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# Association of 5′ estrogen receptor alpha gene polymorphisms with bone mineral density, vertebral bone area and fracture risk

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**This study investigates the influence of genetic variation of the estrogen receptor alpha (ESR1) gene locus on several bone parameters in 2042 individuals of The Rotterdam Study, a prospective population-based cohort study of elderly subjects. We analysed three polymorphic sites in the 5′ region of the ESR1 gene; a $(TA)_n$-repeat in the promoter region, and molecular haplotypes of the *Pvu*II and *Xba*I RFLPs in intron 1, and inferred long-range haplotypes (LRH) thereof. We observed only three of the possible four *Pvu*II–*Xba*I haplotypes in our population. A comparison with other Caucasian populations showed similar haplotype frequencies, while in Asian and African populations these were different. Linkage disequilibrium (LD) analysis between the *Pvu*II–*Xba*I haplotype and the $(TA)_n$ repeat showed strong LD between the two sites. Reconstruction of long range haplotypes over the entire 5′ region, revealed six frequent LRH. In men, we did not observe an association between the ESR1 polymorphisms studied and bone parameters. In women, we demonstrated an allele dose effect of haplotype 'px' ($P = 0.003$) and a low number of $(TA)_n$ repeats ($P = 0.008$) with decreased lumbar spine bone mineral density (BMD) (4.8% lower BMD in women homozygous for haplotype 'px', representing 28% of the population, compared with homozygous non-carriers) and decreased vertebral bone area (2.3% difference between extreme genotypes; $P = 0.016$). Most importantly, we found an increased vertebral fracture risk with evidence for an allele dose effect with an odds ratio of 2.2 (95%CI 1.3–3.5) for haplotype 'px', and 2.0 (1.5–3.2) for a low number of $(TA)_n$ repeats. The ESR1 genotype dependent fracture risk is largely independent of BMD and bone area. Combination of risk alleles at both loci by long-range haplotyping improved the associations slightly, but because of the strong LD between the two polymorphic sites, we were unable to determine if any particular polymorphic site is driving the associations found. We conclude that ESR1 polymorphism in the 5′ (promoter) region is associated with vertebral fracture risk, lumbar spine BMD and vertebral bone area in postmenopausal women, but not in men. The molecular mechanism underlying this association needs further study.**

## INTRODUCTION

Osteoporosis is characterized by low bone mineral density (BMD) and an increased risk of fractures (1). Osteoporosis is considered to be a multifactorial syndrome with environmental and genetic factors interacting. Twin studies have suggested that up to 75% of the variance in BMD is genetically determined (2,3). Genetic variations in several genes are thought to be responsible for this genetic component and one of the potential candidates is the estrogen receptor alpha (ESR1) gene.

Several lines of evidence show the important role of the estrogen endocrine system in the regulation of BMD and the occurrence of osteoporosis. Exposure to low estrogen levels occurring after menopause in women is associated with

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increased risk for osteoporosis. The serum level of estradiol has been shown to be an important predictor of subsequent bone mass (4) and risk for osteoporotic fractures (5,6). In line with this, estrogen replacement treatment in early postmenopausal women decreases the risk of osteoporotic fractures (7–9). Estrogens exerts its effect primarily via the ESR1 and the pivotal role of ESR1 in the regulation of bone mass was suggested by a report of a young adult male with a loss-of-function mutation of the ESR1 gene resulting in a phenotype with low BMD (10). Consistent with this, an ESR1 knockout mouse model showed that both female and male mice had decreased bone mass (11).

Several genetic variations in the ESR1 gene have been described and associations of these polymorphisms with BMD have been reported, but results have been inconsistent (12–21). In part, this is due to the limited sample size of most studies where lack of power can lead to spurious results. In addition, differences between populations can play a role, such as ethnicity, age, environment and genetic make-up. Most studies focused on women and have not analyzed fractures, the clinically most relevant endpoint of osteoporosis.

We analyzed three polymorphic sites in the 5′ end of the ESR1 gene. These were a $(TA)_n$ VNTR located approximately 1 kb upstream of the first exon, and the *Pvu*II and *Xba*I restriction fragment length polymorphisms (RFLPs) (22) in intron 1, about 400 bp upstream of exon 2. Since no functional effect of these sequence variations on expression or function of the ESR1 protein has been established so far, they were treated essentially as anonymous polymorphisms. In association studies they are therefore considered as markers and association can be explained by linkage of marker alleles with a truly functional allele elsewhere in the gene. We hypothesize that genetic variation in the ESR1 gene could lead to differences in mRNA expression, which might result in different responsiveness to circulating levels of its ligand estrogen, which in turn results in genotype-dependent differences in bone mass, bone metabolism and fracture risk.

Therefore, we investigated, in a large and homogeneous population-based sample of Caucasian elderly men and women, the influence of the ESR1 polymorphism on BMD, vertebral bone area and fractures and compared our results with those obtained in other association studies.

## RESULTS

### Baseline characteristics

Table 1 shows the baseline characteristics of the total study population and also for the subgroup for whom vertebral fracture data were available. The individuals in the vertebral fracture study were on average 2 years younger, but none of the other baseline characteristics were significantly different.

### Genotype and allele frequencies

Table 2 shows the genotype distribution according to *Pvu*II–*Xba*I RFLP haplotypes in men and women. Genotypes were found to be in Hardy–Weinberg equilibrium (HWE; $P = 0.96$ and 0.99, respectively, for men and women). Table 3 shows the frequencies of the haplotypes found in our cohort of women, compared with those in other populations of women. The frequencies of the haplotypes were similar in other Caucasian populations, but in two Asian populations haplotype 3 (Px) was more frequent, while the haplotype 2 (PX) allele was less frequent. In the small African population tested (Coriell panel), haplotype 1 was less frequent than in Caucasian and Asian women, and haplotype 2 was more frequently present. In all populations the haplotype 4 (pX) was rare or not present at all.

**Table 1.** Baseline characteristics of the total study population and the subgroup for whom vertebral fracture data are available

| | Study population | Study population vertebral fractures | *P*-value |
|---|---|---|---|
| *Women* | | | |
| Number | 1100 | 657 | |
| Age (years) | 67.0 ± 6.9 | 65.7 ± 6.4 | <0.001 |
| BMI (kg/m$^2$) | 26.2 ± 3.7 | 26.0 ± 3.7 | 0.55 |
| FN-BMD | 0.80 ± 0.12 | 0.81 ± 0.12 | 0.13 |
| LS-BMD | 1.01 ± 0.17 | 1.02 ± 0.17 | 0.73 |
| Age of menopause (years) | 48.7 ± 4.9 | 48.7 ± 5.0 | 0.97 |
| Age at menarche (years) | 13.7 ± 1.7 | 13.7 ± 1.8 | 0.67 |
| *Men* | | | |
| Number | 942 | 527 | |
| Age (years) | 67.3 ± 7.1 | 65.4 ± 6.5 | <0.001 |
| BMI (kg/m$^2$) | 25.5 ± 2.9 | 25.6 ± 2.8 | 0.50 |
| FN-BMD | 0.87 ± 0.13 | 0.88 ± 0.13 | 0.20 |
| LS-BMD | 1.15 ± 0.20 | 1.15 ± 0.19 | 0.81 |

Values are means ± standard deviation.

The frequencies of the $(TA)_n$ VNTR alleles in our total population are shown in Figure 1. The bimodal distribution of VNTR alleles is similar to that found in earlier studies of Caucasians. The $(TA)_n$ genotype data were not available for other ethnic groups or could not be determined because of low power due to small sample size and the large number of alleles.

### LD and haplotype analysis *Pvu*II–*Xba*I and the $(TA)_n$ VNTR

LD-analysis showed strong LD between the $(TA)_n$-repeat and *Pvu*II–*Xba*I haplotype. In order to get more insight into the pattern of linkage disequilibrium between the two polymorphic loci, pairwise disequilibria measures ($D'$) between the three different *Pvu*II–*Xba*I haplotypes and all the different $(TA)_n$-repeat alleles were calculated. Figure 2 shows that haplotype 1 is in strong (although not complete) LD with a low number of $(TA)_n$-repeat, while haplotype 2 is linked to a longer $(TA)_n$ repeat and haplotype 3 is not in LD with the $(TA)_n$ VNTR.

We went on to reconstruct long range haplotypes (LRH) of *Pvu*II–*Xba*I RFLP haplotypes and the $(TA)_n$-VNTR alleles. The $(TA)_n$ VNTR is located at a distance of 35 kb from the two RFLPs so molecular haplotypes of all three polymorphisms cannot easily be determined. Instead, haplotypes frequencies were inferred based on genotype frequencies of individual polymorphisms using a Markov chain–Monte Carlo algorithm (23)

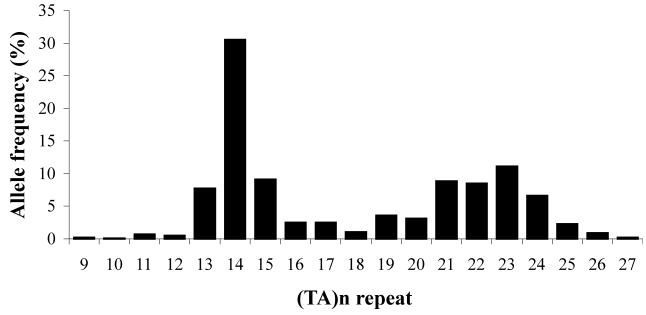


**Figure 1.** Allele frequencies of $(TA)_n$ VNTR polymorphism in the total study population. L = low number of TA repeats; H = high number of TA repeats.

**Table 2.** Genotype distribution according to *Pvu*II–*Xba*I haplotypes

| Genotype | Men number (frequency) | Women number (frequency) |
|---|---|---|
| 11 | 275 (29.2) | 311 (28.3) |
| 12 | 350 (37.2) | 421 (38.3) |
| 13 | 122 (13.0) | 127 (11.5) |
| 22 | 105 (11.1) | 142 (12.9) |
| 23 | 76 (8.1) | 86 (7.8) |
| 33 | 14 (1.5) | 13 (1.2) |
| Total | 942 (100) | 1100 (100) |

1 = px; 2 = PX; 3 = Px.

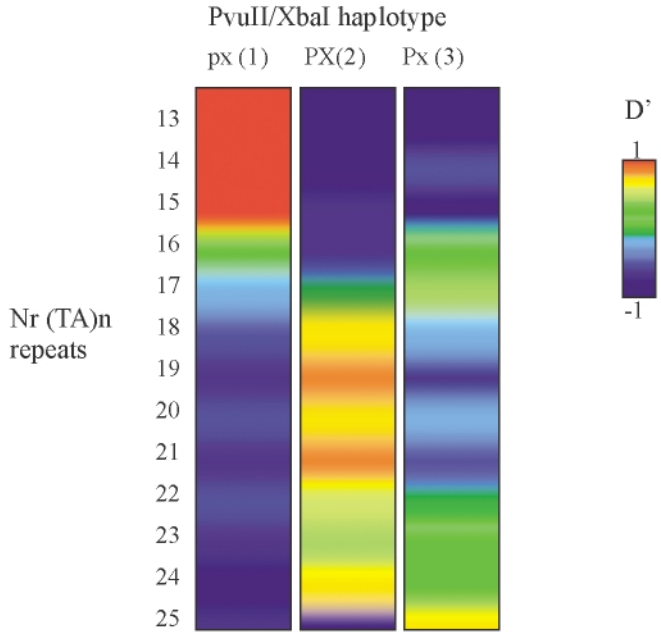


**Figure 2.** Pairwise disequilibrium coefficients ($D'$) between the three *Pvu*II–*Xba*I haplotype alleles and the 13 most common $(TA)_n$ repeat alleles. The coefficient is positive when the alleles are linked, and is negative when the alleles exclude each other. When $D'$ is 1, the two alleles are completely linked, when $D'$ is −1, the two alleles exclude each other completely.

for haplotype reconstruction of each individual (Table 4). Owing to the strong LD, we observed two frequent LRHs (L-1 and H-2) among the six possible haplotypes. While 88% of the L $(TA)_n$ VNTR alleles are linked to the *Pvu*II–*Xba*I haplotype 1, only 69% of H $(TA)_n$ alleles are in LD with the *Pvu*II–*Xba*I haplotype 2. This reflects the differences in $D'$ as shown in Figure 2. We then analyzed association of ESR1 polymorphism with bone characteristics for the *Pvu*II–*Xba*I haplotypes and the $(TA)_n$ VNTR separately and for the combined LRHs.

### *Pvu*II–*Xba*I haplotype and bone characteristics

We investigated the relation between each of the three *Pvu*II–*Xba*I haplotypes and BMD, using linear regression analysis. For each haplotype, subjects were grouped according to the number of copies of the haplotype under investigation. Haplotype 1(px) showed a significant association with decreased BMD at the lumbar spine (LS-BMD) in women (Table 5), while haplotype 2 was associated with increased LS-BMD and haplotype 3 did not show an association. Baseline characteristics according to the haplotype 1 carrier status showed that, in both genders, age and body mass index (BMI) did not differ significantly between the genotypes (results not shown), while, as was previously found in women, age at menopause differed significantly (24). The mean age at menopause was 0.6 years higher for every copy of haplotype 1 ($P = 0.005$, linear regression). No significant difference was found for age at menarche. Table 5 shows also vertebral bone area measures according to haplotype 1 genotype, which was significantly different between the different genotypes in women. In men, no association was found between the haplotype 1 genotype and BMD or bone area measures. In all analyses, additional adjustment for baseline age, BMI and age at menopause did not essentially change the associations.

Table 6 shows the number of fractures and odds ratios according to haplotype 1 carrier status. We found no association of the haplotype 1 with risk for non-vertebral fracture, neither in men nor in women. We did additional analyses for different types of non-vertebral fracture, like hip, upper humerus and wrist, but we could not find a significant association of the haplotype 1 for any of these different types of fractures. For women we found a significantly increased risk for vertebral fractures in haplotype 1 carriers. We found evidence for an allele dose effect in which the risk increased 2.0 (1.4–2.9) times per copy of the haplotype 1. In view of the possible confounding effect of vertebral fractures on vertebral bone area, we adjusted the associations of vertebral bone area for presence or absence of vertebral fractures and found no effect of this adjustment on the association. In an additional analysis, we have excluded the fracture cases and found that the association between bone area and ESR1 polymorphism was still present (data not shown). This strongly suggests that the effect of ESR1 variation on bone area is independent of fractures.

In view of these results and the strong LD between the *Pvu*II–*Xba*I haplotype and the $(TA)_n$ VNTR, we went on to analyze the $(TA)_n$ VNTR associations to bone characteristics, in women only and only for lumbar spine BMD, vertebral bone area and only for vertebral fracture risk.

### The $(TA)_n$ VNTR and bone characteristics

We investigated the relation between each $(TA)_n$ VNTR allele and bone characteristics by grouping the women according to

**Table 3.** *Pvu*II–*Xba*I haplotype frequencies in women with several ethnic backgrounds

| Ethnic origin | Number of subjects | Country (reference) | Haplotype | | | |
|---|---|---|---|---|---|---|
| | | | 1 ( px) | 2 (PX) | 3 (Px) | 4 ( pX) |
| Caucasian | 1100 | The Netherlands (this study) | 53.0 | 36.1 | 10.9 | 0 |
| Caucasian | 610 | Italy (14) | 52.1 | 40.9 | 5.7 | 1.3 |
| Caucasian | 454 | Denmark (16) | 53.0 | 33.7 | 13.3 | 0 |
| Caucasian | 206 | United Kingdom (13) | 56.1 | 33.5 | 9.2 | 1.2 |
| Asian | 238 | Japan (15) | 54.5 | 18.7 | 26.5 | 0.3 |
| Asian | 598 | Korea (18) | 57.7 | 18.5 | 21.5 | 2.3 |
| African | 19 | Coriell panel | 36.8 | 50.0 | 13.6 | 0 |

**Table 4.** Estimated frequencies of long range haplotypes from *Pvu*II–*Xba*I haplotype alleles and $(TA)_n$ alleles[a] in 1100 women

| LRH haplotype | $(TA)_n$ | *Pvu*II–*Xba*I | Number of alleles (%) |
|---|---|---|---|
| A | L | 1 | 1065 (48.4) |
| B | L | 2 | 59 (2.7) |
| C | L | 3 | 54 (2.5) |
| D | H | 1 | 105 (4.8) |
| E | H | 2 | 732 (33.3) |
| F | H | 3 | 185 (8.4) |
| Total | | | 2200 (100) |

[a]The $(TA)_n$ alleles are defined as L ($n < 18$) and H ($n \geq 18$).

carrier status of the individual $(TA)_n$ VNTR allele under investigation. Table 7 shows that all carriers of alleles with a low number of $(TA)_n$ repeats ($n < 18$) have a higher percentage of vertebral fractures rather than one or two individual $(TA)_n$ alleles. The same was observed for the association with lumbar spine BMD. However, for the vertebral bone area we did not observe such a clear pattern of association.

Based on the results described above, and the clear bimodular distribution of the $(TA)_n$ VNTR alleles, the $(TA)_n$ alleles were grouped into a high number of repeats ($n \geq 18$, allele H) and a low number of repeats ($n < 18$, allele L) and association analysis between genotype and bone characteristics was repeated. Similar results were found with the L-allele of the TA-VNTR when compared with the haplotype 1 associations. The short $(TA)_n$ VNTR was associated with decreased BMD at the lumbar spine and decreased lumbar spine bone area in women.

Differences were 4.8 and 2.2%, respectively, between the extreme genotype groups LL and HH. Similar to the haplotype 1, we found a significant association with vertebral fractures in women, and we did not find an association with non-vertebral fractures (results not shown). Again we found evidence for an allele dose effect with vertebral fracture risk increasing 2.2 (95% CI 1.5–3.1) per copy of the L-allele of the $(TA)_n$ VNTR, and this risk was independent of the possible confounding factors age, BMI, lumbar spine BMD, lumbar spine bone area and age at menopause (data not shown).

### Long-range haplotypes of *Pvu*II–*Xba*I haplotypes and the $(TA)_n$-VNTR and bone characteristics

In order to determine which of the polymorphic sites at the 5′ region of the ER gene is driving the associations with bone end-points, we repeated the association analysis with the six observed LRHs. Table 8 shows the difference between carriers and non-carriers of the particular LRH with respect to percentage of vertebral fractures, mean lumbar spine BMD and mean vertebral bone area.

For vertebral fractures, all LRHs with a low number of TA repeats show a higher percentage of vertebral fractures in carriers compared to non-carriers. This pattern was not seen for BMD and bone area measures. However, the largest effect was always observed for the LRH allele A, which is the combination of a low number of TA repeats and *Pvu*II–*Xba*I haplotype 1. This is also reflected in Table 9, where the three different risk alleles (*Pvu*II–*Xba*I haplotype 1, TA-L and LHR-A) are compared for their strength of association. The long-range haplotype A (the combination of the haplotype px and VNTR allele L) shows a small improvement of the associations compared with the individual risk alleles.

## DISCUSSION

This study shows an association between the *Pvu*II–*Xba*I haplotype and the $(TA)_n$ repeat in the ESR1 gene with lumbar spine BMD, vertebral bone area and vertebral fracture risk in postmenopausal women. No association was found with femoral neck BMD and non-vertebral fractures. In men, no significant association with bone characteristics was found. We demonstrated that in our population the *Pvu*II–*Xba*I haplotype and the $(TA)_n$ VNTR are in strong LD, which makes it very difficult to determine which one of the two polymorphic sites is driving the associations with these bone characteristics.

Several studies in women have reported inconsistent associations between polymorphism of the ESR1 gene and BMD (12–21). The existence of ethnic differences between the populations, the case–control designs and a health-based selection bias in several studies could explain the discordant findings. The present study confirms data of a number of previous studies (12,14,20,25). In contrast, two other studies found the *Pvu*II–*Xba*I haplotype 3 (Px) to be associated with decreased BMD (13,15), whereas others showed no association (16,18,26). Statistical reasons (such as lack of power and study design) could contribute to the contradictory results published so far. In addition, our findings together with those of others suggest that there could be allelic heterogeneity at the ESR1 locus among different populations. This should be accompanied with differences in genotype distributions, which we indeed found when we studied the allelic frequencies among

**Table 5.** Bone measures (mean ± SD) according to ERα genotype for *Pvu*II–*Xba*I haplotype 1

| Bone measures | Number of copies of *Pvu*II–*Xba*I haplotype 1 | | | *P*-value |
|---|---|---|---|---|
| | 0 | 1 | 2 | |
| *Women* | $n = 240$ | $n = 548$ | $n = 311$ | |
| Femoral neck BMD (g/cm$^2$) | | | | |
| Crude | 0.81 ± 0.12 | 0.81 ± 0.12 | 0.80 ± 0.13 | 0.36 |
| Adjusted[a] | 0.81 ± 0.11 | 0.81 ± 0.12 | 0.80 ± 0.10 | 0.22 |
| Lumbar spine BMD (g/cm$^2$) | | | | |
| Crude | 1.03 ± 0.17 | 1.02 ± 0.17 | 0.99 ± 0.16 | 0.006 |
| Adjusted[a] | 1.04 ± 0.15 | 1.02 ± 0.16 | 0.99 ± 0.15 | 0.001 |
| Lumbar spine bone area (cm$^2$) | | | | |
| Crude | 43.3 ± 4.3 | 42.5 ± 4.6 | 42.3 ± 4.4 | 0.01 |
| Adjusted[a] | 43.3 ± 4.5 | 42.6 ± 4.5 | 42.3 ± 4.5 | 0.02 |
| *Men* | $n = 195$ | $n = 472$ | $n = 274$ | |
| Femoral neck (g/cm$^2$) | | | | |
| Crude | 0.87 ± 0.14 | 0.87 ± 0.13 | 0.86 ± 0.13 | 0.26 |
| Adjusted[b] | 0.88 ± 0.13 | 0.87 ± 0.13 | 0.86 ± 0.13 | 0.08 |
| Lumbar spine (g/cm$^2$) | | | | |
| Crude | 1.15 ± 0.19 | 1.16 ± 0.20 | 1.15 ± 0.20 | 0.68 |
| Adjusted[b] | 1.16 ± 0.19 | 1.16 ± 0.18 | 1.15 ± 0.18 | 0.41 |
| Lumbar spine bone area (cm$^2$) | | | | |
| Crude | 51.8 ± 5.2 | 51.5 ± 5.3 | 52.2 ± 5.6 | 0.28 |
| Adjusted[b] | 51.9 ± 5.3 | 51.5 ± 5.2 | 52.2 ± 5.3 | 0.45 |

[a]Values are adjusted for age, BMI and age at menopause.
[b]Values are adjusted for age and BMI.
*P*-values were calculated using linear regression analysis.

**Table 6.** Fracture risk according to *Pvu*II–*Xba*I haplotype 1 carrier status

| | Women[a] number of copies of *Pvu*II–*Xba*I haplotype 1 | | | Men[b] number of copies of *Pvu*II–*Xba*I haplotype 1 | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 0 | 1 | 2 |
| *Non-vertebral* | | | | | | |
| No. fractures/total no. (%) | 31/241 (12.9) | 84/548 (15.3) | 35/311 (11.3) | 13/195 (6.7) | 29/472 (6.1) | 16/275 (5.8) |
| OR crude | 1 | 1.2 (0.8–1.8) | 0.9 (0.5–1.4) | 1 | 0.9 (0.5–1.8) | 0.9 (0.4–1.8) |
| OR adjusted | 1 | 1.2 (0.8–1.9) | 0.8 (0.5–1.4) | 1 | 0.9 (0.5–1.8) | 0.8 (0.4–1.7) |
| *Vertebral* | | | | | | |
| No. fractures/total no. (%) | 9/146 (6.2) | 37/318 (11.6) | 41/193 (21.2) | 13/104 (12.4) | 23/254 (9.1) | 29/169 (17.2) |
| OR crude | 1 | 2.0 (0.9–4.3) | 4.1 (1.9–8.8) | 1 | 0.7 (0.3–1.4) | 1.5 (0.7–2.9) |
| OR adjusted | 1 | 2.0 (0.9–4.3) | 4.0 (1.9–8.7) | 1 | 0.6 (0.3–1.4) | 1.3 (0.6–2.8) |

OR = odds ratios; OR are presented with 95% confidence intervals.
[a]Values are adjusted for age, BMI, age at menopause and BMD (non-vertebral type of fractures were adjusted for femoral neck BMD, vertebral fractures for lumbar spine BMD and lumbar spine bone area).
[b]Values are adjusted for age, BMI and BMD (non-vertebral type of fractures were adjusted for femoral neck BMD, vertebral fractures for lumbar spine BMD and lumbar spine bone area).

different ethnic populations. The two Asian populations studied (15,18) showed different frequencies of the haplotypes 3 (Px) and 2 (PX) compared with the Caucasian populations, while in the small African study sample the haplotype 1 ( px) was present at lower frequency. A different degree of LD between the polymorphism studied and the true functional polymorphism in the different populations might be another reason for the inconsistent results concerning association studies between ESR1 variations and bone characteristics. Our findings of the association with vertebral fractures are in line with those of Langdahl *et al.* (12) and Becherini *et al.* (14). These case–control studies reported results using the TA-VNTR in the promoter region and found a lower mean number of TA repeats present in individuals with osteoporotic fractures. Interestingly, the increase in fracture risk we found in the present population-based study was not explained by the relatively modest 0.2 SD difference in BMD between the genotypes. We observed the ESR1 genotype association with vertebral fractures to be independent of lumbar spine BMD and of age at menopause. Whereas the assessment of BMD is to some extent ESR1-dependent, and as such indirectly influences the risk of fractures, the BMD-independent relation of ESR1 genotype with fractures suggests that other underlying biological mechanisms than those reflected in BMD explain the increased

**Table 7.** Differences in mean BMD of the lumbar spine and percentage of vertebral fractures between women carrying the test $(TA)_n$ allele and women not carrying the test allele

| Number of $(TA)_n$ | Number of carriers | Δ percentage vertebral fractures[a] | ΔLS BMD[b] | ΔLS bone area[c] |
|---|---|---|---|---|
| 13 | 153 | +9.6 | −0.022 | −0.50 |
| 14 | 543 | +4.7 | −0.015 | +0.29 |
| 15 | 194 | +2.8 | −0.002 | −0.93 |
| 16 | 54 | +6.2 | −0.014 | −0.76 |
| 17 | 63 | +7.8 | +0.008 | +0.32 |
| 18 | 25 | −5.0 | +0.018 | −1.09 |
| 19 | 78 | −5.4 | +0.012 | −0.27 |
| 20 | 59 | −8.1 | −0.036 | +0.82 |
| 21 | 187 | −7.1 | +0.02 | +0.75 |
| 22 | 170 | −2.2 | +0.011 | −0.10 |
| 23 | 237 | −3.6 | +0.009 | +0.39 |
| 24 | 144 | −5.1 | +0.025 | +0.62 |
| 25 | 37 | −3.9 | −0.033 | −0.66 |

[a]Percentage of vertebral fractures in allele-carriers minus that of non-carriers.
[b]Mean BMD of allele-carriers minus mean BMD of non-carriers.
[c]Mean vertebral bone area of allele-carriers minus that of non-carriers.

fracture risk. This same phenomenon was also observed for the relation between the collagen-type Ialpha1 Sp1 polymorphism, BMD and fracture risk (27), and for VDR polymorphisms (28). This suggests that BMD might not be the most suitable endpoint in genetic association studies for osteoporosis, at least for these candidate genes. In addition, it indicates that these genetic markers might be used to predict fracture risk independently or in combination with BMD measurements.

We also found an association of ESR1 polymorphism with a parameter of bone geometry, total vertebral bone area. This association was independent of the association with vertebral fractures and suggests a relation between ESR1 polymorphism and bone size. Therefore, we hypothesize that ESR1 polymorphisms leads to a difference in bone growth, which might be explained by a genotype-dependent estrogen sensitivity locally at the site of bone growth. In support of this hypothesis, we recently found an association of ESR1 polymorphism with stature (29). Together with our previous observation of the genetic effect of ESR1 polymorphism on menopausal status (24), and associations with cardiovascular disease (30) and cancer (31), this illustrates the pleiotropic nature of the ESR1 protein. The estrogen endocrine system can be simultaneously involved in several different metabolic pathways, such as the reproductive system, bone and cardiovascular function.

Our study has some potential limitations. Vertebral fracture data were only collected in individuals that survived the follow-up period of approximately 7 years. Although this results in a healthy responder bias this is not likely to be genotype-dependent, since we did not find an influence of the ESR1 polymorphisms we studied on survival (data not shown) and, therefore, we do not expect this to influence our results. Genetic association studies can be influenced by population heterogeneity. In this cohort study, all subjects were Dutch Caucasians; to our knowledge no systematic differences were present with respect to the part of The Netherlands in which this study was performed. Therefore our study population might be considered as an ethnically homogeneous and representative sample of the Dutch subjects.

The results from this study clearly show strong linkage disequilibrium between the $(TA)_n$ VNTR in the promoter and the *Pvu*II–*Xba*I haplotypes in the first intron of the gene. Previous studies also showed a strong relationship between the two polymorphic sites, however we determined the pattern of linkage disequilibrium between the two polymorphic sites in detail and tried to assess which one of the polymorphisms is driving the associations. Although some earlier studies suggested one of the polymorphisms to have stronger effects than the others, we were unable to distinguish the different effects of the two polymorphic sites because of the strong linkage between them despite the large population we had available to study. However, the association studies with the long range haplotypes tend to suggest that both sites are contributing, since a particular long range haplotype shows the strongest effects on the different bone parameters and fracture risk.

The *Pvu*II and *Xba*I polymorphic sites are located in an intron, and so far it is not known whether they have functional consequences. However, polymorphisms in introns could affect mRNA production, since introns have been recognized to contain regulatory sequences. A well-known example is the Sp1 polymorphism located in the first intron of the collagen-type Ialpha1 gene, which is known to change the mRNA production of the gene which eventually leads to decreased BMD an increased fracture risk (27,32). The *Pvu*II–*Xba*I polymorphic site in the first intron of the ESR1 gene could influence gene expression in a similar manner. Recently, a report showed that the *Pvu*II polymorphism is located within a potential bMyb binding site, which was able to regulate transcription efficacy of a reporter gene (33). Alternatively, the location of a variable length of the $(TA)_n$ VNTR in the promoter of the ESR1 gene could also affect gene transcription. Previous studies have shown that a VNTR in proximity to a promoter can have a significant influence on transcriptional regulation (34–36). However, it is still possible that yet another third polymorphic site linked to the ones studied here is the true functional sequence variation. The only way to clarify this issue is to identify all polymorphisms in this region of the gene and perform functional studies on these polymorphisms.

A loss of function mutation in ESR1 leading to low BMD was initially reported in a young adult male. However, in the present study we showed that the associations observed were present in women but not in men. For fractures, this might simply be explained by lack of statistical power due to the lower number of fractures observed in men. In that respect one should keep in mind that, although the present population study includes a large number of individuals, exact estimation of the fracture risks and the size-effect of the BMD-association can only be determined by meta-analysis of all data. The difference between men and women with respect to the associations found can be explained by the higher circulating estrogen levels in elderly men compared with postmenopausal women, which may mask the differences between genotypes.

The associations were strongest at the spine (i.e. with lumbar spine BMD and with vertebral fractures). This is in line with previous data showing a higher response to estrogen replacement therapy at the lumbar spine in contrast to the femoral neck (19,37–39). Probably, the effect of the ESR1 is more pronounced

**Table 8.** Association of the six *Pvu*II–*Xba*I $(TA)_n$ repeat LRH alleles in 1100 women with BMD and bone area of the spine

| LRH allele | Haplotype alleles $(TA)_n$, *Pvu*II–*Xba*I | Number of carriers | Δ percentage vertebral fractures[a] | ΔLS BMD (g/cm$^2$)[b] | ΔLS bone area (cm$^2$)[c] |
|---|---|---|---|---|---|
| A | L, 1 | 797 | +9.3 | −0.028 | −0.78 |
| B | L, 2 | 57 | +5.1 | +0.026 | +0.20 |
| C | L, 3 | 51 | +3.0 | −0.005 | +0.16 |
| D | H, 1 | 100 | −3.4 | +0.009 | +0.41 |
| E | H, 2 | 604 | −8.2 | +0.019 | +0.55 |
| F | H, 3 | 174 | −3.8 | +0.010 | +0.06 |

L = low number of $(TA)_n$ repeats ($n < 18$); H = high number of $(TA)_n$ repeats ($n \geq 18$).
[a]Percentage of vertebral fractures in allele-carriers minus that of non-carriers.
[b]Mean BMD of allele-carriers minus mean BMD of non-carriers.
[c]Mean vertebral bone area of allele-carriers minus that of non-carriers.

**Table 9.** Associations in 1100 women with vertebral fracture risk, lumbar spine BMD and vertebral bone area by ESR1 genotype as defined by the *Pvu*II–*Xba*I haplotype, the $(TA)_n$ VNTR, and the combination thereof (the LRH)

| | Odds ratios[a] per copy of the allele | *P*-value[b] | ΔLS-BMD[c] (g/cm$^2$) per copy of the allele | *P*-value[b] | ΔLS bone area[d] (cm$^2$) per copy of the allele | *P*-value[b] |
|---|---|---|---|---|---|---|
| *Pvu*II–*Xba*I haplotype 1 | 2.0 [1.4–2.9] | <0.001 | −0.0208 | 0.003 | −0.465 | 0.016 |
| $(TA)_n$ VNTR L | 2.2 [1.5–3.1] | <0.001 | −0.0183 | 0.008 | −0.474 | 0.013 |
| LRH allele A | 2.2 [1.5–3.1] | <0.001 | −0.0220 | 0.002 | −0.540 | 0.005 |

[a]Odds ratios are presented with 95% confidence intervals; odds ratios are adjusted for age, BMI and lumbar spine BMD.
[b]*P*-values are calculated using linear regression.
[c]BMD values are adjusted for age and BMI.
[d]Vertebral bone area values are adjusted for age and BMI.

in the vertebral body, which is rich in trabecular bone, due to a higher bone turnover rate. Trabecular bone has a higher rate of bone turnover than cortical bone because trabecular bone presents relatively more surface per unit of bone volume.

We conclude that the *Pvu*II–*Xba*I RFLP-haplotype and the $(TA)_n$-VNTR in the ER gene are associated with lumbar spine BMD, vertebral bone area, and vertebral fracture risk in postmenopausal women. This risk is independent of BMD differences or other confounding factors. Combination of risk alleles at both loci by long-range haplotyping improved the associations slightly, but because of the strong linkage disequilibrium between the two polymorphic sites, we were unable to determine if any particular site is driving the associations. Further studies are needed to elucidate the exact molecular mechanism underlying this association.

## MATERIAL AND METHODS

### Study population

Subjects were participants of the Rotterdam Study, a prospective population based cohort study of individuals aged 55 years and over. The study was designed to investigate the incidence of, and determinants of, chronic disabling diseases. Rationale and design have been described previously (40). The Rotterdam Study was approved by the Medical Ethics Committee of Erasmus University Medical School and written informed consent was obtained from each subject. All 10 275 inhabitants aged 55 years and over of a district in Rotterdam, The Netherlands, were invited for baseline examination between August 1990 and June 1993. Of those, 7983 participated. Baseline measurements of bone mineral density were available for 5931 independently living subjects from the study, but 1453 of these were excluded on the basis of age (>80 years), use of a walking aid, known diabetes mellitus or use of diuretic, estrogen, thyroid hormone or cytostatistic drug therapy. From the 4478 remaining subjects, we studied a random sample of 2042 subjects.

In addition, we determined allele frequencies in a panel of subjects of African origin from the Coriell Institute (Camden, NJ, USA), which consists of 10 African-American subjects (HD04) and nine African subjects from south of the Sahara (HD12).

### Clinical examination

At baseline, BMD (expressed in g/cm$^2$) was measured at the femoral neck and BMD and average vertebral bone area (cm$^2$) was measured over the L2–L4 of the lumbar spine by dual energy X-ray absorptiometry (DEXA, Lunar DPX-L densitometer, Lunar Corp., Madison, WI, USA) as described previously (41). Height and weight were measured in standing position in indoor clothing without shoes. BMI was computed as weight in kilograms divided by height in meters squared (kg/m$^2$). Menopause status and age at menarche was assessed and validated as described previously (24).

### Vertebral fracture assessment

Both at baseline and at a follow-up visit, between 1997 and 1999, thoracolumbar radiographs of the spine were obtained. The follow-up radiographs were available for 1184 individuals,

who survived after an average 7.4 years after baseline center visit and who were still able to come to our research center. All follow-up radiographs were scored for the presence of vertebral fracture by the McCloskey/Kanis method (42) as described earlier (43). If a vertebral fracture was detected, the baseline radiograph was evaluated as well. If the vertebral fracture was already present at baseline, it was considered a baseline prevalent fracture. If it was not present at baseline, the fracture was defined to be incident.

### Assessment of incident non-vertebral fracture

Follow-up started either at 1 January 1991 or, when later, at the time of inclusion into the study. For this analysis follow-up ended either at December 1999 or, when earlier, at the participant's death. The general practitioners of the participants provided data on morbidity including non-vertebral fractures and mortality. For approximately 80% of the study population, medical events were reported through computerized general practitioner diagnosis registers. For the remaining 20%, research physicians collected data from the general practitioners' medical records of the study participants. All collected fractures were verified by reviewing discharge reports and letters from medical specialists. Fracture events were coded independently by two research physicians according to the International Classification of Diseases, 10th revision (ICD-10). In case of discrepancy, consensus was attained in a separate session. A medical expert in the field reviewed all coded events for final classification.

### Genotyping

Genomic DNA was isolated from peripheral leucocytes by standard procedures. The molecular haplotyping of the *Pvu*II and *Xba*I RFLPs was performed as shown in Figure 1. A 346 bp PCR fragment was generated by a forward primer (ER-F: 5′-GATATCCAGGGTTATGTGGCA-3′) and a reverse primer (ER-R: 5′-AGGTGTTGCCTATTATATTAACCTTGA-3′) in a reaction mixture of 10 µl containing 10 ng of genomic DNA, 50 mM KCl, 10 mM Tris–HCl ( pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM deoxy-NTP, 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) with a cycling protocol of 94, 60 and 72°C for 45 s each for 30 cycles. Ten microliters of PCR product were digested by addition of 5 µl of digestion mixture containing 5 U *Pvu*II, 7 U *Xba*I restriction enzyme (MBI Fermentas) and 1.5 µl of ReactBuffer 2 (Life Technologies Inc.) 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 $Na_2EDTA$) for 80 min at 125 V. Separation patterns were documented with a digital camera (DC120, Kodak Company, Rochester, NY, USA) under UV illumination (302 nm). Genotypes were defined as haplotype numbers 1, 2, etc. by decreasing frequency in the population. The correspondence between haplotype numbers, RFLP alleles and nucleotides at positions −397int1 and −351int1 is shown in Figure 1.

A 160–194 bp PCR fragment was generated containing the $(TA)_n$ VNTR using a FAM-labeled forward primer (5′-GACGCATGATATACTTCACC-3′) and reverse primer (5′-GCAGAATCAAATATCCAGATG-3′) in a reaction mixture of 10 µl containing 10 ng of genomic DNA, 50 mM KCl, 10 mM Tris–HCl ( pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM deoxy-NTP, 5 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) with a cycling protocol of 94, 59 and 72°C for 30 s each for 28 cycles. The labeled PCR products were analyzed on an ABI 3100 automated capillary DNA sequencer using Genescan software (Applied Biosystems, Perkin Elmer, Capelle a/d IJssel, The Netherlands). The length of alleles was determined using internal size standards and genotypes were expressed as size-allele combinations.

### Statistical analysis

*Estimation of PvuII–XbaI haplotype frequency.* In our own study, the *Pvu*II–*Xba*I haplotypes were derived from direct molecular haplotyping, as described above. Assessment of the *Pvu*II–*Xba*I haplotypes in other study populations, described in previous manuscripts (12–15,17) was possible when the combined genotype per individual was known. We used these data to infer the frequency of the haplotypes present in the population using the program 3LOCUS.pas (44).

*Linkage disequilibrium analysis.* The linkage disequilibrium coefficient ($D'$) between each pair of alleles at both polymorphic loci was calculated. The coefficient is positive when the alleles co-occur and is negative when alleles exclude each other. $D'$ is calculated from the disequilibrium measure $D = h - pq$, where $h$ is the frequency of the haplotype present in the population and $p$ and $q$ are frequencies of the alleles under investigation where $p < q$. In order to compare the degree of disequilibrium between pairs of alleles with different allele frequencies, the allele frequency-independent $D'$ was calculated, where $D' = D/D_{\max}$. If $D < 0$ then $D_{\max} = pq$, and if $D > 0$ then $D_{\max} = p(1 - q)$. We used the program PHASE (24) for determining the frequencies of the TA–*Pvu*II–*Xba*I haplotypes in different subpopulations.

*Association analysis.* Subjects were grouped according to genotype. We grouped subjects by allele copy number (0, 1, 2) for the *Pvu*II–*Xba*I RFLP haplotypes 1 ( px), 2 (PX) and 3 (Px). For the $(TA)_n$ VNTR, subjects were first grouped according to carrier status of each allele separately. In a second analysis, the $(TA)_n$ VNTR alleles were grouped according to the number of TA-repeats: group 'H' includes alleles with a high number of TA repeats [$(TA)_n \geq 18$] and group 'L' includes alleles with a low number of TA repeats [$(TA)_n < 18$]. The cut-off point was based on the bimodal allele frequency distribution (Fig. 2). Subjects were genotyped as 'LL', 'LH' or 'HH'.

We allowed for three possible genetic 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 linear regression analysis to quantify the association. In case of a dominant or a recessive effect of

the test allele, analysis of (co)variance [AN(C)OVA] was performed to test for differences between two genotype groups. For dominant alleles we compared test-allele carriers versus non-carriers, while for recessive effects homozygous subjects for the test allele were compared to heterozygous carriers combined with non-carriers.

HWE was calculated according to standard procedures using the chi-square analysis. To estimate non-vertebral fracture risk we used Cox proportional hazard models, thereby taking potential differences in follow-up time into account. *P*-values were two-sided and 0.05 or less was considered significant. To estimate the risk of vertebral fractures, odds ratios with 95% confidence intervals (95% CI) were calculated using logistic regression models. We were not able to use Cox proportional hazard models since the exact time of event was not known. We performed all vertebral fracture analysis seperately for both prevalent and incident fractures, and always found the same trends. Therefore, for reasons of power, all vertebral fracture analysis presented were done with combined prevalent and incident vertebral fractures. To calculate risk estimates and 95% CI for incremental classes of haplotypes (allele–dose effects), the genetic variable was used as a continuous measure in the model. All statistical analysis was performed using SPSS version 10.1.0 (SPSS Inc., Chicago, IL, USA).

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