of patients who had a past medical history of diabetes, carcinoma or renal failure was also excluded. Menopause was defined as amenorrhoea of at least 6 months duration.

Blood and urine samples were collected from the participants for DNA analysis and for the determination of biochemical markers of bone turnover.

The age range of the women enrolled in this study was 40–75 years, with a mean (\pm SD) age of 54.9 ± 6.8 years. According to WHO criteria [29] for both lumbar and femoral BMD, 20 (19.2%) of the participants were osteoporotic at the lumbar spine (t -score less than -2.5), 21 (20.2%) were osteopenic (t -score less than -1.5 to -2.5) and 63 (60.6%) were normal. At the femoral neck, 4 (3.8%) individuals were osteoporotic and 14 (13.5%) were osteopenic.

VDR genotyping

Genomic DNA was extracted and purified from peripheral blood leucocytes by salting out [30]. The regions of the BsmI and FokI restriction sites were analysed as previously described [2,24]. Genomic DNA was amplified by polymerase chain reaction (PCR) using 1 μ M of each primer (BsmI: 5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3', 5'-AAC CAG CGG GAA GAG GTC AAG GG-3' and FokI: 5'-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3', 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3') in a final 50 μ l reaction mixture containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100 and 200 μ M of each dNTP (Promega, Madison, Wisc., USA). An initial "hot start" cycle at a temperature of 95°C for 5 min was performed followed by the addition of 2.5 U Taq DNA polymerase (Promega). Polymerase chain reaction conditions were 60 s at 94°C, 30 s at 60°C and 90 s at 72°C for BsmI, and 30 s at 94°C, 30 s at 60°C and 30 s at 72°C for FokI, for 35 cycles followed by a final extension at 72°C for 3 min. The PCR products were then digested with endonucleases at 65°C for BsmI and at 37°C for FokI (New England Biolabs, Beverly, Mass., USA) for 16 h followed by agarose gel electrophoresis (BsmI on 1.5%, FokI on 3%). After staining with ethidium bromide, the digested products were scanned and visualised with UV irradiation (Fluor-S multi imager; Bio-RAD Laboratories Inc., Calif., USA). Absence of the restriction sites for BsmI and FokI, previously denoted by capital letters B or F were denoted as G and C, respectively, while presence of the cleavage sites previously denoted by lower case letters b or f were denoted as A and T, respectively, resulting in genotypes GG/GA/AA for BsmI and CC/CT/TT for FokI polymorphism. This nomenclature refers to the actual nucleotide changes in the respective polymorphisms. Re-analysis of 20% of samples was performed for both BsmI and FokI to check for accuracy of genotyping. No discrepancies were found with the original genotyping.

Biochemical markers of bone turnover

Blood and urine samples collected were also analysed for biochemical markers of bone turnover. Urinary deoxypyridinoline (Dpd), an indicator of bone resorption, was measured using enzyme-immunosorbent assay kits (Metra Biosystems Inc., Calif., USA). The minimum detection limit of this method was 1.1 nmol/l. Urine creatinine estimation was also carried out to correct variations in urine concentration by dividing the Dpd value (nmol/l) by the urine creatinine value (mmol/l), expressing the final results as nmol Dpd/mmol creatinine. The method used for urine creatinine estimation was based on the Jaffé reaction and was carried out using the Roche/Hitachi 902 automated analyser (Boehringer Mannheim, Germany). Serum procollagen type I carboxy-terminal extension peptide (CICP) was measured using a sandwich enzyme immunoassay (Metra Biosystems). Results obtained were analysed using quantitation software with a four-parameter calibration curve (MetraFIT Version 1.0; Metra Biosystems).

Bone densitometry

Bone mineral density was measured at the lumbar spine (L2–L4) and femoral neck using a Norland 486 dual-energy X-ray absorptiometer (Norland, Medical Systems Inc., New York, USA).

Statistical analysis

Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) for Windows student version 9.0 (SPSS, Chicago, Ill., USA). The normality of the population was tested using the non-parametric test Kolmogorov-Smirnov while the Levene's statistic was used to test for homogeneity of vari-

ances. Since the population showed a normal distribution, comparisons between genotypes were performed using one-way analysis of variance (ANOVA). Adjustments for age, BMI and years since menopause (YSM) were performed using a generalised linear model (GLM) univariate analysis of variance.

The unpaired independent sample *t*-test was used to analyse data in terms of genetic models: dominant (A) or recessive (B) allele, while a linear regression analysis was used to test for an allele-dose effect (C) of a risk or protective allele.

The chi-squared test was used to compare observed genotype frequencies with those expected under Hardy-Weinberg equilibrium and to test for linkage between BsmI and FokI polymorphisms by examining the actual and expected distribution of genotypes. All statistical tests were considered two-tailed at a level of significance of 0.05 where the null hypothesis (no difference between means) was rejected at $P < 0.05$.

Results

BsmI VDR genotypes

The distribution of the VDR genotypes detected by endonuclease BsmI in postmenopausal women is shown in Table 1. The frequencies of the genotypes obtained within this population ($n=104$) were 16.4% GG homozygotes, 51.9% GA heterozygotes and 31.7% AA homozygotes. Allele frequencies observed in the Maltese population were 42.3% for the G allele and 57.7% for the A, and were in Hardy-Weinberg equilibrium ($\chi^2=0.42$; $P=0.52$).

Mean values for lumbar BMD in postmenopausal women were observed to be highest in GG and AA homozygotes (3.0% higher when compared to GA heterozygotes) while femoral BMD was observed to be highest only in GG homozygotes (1.2% higher when compared to GA and AA genotypes). However, differences in lumbar and femoral BMD were not observed to be statistically significant between genotypes, even after adjusting BMD for age, BMI and YSM. Although not statistically significant, procollagen was observed to be

Table 1 Mean (SD) characteristics of Maltese postmenopausal women by vitamin D receptor genotype (BsmI)

	GG	GA	AA	<i>P</i>
<i>n</i>	17	54	33	–
%	16.4	51.9	31.7	–
Age (years) ^a	55 (7.8)	55.4 (7.3)	54.0 (5.6)	0.66
BMI (kg/m ²) ^a	27.5 (3.3)	28.4 (4.6)	29.2 (4.3)	0.42
BMD L2–L4 (g/cm ²) ^a	0.99 (0.15)	0.96 (0.20)	0.99 (0.19)	0.77
BMD L2–L4 (adjusted) ^b	1.00	0.97	0.97	0.73
BMD femoral (g/cm ²) ^a	0.86 (0.14)	0.85 (0.14)	0.85 (0.11)	0.97
BMD femoral (adjusted) ^b	0.87	0.86	0.84	0.52
Procollagen I (ng/ml) ^{a,c}	93.2 (27.6)	92.9 (43.8)	83.2 (36.2)	0.59
Deoxypyridinoline crosslinks nmol/mmol creatinine) ^{a,c}	8.6 (2.9)	9.3 (3.4)	9.0 (3.4)	0.81

^aValues are means \pm SD in parentheses; *P*-values by ANOVA

^bValues are adjusted for age, BMI and YSM; 95% CI given by univariate analysis after adjustment (not shown)

^cProcollagen I: GG $n=14$; GA $n=41$; AA $n=25$. Pyridinoline: GG $n=11$; GA $n=36$; AA $n=22$

highest in the GG genotype, which also showed the lowest value for deoxypyridinoline crosslinks.

Neither the G nor the A alleles were significantly associated with BMD at both anatomical sites, using genetic models for dominant and recessive alleles (models A and B) (Table 3). Also, no allele dose effect (model C) was observed for lumbar ($P=0.90$) and femoral BMD ($P=0.86$) in postmenopausal women.

VDR start codon polymorphism

Table 2 shows the distribution of the VDR genotypes in postmenopausal women ($n=101$) as identified by FokI. The genotype frequencies observed were 60.4% CC homozygotes, 30.7% CT heterozygotes and 8.9% TT homozygotes. Allele frequencies were also in Hardy-Weinberg equilibrium ($\chi^2=2.74$; $P=0.10$), with 75.7% of the population having the C allele and 24.3% the T allele. Lumbar and femoral BMD was highest in CC homozygotes, being 7.1% and 6.9%, respectively, higher when compared to TT homozygotes and 4.0% and 3.4%, respectively, higher when compared to CT heterozygotes. These differences were not statistically significant when tested by ANOVA, even after adjusting for age, BMI and YSM. No association between lumbar or femoral BMD and genotype was observed for genetic models A and B (Table 3), while there was an allele dose effect for lumbar spine ($P=0.21$) and femoral neck ($P=0.18$) but this did not reach significance.

Analysis of linkage disequilibrium (LD) between BsmI and FokI RFLPs

No evidence of linkage disequilibrium between the two alleles was observed ($n=101$; $\chi^2=6.63$; $P=0.156$; $df=4$) (Table 4), showing that the BsmI and FokI genotypes are independent of each other.

Table 2 Mean (SD) characteristics of Maltese postmenopausal women by vitamin D receptor genotype (FokI)

	CC	CT	TT	<i>P</i>
<i>n</i>	61	31	9	–
%	60.4	30.7	8.9	–
Age (years) ^a	54.7 (7.3)	55.0 (6.8)	54.2 (2.8)	0.95
BMI (kg/m ²) ^a	28.3 (4.4)	29.5 (4.4)	26.7 (3.5)	0.19
BMD L2–L4 (g/cm ²) ^a	0.99 (0.19)	0.95 (0.20)	0.92 (0.17)	0.38
BMD L2–L4 (adjusted) ^b	0.99	0.95	0.95	0.46
BMD femoral (g/cm ²) ^a	0.87 (0.14)	0.84 (0.12)	0.81 (0.13)	0.41
BMD femoral (adjusted) ^b	0.87	0.84	0.83	0.38
Procollagen I (ng/ml) ^{a,c}	88.6 (42.5)	91.4 (38.7)	87.5 (11.2)	0.95
Deoxypyridinoline crosslinks (nmol/mmol creatinine) ^{a,c}	9.2 (3.4)	9.1 (3.3)	8.0 (3.1)	0.67

^aValues are means \pm SD in parentheses; *P*-values given by ANOVA

^bValues are adjusted for age, BMI and YSM; 95% CI given by univariate analysis (not shown)

^cProcollagen I: CC $n=47$; CT $n=24$; TT $n=6$. Pyridinoline: CC $n=40$; CT $n=20$; TT $n=7$

Table 3 Effects of VDR alleles (BsmI and FokI) on lumbar and femoral BMD in postmenopausal Maltese women

BsmI	<i>n</i>	LS BMD (g/cm ²)	Fem BMD (g/cm ²)	FokI	<i>n</i>	LS BMD (g/cm ²)	Fem BMD (g/cm ²)
A				A			
GG	17	0.99±0.15	0.86±0.14	CC	61	0.99±0.19	0.87±0.14
GA and AA	87	0.97±0.19	0.85±0.13	CT and TT	40	0.95±0.18	0.83±0.12
<i>P</i>	–	0.71	0.80	<i>P</i>	–	0.24	0.24
B				B			
GG and GA	71	0.97±0.19	0.85±0.14	CC and CT	92	0.98±0.19	0.86±0.13
AA	33	0.99±0.19	0.85±0.11	TT	9	0.92±0.17	0.81±0.13
<i>P</i>	–	0.63	0.96	<i>P</i>	–	0.36	0.29

Mean lumbar (LS) and femoral BMD compared between genotype groups assuming hypothesis (A) dominant G or C alleles; (B) recessive G or C alleles. BMD values are given as means±SD.

Probability (*P*) given from an unpaired independent sample *t*-test (two-tailed)

Table 4 Analysis of LD between BsmI and FokI genotypes

BsmI genotype	FokI genotype		
	CC	CT	TT
GG	8 (10.3)	6 (5.2)	3 (1.5)
GA	37 (31.4)	13 (16.0)	2 (4.6)
AA	16 (19.3)	12 (9.8)	4 (2.9)

Values are observed frequencies and expected frequencies (obtained by chi-squared test) shown in parentheses

Discussion

The active form of vitamin D plays a very important role as an endocrine hormone in intestinal calcium absorption, and therefore it is an important determinant of BMD. The effects of vitamin D are mediated by its attachment to the VDR found in all target tissues, making the gene a good candidate in the determination of BMD and therefore in osteoporosis. Polymorphisms at both the 3' end of the VDR gene and at the start codon were extensively studied in various populations, to try and establish an association with BMD [2,24].

This study was the first to be carried out in the Maltese population to determine whether there is any correlation between VDR genotypes and BMD. Allele and VDR genotype frequencies identified by endonuclease BsmI in postmenopausal women were very similar to those observed in other Caucasians such as French [31] and Slovenian [32] female populations, but differed from those observed in Japanese [20] and Taiwanese women [33]. Although not statistically significant, mean lumbar and femoral BMD was observed to be highest in GG homozygotes when compared to the other genotypes. These results agree with those observed in Greek postmenopausal women, where individuals having the GG genotype had a non-significant highest lumbar BMD [27]. Houston et al. [34] observed a significant association between GG genotypes and high BMD in pre- and postmenopausal women from Scotland. Also, results obtained by Uitterlinden and colleagues [35] showed that a VDR haplotype allele, representative of the A allele group, was weakly associated with low BMD

in a sample of approximately 1782 Dutch men and women. These results contrast with those observed in most studies where the G allele was associated with low BMD [2,19,20,21,33].

The VDR SCP allele and genotype frequencies observed in the Maltese population differed from those observed in Danish [21], Italian [25], Swiss [36], Mexican-Americans [24] and oriental populations [26,37], but agreed with those observed in Greeks [27], African-Americans [38] and black American women [39]. Unadjusted lumbar and femoral BMD was highest in CC homozygotes and lowest in TT homozygotes, with CT heterozygotes having intermediate BMD, indicating an allele dose effect on both lumbar and femoral BMD. Although these observations were not statistically significant, they were consistent with those observed by Ferrari et al. [36] where it was determined that a non-significant association exists between FokI polymorphisms and BMD. Also, Gross and co-workers [24] showed that BMD was significantly associated with the VDR SCP. BsmI and FokI genotypes do not seem to be in linkage disequilibrium with each other, agreeing with what was observed in a similar analysis in other populations [36,38].

The reasons for the inconsistency in results obtained with regard to BMD and VDR genotypes from different studies may be various, including differences in selection criteria of the population, sample size and power, as well as gene-environment and gene-gene interactions. Our study population was made up of individuals referred to the Bone Density Unit for an osteoporosis risk evaluation. This may increase the chance of introducing a selection bias into this study, although the incidence of osteoporosis in this sample population (19.2%) was less than that of the worldwide frequency of osteoporosis for postmenopausal women (30%) [29]. The differences in lumbar and femoral BMD (1.2–3.0%) were too small to detect with statistical significance between the three BsmI genotypes in a sample of this size. Fleet and co-workers [19] detected with statistical significance, differences of 8.1–13.4% at both anatomical sites between BsmI genotypes in a sample of 155 subjects. Also, in our study lumbar BMD was 7.1% higher in CC homozygotes when compared with TT. This difference was smaller than that observed by Gross et al. [24] (lumbar: 12.8%) where a

statistical significant difference between genotypes was observed at the lumbar spine when carrying out a similar study of 100 participants, whereas a similar percentage difference in lumbar BMD was only weakly associated ($P=0.06$) to FokI VDR polymorphism in postmenopausal Italian women when using a sample of 400 individuals [25]. After calculating statistical power for this study, it was found that it would take a sample size larger than 2000 to show significant differences in lumbar and femoral BMD for the BsmI genotype and a sample larger than 500 for the FokI genotype.

Gene-gene interactions may also be responsible for the discordant results obtained so far, where in a polygenic disease as osteoporosis, the accumulated effects of a number of genes will result in the observed phenotype. A marked degree of allelic heterogeneity exists for the BsmI VDR polymorphism, since different alleles in different populations are responsible for the same phenotype (low BMD) [2,20,34,35]. Genes involved in the various processes of bone metabolism may interrelate to each other to determine the individual's susceptibility to osteoporosis. Interactions between the VDR gene and the oestrogen receptor (ER) gene were also reported [40] and may also be responsible for the inconsistent results obtained. Uitterlinden and co-workers [41] reported another interlocus interaction between the VDR gene and the Col1A1 gene, where they concluded that interactions between these two loci are responsible for an increased fracture risk. These observations show that the influence of the VDR genotypes on BMD may depend upon the presence or the absence of other allelic variations at a distinct locus.

Future studies must be addressed to evaluate better the influences of the various environmental factors on the genetic component of BMD. This study does not exclude the possible influences of the VDR gene on BMD and the increased risk for osteoporosis, influences that might be too small to detect with statistical significance in a study of this size and that may be masked by the effects of various environmental factors and by the influences of other genes.

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References

- Riggs BL, Melton LJ (1986) Involutional osteoporosis. *N Engl J Med* 314:1676–1686
- Morrison NA, Cheng QJ, Tokita A et al. (1994) Prediction of bone density from vitamin D receptor alleles. *Nature* 367:284–287
- Grant SF, Reid DM, Blake G, Herd R, Fogelman I, Ralston SH (1996) Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I alpha 1 gene. *Nat Genet* 14:203–205
- Kobayashi S, Inoue S, Hosoi T, Ouchi Y, Shiraki M, Orimo H (1996) Association of bone mineral density with polymorphism of the estrogen receptor gene. *J Bone Miner Res* 11:306–311
- Spotila LD, Rodriguez H, Koch M et al. (2000) Association of a polymorphism in the TNFR2 gene with low bone mineral density. *J Bone Miner Res* 15:1376–1383
- Langdahl BL, Carstens M, Stenkjaer L, Eriksen EF (2002) Polymorphisms in the osteoprotegerin gene are associated with osteoporotic fractures. *J Bone Miner Res* 17:1245–1255
- Ota N, Nakajima T, Nakazawa I et al. (2001) A nucleotide variant in the promoter region of the interleukin-6 gene associated with decreased bone mineral density. *J Hum Genet* 46:267–272
- Kim JG, Roh KR, Lee JY (2002) The relationship among serum insulin-like growth factor-1, insulin-like growth factor-1 gene polymorphism and bone mineral density in postmenopausal women in Korea. *Am J Obstet Gynecol* 186:345–350
- Langdahl BL, Carstens M, Stenkjaer L, Eriksen EF (2003) Polymorphisms in the transforming growth factor beta-1 gene and osteoporosis. *Bone* 32:297–310
- Duncan EL, Brown MA, Sinsheimer J et al. (1999) Suggestive linkage of the parathyroid receptor type 1 to osteoporosis. *J Bone Miner Res* 14:1993–1999
- Devoto M, Shimoya K, Caminis J et al. (1998) First-stage autosomal genome screen in extended pedigrees suggests genes predisposing to low bone mineral density on chromosomes 1p, 2p and 4q. *Eur J Hum Genet* 6:151–157
- Takacs I, Koller DL, Peacock M et al. (2000) Sib pair linkage and association studies between bone mineral density and interleukin-6 gene locus. *Bone* 27:169–173
- Deng HW, Shen H, Xu FH et al. (2002) Tests of linkage and/or association of genes for vitamin D receptor, osteocalcin, and parathyroid hormone with bone mineral density. *J Bone Miner Res* 17:678–686
- Wilson SG, Reed PW, Bansal A et al. (2003) Comparison of genome screens for two independent cohorts provides replication of suggestive linkage of bone mineral density to 3p21 and 1p36. *Am J Hum Genet* 72:144–155
- Zmuda JM, Cauley JA, Ferrell RE (2000) Molecular epidemiology of vitamin D receptor gene variants. *Epidemiol Rev* 22:203–217
- Eisman JA (1995) Vitamin D receptor gene alleles and osteoporosis: an affirmative view. *J Bone Miner Res* 10:1289–1293
- Morrison NA, Yeoman R, Kelly PJ, Eisman JA (1992) Contribution of trans-acting factor alleles to normal physiological variability: vitamin D receptor gene polymorphisms and circulating osteocalcin. *Proc Natl Acad Sci* 89:6665–6669
- Morrison NA, Cheng QJ, Tokita A et al. (1997) Prediction of bone density from vitamin D receptor alleles (correction). *Nature* 387:106
- Fleet JC, Harris SS, Wood RJ, Dawson-Hughes B (1995) The BsmI vitamin D receptor restriction fragment length polymorphism (BB) predicts low bone density in premenopausal black and white women. *J Bone Miner Res* 10:985–990
- Kikuchi R, Uemura T, Gorai I, Ohno S, Minaguchi H (1999) Early and late postmenopausal bone loss is associated with BsmI vitamin D receptor gene polymorphism in Japanese women. *Calcif Tissue Int* 64:102–106
- Langdahl BL, Gravholt CH, Brixen K, Eriksen EF (2000) Polymorphisms in the vitamin D receptor gene and bone mass, bone turnover and osteoporotic fractures. *Eur J Clin Invest* 30:608–617
- Kung AWC, Yeung SSC, Lau KS (1998) Vitamin D receptor gene polymorphisms and peak bone mass in southern Chinese women. *Bone* 22:389–393
- Fountas L, Moutsatsou P, Kastanias I et al. (1999) The contribution of vitamin D receptor gene polymorphisms in osteoporosis and familial osteoporosis. *Osteoporos Int* 10:392–398
- Gross C, Eccleshall TR, Malloy PJ, Villa ML, Marcus R, Feldman D (1996) The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. *J Bone Miner Res* 11:1850–1855

25. Gennari L, Becherini L, Mansani R et al. (1999) FokI polymorphism at the translation initiation site of the vitamin D receptor gene predicts bone mineral density and vertebral fractures in postmenopausal Italian women. *J Bone Miner Res* 14:1379–1386
26. Choi YM, Jun JK, Choe J et al. (2000) Association of the vitamin D receptor start codon polymorphism (FokI) with bone mineral density in postmenopausal Korean women. *J Hum Genet* 45:280–283
27. Efstathiadou Z, Kranas V, Ioannidis JPA, Georgiou I, Tsatsoulis A (2001) The Sp1 COLIA1 gene polymorphism, and not the vitamin D receptor gene polymorphisms, determines bone mineral density in postmenopausal Greek women. *Osteoporos Int* 12:326–331
28. Wettinger G (2002) Slavery in the Islands of Malta and Gozo ca 1000–1812. Publishers Enterprises Group (PEG) Ltd, San Gwann, Malta
29. Kanis JA, Melton LJ, Christiansen C, Johnston CC, Khaltaev N (1994) The diagnosis of osteoporosis. *J Bone Miner Res* 9:1137–1141
30. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215
31. Garnero P, Borel O, Sornay-Rendu E, Delmas PD (1995) Vitamin D receptor gene polymorphisms do not predict bone turnover and bone mass in healthy premenopausal women. *J Bone Miner Res* 10:1283–1288
32. Marc J, Prezelj J, Komel R, Kocijancic A (2000) Association of vitamin D receptor gene polymorphism with bone mineral density in Slovenian postmenopausal women. *Gynecol Endocrinol* 14:60–64
33. Chen HY, Chen WC, Hsu CD, Tsai FJ, Tsai CH, Li CW (2001) Relationship of BsmI vitamin D receptor polymorphism to bone mineral density and occurrence of osteoporosis in postmenopausal Chinese women in Taiwan. *Osteoporos Int* 12:1036–1041
34. Houston LA, Grant SF, Reid DM, Ralston SH (1996) Vitamin D receptor polymorphism, bone mineral density, and osteoporotic vertebral fracture: studies in the UK population. *Bone* 18:249–252
35. Uitterlinden AG, Pols HAP, Burger H et al. (1996) A large-scale population-based study of the association of the vitamin D receptor gene polymorphisms with bone mineral density. *J Bone Miner Res* 11:1241–1248
36. Ferrari S, Rizzoli R, Manen D, Slosman D, Bonjour JP (1998) Vitamin D receptor gene start codon polymorphisms (FokI) and bone mineral density: Interactions with age, dietary calcium, and 3'-end region polymorphisms. *J Bone Miner Res* 13:925–930
37. Cheng WC, Tsai KS (1999) The vitamin D receptor start codon polymorphism (FokI) and bone mineral density in premenopausal women in Taiwan. *Osteoporos Int* 9:545–549
38. Zmuda JM, Cauley JA, Danielson ME, Theobald TM, Ferrell RE (1999) Vitamin D receptor translation initiation codon polymorphism and markers of osteoporotic risk in African-American women. *Osteoporos Int* 9:214–219
39. Harris SS, Eccleshall TR, Gross C, Dawson-Hughes B, Feldman D (1997) The vitamin D receptor start codon polymorphism (FokI) and bone mineral density in premenopausal American black and white women. *J Bone Miner Res* 12:1043–1048
40. Gennari L, Becherini L, Masi L et al. (1998) Vitamin D and estrogen receptor allelic variants in Italian postmenopausal women: evidence of multiple gene contribution to bone mineral density. *J Bone Miner Res* 83:939–944
41. Uitterlinden AG, Weel AEAM, Burger H et al. (2001) Interaction between the vitamin D receptor gene and collagen type I $\alpha 1$ gene in susceptibility for fracture. *J Bone Miner Res* 16:379–385