0021-972X/03/$15.00/0
*Printed in U.S.A.*

The Journal of Clinical Endocrinology & Metabolism 88(8):3777–3784

doi: 10.1210/jc.2002-021861

# Interaction between Vitamin D Receptor Genotype and Estrogen Receptor α Genotype Influences Vertebral Fracture Risk

E. M. COLIN, A. G. UITTERLINDEN, J. B. J. MEURS, A. P. BERGINK, M. VAN DE KLIFT, Y. FANG, P. P. ARP, A. HOFMAN, J. P. T. M. VAN LEEUWEN, AND H. A. P. POLS

*Departments of Internal Medicine (E.M.C., A.G.U., J.B.J.M., A.P.B., Y.F., P.P.A., J.P.T.M.v.L., H.A.P.P.) and Epidemiology and Biostatistics (A.G.U., M.v.d.K., Y.F., A.H., H.A.P.P.), Erasmus Medical Center, 3015 GD Rotterdam, The Netherlands*

**In view of the interactions of vitamin D and the estrogen endocrine system, we studied the combined influence of polymorphisms in the estrogen receptor (ER) α gene and the vitamin D receptor (VDR) gene on the susceptibility to osteoporotic vertebral fractures in 634 women aged 55 yr and older. Three VDR haplotypes (1, 2, and 3) of the *Bsm*I, *Apa*I, and *Taq*I restriction fragment length polymorphisms and three ERα haplotypes (1, 2, and 3) of the *Pvu*II and *Xba*I restriction fragment length polymorphisms were identified. We captured 131 nonvertebral and 85 vertebral fracture cases during a mean follow-up period of 7 yr. ERα haplotype 1 was dose-dependently associated with increased vertebral fracture risk ($P < 0.001$) corresponding to an odds ratio of 1.9 [95% confidence interval (CI), 0.9–4.1] per copy of the risk allele. VDR haplotype 1 was overrepresented in vertebral fracture cases. There was a significant interaction ($P = 0.01$) between ERα haplotype 1 and VDR haplotype 1 in determining vertebral fracture risk. The association of ERα haplotype 1 with vertebral fracture risk was only present in homozygous carriers of VDR haplotype 1. The risk of fracture was 2.5 (95% CI, 0.6–9.9) for heterozygous and 10.3 (95% CI, 2.7–40) for homozygous carriers of ERα haplotype 1. These associations were independent of bone mineral density. In conclusion, interaction between ERα and VDR gene polymorphisms leads to increased risk of osteoporotic vertebral fractures in women, largely independent of bone mineral density. (*J Clin Endocrinol Metab* 88: 3777–3784, 2003)**

OSTEOPOROSIS IS CHARACTERIZED by low bone mineral density (BMD), deterioration of the microarchitecture of bone, and subsequent increased fracture (1, 2). Twin and family studies have suggested that BMD has a strong genetic component, besides being influenced by nutritional and lifestyle factors (3–6). Osteoporosis is regarded as a complex genetic trait, which means that variants of several genes underlie the variability of the phenotype. Among the candidate genes in relation to BMD are the genes for collagen type Iα1 (COLIα1), the VDR, and the estrogen receptor (ER) α (7–11). Polymorphisms in the genes for the VDR and the ERα have been examined in relation to BMD. Although contrary reports have been published, two meta-analyses have shown a weak relation between the VDR gene and BMD (12, 13). We and others have found a significant association between VDR polymorphisms and fracture risk (11, 14), although other studies could not confirm such an association (15, 16). Also, contrary reports regarding the contribution of polymorphisms in the ERα gene to BMD and fracture risk have been published (17–26). A recent meta-analysis, however has shown a relation between the ERα gene and BMD and fracture risk (27).

The VDR gene and the ERα are interesting because the encoded proteins are important transcription factors as key players in the respective signal transduction pathways. Indeed, several interactions between the vitamin D and estrogen endocrine system have been described. 1,25-Dihydroxyvitamin $D_3$ (1,25-$(OH)_2D_3$) and 17β-estradiol ($E_2$) have a mutual effect on their biosynthesis (28–30) and receptor expression (31, 32). Also, some genetic studies found an interaction between ERα and VDR genotypes with respect to BMD (33–35). Suarez *et al.* (36) found an interactive effect of ERα and VDR gene polymorphisms on growth in infants.

So far, most genetic studies on osteoporosis have focused on BMD as the primary end point and not on the clinically more relevant end point of fractures. In the current study, we focus on the interaction between ERα and VDR genotypes in relation to the most typical osteoporotic fracture, the vertebral fracture.

## Subjects and Methods

### *Study subjects*

All women included in this study were part of a population-based cohort study of subjects aged 55 yr or older, living in the Ommoord district of the city of Rotterdam in The Netherlands. The objective of the study is to document the occurrence of disease in the elderly in relation to several potential determinants (37). A total of 10,275 persons were invited for baseline examination in 1990. Of those, 7,983 (61.1% women) participated, bringing the overall response rate to 78%. The baseline assessments included the measurement of anthropometric characteristics, femoral and lumbar spine BMD. Subjects were excluded according to the following criteria: age 80 yr or older; use of a walking aid; use of estrogen or hormone replacement therapy, diuretic, thyroid hormone, or cytostatics; or known diabetes mellitus. After genotyping, women with the rare VDR haplotypes 4 and 5 (n = 16) were excluded. Anthropometric data, DNA samples, and genotype data for both loci were finally

Abbreviations: ANCOVA, Analysis of covariance; BMD, bone mineral density; BMI, body mass index; CI, confidence interval(s); $E_2$, 17β-estradiol; ER, estrogen receptor; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin $D_3$; OR, odds ratio; RFLP, restriction fragment length polymorphisms; VDR, vitamin D receptor.

available in a sample of 1062 women. Data on incident vertebral fractures were available for a subgroup of 634 women.

### *Measurements*

At baseline, height and weight were measured. BMD (in grams per square centimeter) was measured at the femoral neck and lumbar spine by dual energy x-ray absorptiometry (Lunar DPX-L densitometer, Lunar Corp., Madison, WI), as reported earlier (38). Body mass index (BMI) was computed as weight in kilograms divided by height in square meters. Age at menopause was assessed by questionnaire. Dietary intake for calcium (milligrams per day) and vitamin D (milligrams per day) were assessed by food frequency questionnaire and adjusted for energy intake. Both at baseline, between 1990 and 1993, and at the follow-up visit, between 1997 and 1999, radiographs of the spine were taken from the fourth thoracic to the fifth lumbar vertebrae. All follow-up photos were analyzed for the presence of vertebral fractures by the McCloskey/Kanis method (39). The occurrence of nonvertebral fractures was recorded, confirmed, and classified by a physician. All nonvertebral fractures were reported by general practitioners in the research area (covering 80% of the cohort) by means of a computerized system. Information from general practitioners outside the research area was obtained by regular checking of the patient records by research physicians. All reported events were verified by research physicians who independently reviewed and coded the information subsequently. All coded nonvertebral fractures were reviewed by a medical expert in the field for final classification.

### *Determination of VDR and ERα genotypes*

For genotyping, we determined haplotypes of the *Bsm*I, *Apa*I, and *Taq*I restriction fragment length polymorphisms (RFLPs) at the 3′ end of the VDR gene and haplotypes of the *Pvu*II and *Xba*I RFLPs in the first intron of the ERα gene by direct molecular haplotyping methods as described previously (9). Three frequent VDR haplotypes are discerned and encoded 1 (baT), 2 (BAt), and 3 (bAT) (Fig. 1). The less frequent haplotypes 4 and 5 were excluded from the analysis (n = 16). Women carrying these genotypes represent 1.5% of the population. For direct molecular haplotyping of the *Pvu*II and *Xba*I RFLPs, a 346-bp PCR fragment was generated by a forward primer (ER-F, 5′-GATATC-CAGGGT TATGTGGCA-3′) and a reverse primer (ER-R, 5′-AGGTGT-TGCCTATTATATTAACCTTGA-3′) in a reaction mixture of 10 μl containing 20 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM deoxy-nucleoside triphosphate, 2 pM of each primer, and 0.2 U Super *Taq* polymerase (HT Biotechnology Ltd., Cambridge, UK). The reactions were performed in 384-well format in a thermocycler (MJ-Tetrad, MJ Research, Incline Village, NV) with a cycling protocol of 94, 60, and 72 C for 45 sec each for 30 cycles. Ten microliters of PCR product were digested by simultaneous addition of 5 μl digestion mixture containing 5 U *Pvu*II, 7 U *Xba*I restriction enzyme (MBI Fermentas, Hanover, MD), and 1.5 μl ReactBuffer 2 (Life Technologies, Breda, The Netherlands) and incubating for 90 min at 37 C. The digestion products were analyzed by electrophoresis in a 3% agarose gel in 0.5× TBE (1× TBE = 89 mM Tris, 89 mM boric acid, 2 mM $Na_2$ EDTA) for 80 min at 125 V. Separation patterns were documented with a digital camera (DC120, Eastman Kodak, Rochester, NY) under UV illumination (302 nm). Three ERα haplotype alleles are identified, encoded 1 (px/T-A), 2 (PX/C-G), and 3 (Px/C-A) combining to six genotypes 11, 12, 13, 22, 23, and 33 (Fig. 2). We did not observe the fourth possible haplotype (pX; −397int1T and −351int1G) in our population.

### *Statistical analysis*

Differences in mean age at baseline between the study group and the Rotterdam study were evaluated by means of ANOVA. All other differences in baseline characteristics were compared by analysis of covariance (ANCOVA) testing with age to adjust for possible confounding effects. Differences in baseline characteristics between the different genotype groups of the ERα gene were compared as follows. We grouped subjects by allele copy number (0, 1, 2) for the haplotype alleles of interest. We allowed for three possible models to explain differences between groups, *i.e.* an allele dose effect, a dominant effect or a recessive effect. Allele dose was defined as the number of copies of a certain allele in the genotype. In case of a consistent trend reflected as an allele dose effect, we performed a (multiple) linear or logistic regression analysis to quantify the association. In case of a dominant or recessive effect of the test allele, ANOVA and ANCOVA tests were performed. For dominant effects, we compared test-allele carriers *vs.* noncarriers, whereas for recessive effects, subjects homozygous for the test allele were compared with heterozygous carriers and noncarriers.

Odds ratios (ORs) with 95% confidence intervals (CI) were calculated by (multiple) logistic regression analyses to estimate the relative risk of fractures at baseline by genotypes of the risk allele, with no copies of the risk allele as the reference group. First, we calculated crude ORs, and, secondly, we adjusted for potentially confounding factors (age, BMI, BMD, and age at menopause). We used SPSS version 9.0 (SPSS Inc., Chicago, IL) for all our analyses.

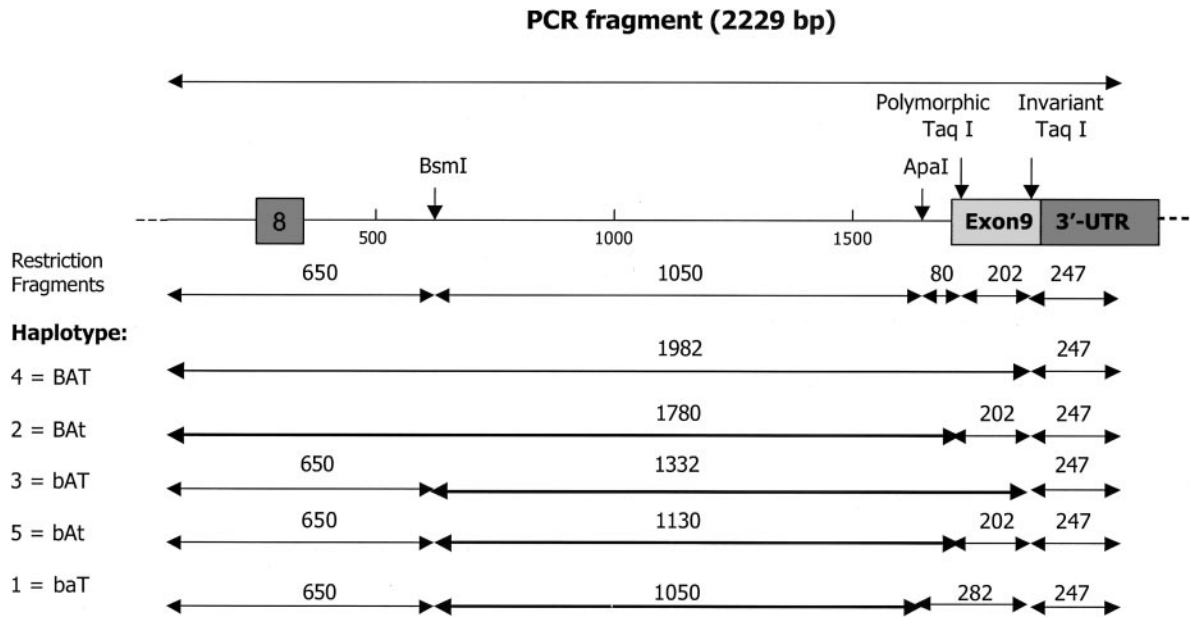


FIG. 1. VDR gene: direct molecular haplotyping. For details, see *Materials and Methods*.

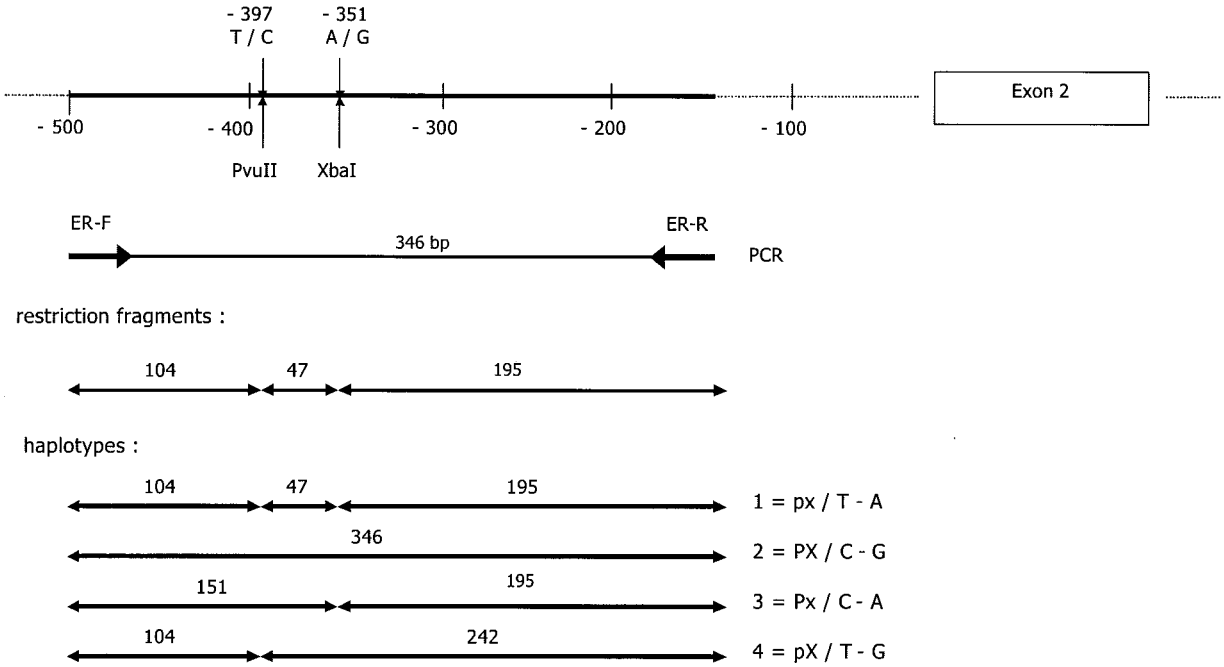


FIG. 2. ERα gene: direct molecular haplotyping. For details, see *Materials and Methods*.

## Results

### *Baseline characteristics*

The study population (n = 1062) was on average 67.0 (SD 6.9) yr old, had an average BMI of 26.1 (SD 3.7) kg/m$^2$, and age at menopause at 48.7 (SD 4.9) yr. Dietary calcium and vitamin D intake were on average 1093 (SD 326) mg/d and 1.96 (SD 1.15) mg/d, respectively. Lumbar spine BMD was on average 1.01 (SD 0.17) g/cm$^2$, and femoral neck BMD was on average 0.81 (SD 0.12) g/cm$^2$, respectively. All women were living independently. In our study population, 85 of 634 [13.4%; mean follow-up period is 6.5 (SD 0.4) yr; range, 2.7–8.4 yr] incident vertebral fractures were captured, whereas 131 of 1062 [12.3%; mean follow-up period is 7.0 (SD 2.0) years; range, 0.2–10.1 yr] subjects had a nonvertebral.

Table 1 shows allele and genotype frequencies for ERα and VDR polymorphisms. The genotype distribution was found to be in Hardy Weinberg equilibrium. When we analyzed for known risk factors for osteoporosis by ERα and VDR genotypes, no differences were shown apart from ERα haplotype 1 that appeared to be dose dependently associated with later onset of menopause, as we have reported earlier (40) (Table 2). Similar data were found for the subgroup of 634 women participating in the analysis for vertebral fractures (data not shown).

### *Association of ERα and VDR with BMD*

In Table 3 women are grouped according to carrier status for the ERα and VDR haplotypes as homozygous carriers (consisting of genotype 11) and heterozygous carriers (including the genotypes 12 and 13) of the ERα haplotype 1 and VDR haplotype 1, respectively, and women not carrying these haplotypes (reference group, including genotypes 22, 23, and 33).

ERα haplotype 1 was dose dependently associated with decreased lumbar spine BMD corresponding with 0.1 SD per copy ERα haplotype 1 (Table 3, Total column). No association was found with femoral neck BMD (Table 3, Total column). ERα haplotype 2 was associated with increased lumbar BMD, corresponding with 0.1 SD per copy ERα haplotype 2 (data not shown). These associations did not change after adjustment for potential confounders such as age, BMI, and age at menopause. No associations were found between ERα haplotype 3 and lumbar spine or femoral neck BMD (data not shown). On the basis of these data, ERα haplotype 1 was considered as risk allele. In the sample of 634 women in whom data on incident vertebral fractures were available, the association between ERα haplotype 1 and lumbar spine BMD showed a similar trend ($P = 0.11$).

On the basis of our previous analyses (11), we selected VDR haplotype 1 as risk allele. In the present study, no association between VDR haplotype 1 and lumbar spine or femoral neck BMD was observed (Table 3, Total rows).

**TABLE 1.** Genotype and allele frequencies of ERα and VDR polymorphisms in the study population

| | ERα | VDR |
|---|---|---|
| Genotype | | |
| 11 | 297 (28.0) | 271 (25.5) |
| 12 | 409 (38.5) | 401 (37.8) |
| 13 | 124 (11.7) | 105 (9.9) |
| 22 | 138 (13.0) | 183 (17.2) |
| 23 | 82 (7.7) | 89 (8.4) |
| 33 | 12 (1.1) | 13 (1.2) |
| Total | 1062 (100) | 1062 (100) |
| *P* value HWE | 0.81 | 0.13 |
| Haplotype | | |
| 1 | 1127 (53.1) | 1048 (49.3) |
| 2 | 767 (36.1) | 856 (40.3) |
| 3 | 230 (10.8) | 220 (10.4) |
| Total | 2124 (100) | 2124 (100) |

Data represent number (%). HWE, Hardy Weinberg equilibrium.

**TABLE 2.** Characteristics of 1062 postmenopausal women according to ERα haplotype 1 and VDR haplotype 1

| | Reference | Heterozygotes | Homozygotes |
|---|---|---|---|
| ERα haplotype 1 characteristics[a] | (n = 232) | (n = 533) | (n = 297) |
| Age (yr) | 67.6 ± 7.1 | 66.9 ± 6.9 | 66.8 ± 6.9 |
| BMI (kg/m$^2$) | 26.2 ± 3.8 | 26.1 ± 3.5 | 26.1 ± 4.0 |
| Age at menopause (yr) | 47.9 ± 5.1 | 48.7 ± 5.0 | 49.2 ± 4.6[b] |
| Dietary calcium intake (mg/d) | 1098 ± 364 | 1097 ± 319 | 1081 ± 306 |
| Dietary vitamin D intake (mg/d) | 2.04 ± 1.32 | 1.96 ± 1.08 | 1.89 ± 1.12 |
| VDR haplotype 1 characteristics[a] | (n = 285) | (n = 506) | (n = 271) |
| Age (yr) | 66.6 ± 6.6 | 67.3 ± 7.0 | 67.0 ± 6.8 |
| BMI (kg/m$^2$) | 26.0 ± 3.4 | 26.2 ± 3.7 | 26.3 ± 4.1 |
| Age at menopause (yr) | 48.9 ± 4.8 | 48.3 ± 5.1 | 49.0 ± 4.7 |
| Dietary calcium intake (mg/d) | 1078 ± 334 | 1104 ± 331 | 1086 ± 306 |
| Dietary vitamin D intake (mg/d) | 2.03 ± 1.26 | 1.91 ± 1.08 | 1.97 ± 1.17 |

Data shown are means ± SD.
[a] Reference includes ERα or VDR genotypes 22, 23, and 33; heterozygotes include 12 and 13; homozygotes include 11.
[b] $P = 0.02$ (allele-dose association, tested by linear regression analysis).

**TABLE 3.** Lumbar spine BMD and femoral neck BMD (mean ± SD) according to combined ERα haplotype 1 genotype and VDR haplotype 1 genotype

| ERα haplotype 1[a] | Total | VDR haplotype 1[b] | | | |
|---|---|---|---|---|---|
| | | Reference | Heterozygotes | Homozygotes | *P* value |
| Lumbar spine BMD | | | | | |
| Total | 1.01 ± 0.29 (1062)[c] | 1.00 ± 0.17 (285) | 1.02 ± 0.16 (506) | 1.02 ± 0.16 (271) | NS |
| Reference | 1.04 ± 0.17 (232) | 1.01 ± 0.16 (59) | 1.04 ± 0.16 (111) | 1.05 ± 0.16 (62) | NS |
| Heterozygotes | 1.02 ± 0.16 (533) | 1.00 ± 0.16 (134) | 1.02 ± 0.16 (260) | 1.03 ± 0.17 (139) | NS |
| Homozygotes | 0.99 ± 0.16 (297) | 0.99 ± 0.16 (92) | 1.01 ± 0.16 (135) | 0.95 ± 0.16 (70) | 0.05[d] |
| *P* value | 0.003[e] | NS | NS | <0.001[f] | 0.09[f] |
| Femoral neck BMD | | | | | |
| Total | 0.81 ± 0.23 (1062) | 0.80 ± 0.12 (285) | 0.81 ± 0.11 (506) | 0.81 ± 0.12 (271) | NS |
| Reference | 0.81 ± 0.11 (232) | 0.79 ± 0.12 (59) | 0.82 ± 0.12 (111) | 0.81 ± 0.11 (62) | NS |
| Heterozygotes | 0.81 ± 0.12 (533) | 0.80 ± 0.12 (134) | 0.80 ± 0.11 (260) | 0.82 ± 0.12 (139) | NS |
| Homozygotes | 0.80 ± 0.12 (297) | 0.81 ± 0.12 (92) | 0.80 ± 0.12 (135) | 0.78 ± 0.12 (70) | NS |
| *P* value | NS | NS | NS | NS | 0.13[g] |

Values are adjusted for age, BMI; *P* values as tested by ANCOVA. NS, Not significant.
[a] Reference includes ERα genotypes 22, 23, and 33; heterozygotes include 12 and 13; homozygote includes 11.
[b] Reference includes VDR genotypes 22, 23, and 33; heterozygotes include 12 and 13; homozygote includes 11.
[c] No. of women.
[d] $P = 0.02$ for recessive association as analyzed by ANCOVA.
[e] $P = 0.001$ for allele dose association as analyzed by linear regression analysis.
[f] $P < 0.001$ for allele dose association as analyzed by linear regression analysis.
[g] *P* value for the interaction term ERα haplotype 1*VDR haplotype 1.

### *Interaction of ERα and VDR genotypes with respect to BMD*

When the association of ERα haplotype 1 with BMD was analyzed according to the carrier status for VDR haplotype 1, there was a significant allele-dose effect of ERα haplotype 1 being associated with decreased lumbar spine BMD only for women homozygous for VDR haplotype 1 (Table 3, homozygotes column; $P < 0.001$). This association was not influenced by age, BMI, and age at menopause. When age, BMI, ERα genotype, and VDR genotype were taken together in a multivariate regression model, there appeared to be a borderline significant interaction between ERα haplotype 1 and VDR haplotype 1 ($P = 0.09$ for the interaction term). In the subgroup of 634 women, in which data on incident vertebral fractures were available, similar associations were found (data not shown). No interaction between ERα and VDR genotypes was found for femoral neck BMD (Table 3; $P = 0.13$ for the interaction term).

### *Association of ERα and VDR with fracture*

When we analyzed the distribution of fractures in women according to the ERα genotype, we observed an overrepresentation of vertebral fractures in women carrying the ERα haplotype 1 (Table 4). Figure 3 shows separately the distribution of vertebral fractures according to the ERα haplotype 1 and VDR haplotype 1 status. Vertebral fractures were overrepresented in women carrying ERα haplotype 1. This association appeared to be dose dependent, with 6.4% in noncarriers of ERα haplotype 1, 12% vertebral fractures (OR, 1.9; 95% CI, 0.9–4.1) in women heterozygous for ERα haplotype 1, and 21% vertebral fractures (OR, 3.9; 95% CI, 1.7–8.2) in women homozygous for ERα haplotype 1. For women carrying ERα haplotype 2, there was an allele dose association with decreased vertebral fracture risk ($P < 0.001$), whereas for ERα haplotype 3 no differences were observed ($P = 0.53$) (data not shown).

**TABLE 4.** Number of women with vertebral fractures according to ERα genotype

| ERα genotype | No. with fracture/total no. (%) |
|---|---|
| 11 | 40/187 (21.4) |
| 12 | 26/236 (11.0) |
| 13 | 10/71 (14.1) |
| 22 | 3/79 (3.8) |
| 23 | 6/53 (11.3) |
| 33 | 0/8 (0) |
| $x^2$ | 19.2 |
| *P* value | 0.002 |

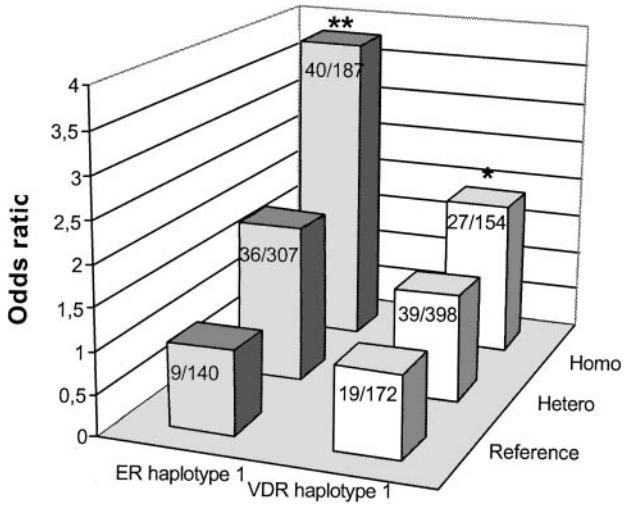


FIG. 3. ORs and numbers of vertebral fractures according to ERα haplotype 1 and VDR haplotype 1 genotype. Reference includes ERα and VDR genotypes 22, 23, and 33. Hetero includes 12 and 13 genotypes. Homo includes 11 genotype. **, $P < 0.001$ for allele dose association of ERα haplotype 1. *, $P = 0.06$ for allele dose association of VDR haplotype 1.

In a previous study, VDR haplotype 1 was found to be associated with increased fracture risk (11). When women were grouped by VDR haplotype 1 genotype also, an allele dose association was observed (Fig. 3). Noncarriers of VDR haplotype 1 had 11% vertebral fractures, women heterozygous for VDR haplotype 1 had 13% vertebral fractures (OR, 1.3; 95% CI, 0.7–2.3), whereas women homozygous for VDR haplotype 1 had 18% fractures (OR, 1.9; 95% CI, 1.0–3.7). VDR haplotypes 2 and 3 were not associated with vertebral fracture risk (data not shown).

When the risk for incident nonvertebral fractures was analyzed, no genotype-dependent effects could be observed (Table 5).

### *Interaction of ERα and VDR with respect to fracture risk*

When we further stratified by VDR haplotype 1 genotype, we observed the ERα haplotype 1 association to be modified by VDR haplotype 1 genotype (Fig. 4). Significant ERα haplotype 1 genotype-dependent differences were only observed in women homozygous for VDR haplotype 1. Logistic regression analysis showed that, compared with the double reference group, within the group of VDR genotype [1, 1] women have a 2-fold (95% CI, 0.5–7.9) and 10-fold (95% CI, 2.7–38) increased risk for vertebral fractures when being heterozygous or homozygous for ERα haplotype 1, respectively. In noncarriers and heterozygous carriers of VDR haplotype 1, no significant ERα haplotype 1 genotype-dependent differences were observed. When age, BMI, ERα genotype, and VDR genotype were taken together in a multivariate regression model, there appeared to be a significant interaction between ERα haplotype 1 and VDR haplotype 1 ($P = 0.01$ for the interaction term). After adjustment for lumbar spine BMD and age at menopause, the results did not change (data not shown).

## Discussion

The current study in postmenopausal women demonstrates for the first time interaction of polymorphisms in the VDR and ERα gene in relation to the risk of incident vertebral fracture risk. Women homozygous for both the VDR haplotype 1 and ERα haplotype 1 had a 10 times higher vertebral fracture risk than noncarriers and a three to four times higher risk than carriers of either one of the risk haplotypes.

So far, most association studies focused on single genes. Two metaanalyses showed a weak association of VDR genotypes with BMD, which supported our own findings in a sample of 2000 men and women from the Rotterdam study (9, 12, 13). A recent metaanalysis showed an association between ERα genotypes and lumbar and femoral BMD (27). Most genetic association studies for osteoporosis have been performed with BMD as the end point, whereas the clinically more relevant end point of osteoporosis is fracture. A limited number of studies have yet been able to address the association of specific gene polymorphisms with fractures. Previously, we have shown that VDR haplotype 1 is the risk allele for osteoarthritis and for vertebral and nonvertebral fractures (11, 41). Ioannidis *et al.* (27) showed in a metaanalysis that the x-allele of the *Xba*I polymorphism (and not the *Pvu*II polymorphism) was associated with increased combined risk for vertebral and nonvertebral fractures. In the present study, we used direct haplotyping methods to increase genetic resolution. We demonstrate an association of ERα haplotype 1 with lumbar spine BMD and vertebral fracture risk. Haplotype 1 corresponds to px, which includes the x-allele found in the metaanalyses to be associated with low BMD and increased fracture risk. Also, at the lumbar spine a synergistic interaction between ERα and VDR genotype for BMD and fractures was detected. No interaction effect between ERα and VDR genotypes was found for femoral neck BMD and for nonvertebral fracture risk. This is in line with previous data, which show a higher response to hormonal replacement therapy at the lumbar spine in contrast to the femoral neck (42–45). The ERα effect may be more pronounced in the spine, which contains more trabecular bone, resulting in a higher rate of bone turnover compared with cortical bone, as present for example in the femoral neck.

We and others previously observed that ERα genotype is associated with differences in age at menarche (46) and age at menopause (40). However, in our current analyses age of menopause did not influence the interaction we observed. This suggests that differences in the age of menopause are small and do not explain the interaction. However, because of the relatively small effect, such influences might only be observed in studies of sufficient power.

**TABLE 5.** Number of women with nonvertebral fractures according to ERα genotype and VDR genotype

| ERα genotype | No. with fracture/total no. (%) | VDR genotype | No. with fracture/total no. (%) |
|---|---|---|---|
| 11 | 32/297 (10.8) | 11 | 35/271 (12.9) |
| 12 | 61/409 (14.9) | 12 | 55/401 (13.7) |
| 13 | 13/124 (10.5) | 13 | 13/105 (12.4) |
| 22 | 10/138 (7.2) | 22 | 16/183 (8.7) |
| 23 | 14/82 (17.1) | 23 | 9/89 (10.1) |
| 33 | 1/12 (8.3) | 33 | 3/13 (23.1) |
| $x^2$ | 8.8 | $x^2$ | 4.8 |
| *P* value | 0.12 | *P* value | 0.45 |

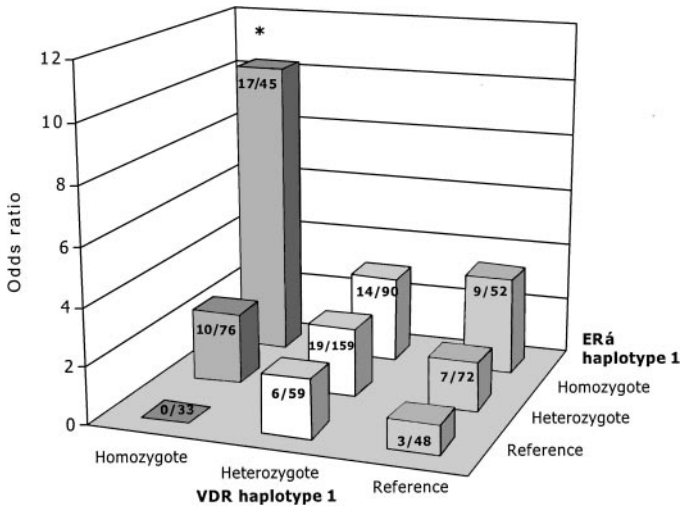


FIG. 4. ORs compared with the double reference group and numbers of vertebral fractures according to combined ERα and VDR genotypes. Reference includes ERα and VDR genotypes 22, 23, and 33. Heterozygotes include 12 and 13. Homozygote includes 11. *, $P < 0.001$ for allele dose association of ERα haplotype 1 in VDR haplotype 1 homozygous carriers. $P = 0.01$ for the interaction term.

An interesting observation was that the association of both ERα and VDR genotypes with vertebral fracture incidence was independent of BMD. This indicates the significance of other bone characteristics for the risk of fracture. But it also pointed to the involvement of ERα and VDR genes in pathways (*e.g.* bone matrix synthesis and bone turnover) other than those directly reflected in BMD, and which also determine strength of bone and thereby fracture risk (47, 48). For example, estrogen deficiency may increase the numbers of remodeling sites with deeper resorption lacunae, resulting in a higher chance of perforating trabeculae with loss of connectivity and ultimately an increased risk for fractures (49).

A limitation of the present study may be health selection bias. However, genotype and allele frequencies are similar to those observed in other Caucasian study populations and so health (apart from the risk for fractures) seems not to be genotype dependent and, therefore, we do not expect this to influence the results. Furthermore, potential selection bias was avoided by deriving cases and noncases from the same source population. Despite the relatively large number of subjects in our study population, the number of fractures is relatively small, and therefore the power to detect interaction is still limited. Consequently, the point estimates could be unstable as is reflected in the relatively wide 95% CI. Therefore, additional larger studies are required to substantiate the present findings and determine more accurate point estimates.

An aspect that should be realized is that the polymorphisms in the ERα and VDR are anonymous. There is no direct known functional consequence for the ERα and VDR protein. Therefore, when association is found, it is assumed that allele(s) of these single nucleotide polymorphisms are in linkage disequilibrium with one or more of the truly functional polymorphisms elsewhere in the gene. These functional polymorphisms could alter VDR (50, 51) or ERα protein structure or might affect the activity of the VDR and ERα 5′ promoter and 3′ untranslated region, leading to the expression of altered quantities of VDR47 and ERα proteins (52) under physiological conditions. Differential transcriptional activity of the VDR and ERα receptor proteins could then preferentially modulate subsets of target genes in vitamin D and estrogen responsive pathways.

Although the mechanism(s) for the gene-gene interaction we observe is so far unknown, it is conceivable from a physiological point of view. 1,25-$(OH)_2D_3$ is an important factor in estrogen biosynthesis (25) and might thus influence local equilibrium between estrogens and androgens. Furthermore, 1,25-$(OH)_2D_3$ regulates ER expression in osteoblast-like cells (31). In this way 1,25-$(OH)_2D_3$ might regulate the effect of $E_2$ on bone metabolism. *In vitro* and *in vivo* studies have shown that several biological responses to treatment with vitamin D, such as intestinal calcium absorption and osteocalcin production, are VDR genotype dependent (53–56). If 1,25-$(OH)_2D_3$ influences the effect of $E_2$ on bone metabolism, this effect might also be VDR genotype dependent.

On the other hand, $E_2$ influences vitamin D metabolism and VDR expression. Sex hormone replacement therapy increases total and free serum 1,25-$(OH)_2D_3$ levels (29, 30). In human fetal osteoblasts, $E_2$ up-regulates VDR expression (57). Also, in rat duodenal mucosa $E_2$ increases VDR expression and bioresponse (32). In this way, $E_2$ might influence vitamin D-regulated processes, like intestinal calcium absorption and osteocalcin production in bone. Several studies have demonstrated that the response to hormonal replacement therapy is ERα genotype dependent (19, 25, 58). Therefore, the effect of estrogen replacement on vitamin D-regulated processes might also be ERα genotype dependent.

In conclusion, the present study shows an interlocus interaction in relation to BMD and fractures between two important candidate genes in osteoporosis. Recently, we also demonstrated an interaction between VDR and another candidate gene, the COLIA1 gene, with respect to fracture risk (11). Together, these findings underscore the polygenic char-

acter of osteoporosis and the importance of the contribution of gene interactions in determining fracture risk. At the same time, our findings highlight the necessity of large (multicenter) studies to achieve sufficient statistical power to further elucidate the complex, multigenic character of osteoporosis.

## Acknowledgments

Received November 26, 2002. Accepted April 21, 2003.

Address all correspondence and requests for reprints to: Prof. Dr. H. A. P. Pols, Erasmus Medical Center, Department of Internal Medicine, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands. E-mail: h.pols@erasmusmc.nl.

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