Estrogen receptor alpha gene polymorphisms are associated with estradiol levels in postmenopausal women

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Abstract

Objective: Postmenopausal estradiol (E2) levels vary widely between individuals and this variation is an important determinant of diseases such as osteoporosis. It has been suggested that the estrogen receptor alpha (ESR1) gene may influence peripheral E2 levels, but the role of common sequence variations in the ESR1 gene is unclear.

Methods: In 631 postmenopausal women and 528 men from the Rotterdam Study, a population-based, prospective cohort study of individuals aged 55 years and over, ESR1 PvuII-XbaI haplotypes were determined and correlated with plasma E2 levels.

Results: In women, haplotype 1 (T-A) was significantly associated with an allele-dose-dependent decrease in E2. After adjusting for age, body mass index, years since menopause and testosterone levels, plasma E2 levels decreased by 1.90 pmol/l per allele copy of this haplotype (P < 0.05). Extreme genotypes, representing 23 and 27% of the population, varied by 3.93 pmol/l. No association with plasma testosterone was observed. In a subset of 446 women, no association of genotype with plasma concentrations of dehydroepiandrosterone sulfate, androstenedione or estrone was seen. In men, none of the sex hormone levels was associated with the ESR1 PvuII-XbaI haplotypes.

Conclusion: We have demonstrated a role for genetic variations in the ESR1 gene in determining postmenopausal E2 levels in women.


Introduction

Estradiol (E2) has a general metabolic role that reaches far beyond reproductive processes. E2 levels play an important role in a number of diseases. For example, withdrawal of the effects of E2 after menopause from non-reproductive tissues such as the skeleton, the cardiovascular system and the brain constitutes a major risk factor for the development of osteoporosis, coronary artery disease and stroke in women. On the other hand, continuous exposure to E2 during the post-reproductive part of life in the form of hormone replacement therapy (HRT) has been shown to be a risk factor for the development of coronary artery disease, stroke and breast cancer (1). Furthermore, individual variation in E2 levels has been associated with differences in the risk of osteoporosis (2, 3) and breast cancer (4). It is anticipated that this individual variation partially results from genetic variation (i.e. polymorphisms) in crucial genes that control hormone biosynthesis, metabolism and signal transduction.

One important candidate gene in determining peripheral E2 levels is the estrogen receptor alpha (ESR1, also known as estrogen receptor 1, ERα) gene. At first glance, the importance of the most common of the two estrogen receptor genes (the other being the estrogen receptor beta) in E2 biosynthesis may not be obvious. However, E2 exerts its effects by binding to estrogen receptors that, once activated, regulate the expression of multiple genes. One of the genes E2 and ESR1 may regulate is the aromatase (also known as CYP19) gene, as recently found by Kinoshita & Chen (5). Aromatase catalyzes the conversion of C19-steroids to estrogens and is essential for E2 biosynthesis. Kinoshita & Chen found that E2, through ESR1, can modulate CYP19 gene expression in human breast cancer cells. Modulation of the CYP19 expression by E2 has also been shown in other vertebrates (6).

Several single-nucleotide polymorphisms (SNPs) and variable-number tandem repeat polymorphisms have been identified in the ESR1 gene (http://www.ncbi.nlm.nih.gov). Of the polymorphisms identified so far, the PvuII and Xbai SNPs, located in the first intron 397 and 351 bp upstream of exon 2, are the most widely studied. These polymorphisms have previously been associated with disease phenotypes such as
osteooporosis (7–10), cardiovascular disease (11, 12) and cancer (13). Recently a potential functional significance of the PvuII polymorphisms was reported (14). The aim of the present study was to determine if these common ESR1 polymorphisms are associated with plasma E2 levels in a population of both men and postmenopausal women aged 55 years and older.

Subjects and methods

Study population

The Rotterdam Study is a population-based, prospective cohort study of men and women aged 55 years and over. Rationale and design have been described previously (15). All residents aged 55 or older of Ommoord, a district of Rotterdam, The Netherlands, were invited to participate. A total of 7983 men and women (78% of those eligible) entered the study: Baseline examinations, including a home interview and an extensive physical examination at the research center, took place between 1990 and 1993. The Rotterdam Study was approved by the medical ethics committee of the Erasmus Medical Center and written informed consent was obtained from all participants.

We determined plasma hormone levels in a gender-stratified random sample of 1159 subjects (631 women) who were able to visit the research center and for whom blood samples were available. Only subjects of Caucasian origin, as identified by having four Caucasian grandparents, were included. All women selected were postmenopausal. Participants who used hormone supplements or corticosteroids at the time of blood drawing were excluded. Due to the limited amount of plasma per participant, not all hormone levels could be measured in all subjects. Plasma E2 and testosterone were determined in all of these participants. Levels of additional sex hormones were available for 808 subjects (446 women).

Clinical examination

At baseline, interview information, including smoking habits, use of medication and age at menopause, was obtained by a trained research assistant. Smoking was categorized as current smokers, past smokers or participants who had never smoked. Age at menopause responses were validated as described previously (16). Anthropometric measurements were obtained at the research center. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared.

Hormone assays

Non-fasting blood samples were drawn by venepuncture at the baseline examination in the research center between 0830 and 1600 h and time of blood draw was noted. Levels of steroid hormones were measured in plasma. For the collection of plasma, blood was collected in 5 ml tubes containing 0.5 ml sodium citrate solution. All tubes were stored on ice before and after blood sampling. Platelet-free plasma was obtained by two-stage centrifugation, first for 10 min at 1600 g at 4°C and then for 30 min at 7000 g at 4°C. Platelet-free samples were immediately frozen in liquid nitrogen and transferred to the laboratory. At the laboratory, plasma samples were stored at −80°C until hormone measurements. For the purpose of the present study, plasma levels of E2, testosterone, androstenedione, estrone (E1), dehydroepiandrosterone sulfate (DHEAS), and sex hormone-binding globulin (SHBG) were estimated in 12 separate batches of samples using coated-tube or double-antibody RIAs purchased from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA). The mean minimum detection limit for E2 was 4.8 pmol/l, which enabled us to study the association with ESR1 polymorphism at very low levels. Non-detected E2 was scored as zero.

Due to the relatively small volumes of plasma available, all values reported are single-sample estimations. Intra-assay coefficients of variation, determined on the basis of duplicate results of internal quality control (QC) plasma pools with three different levels of each analyte, were below 15% for all assays, with the exception of E2 (18%) and E1 (21%). Since inter-assay variations were relatively large (between 20 and 30%, with the exception of SHBG (14%) and testosterone (19%)) results of all batches were normalized by multiplying all concentrations within a batch with a factor which made results for the internal QC pools comparable (17). This reduced inter-assay variations and was considered justified because the patterns of the results of these pools and the mean results for male and female sera in one assay batch were very similar. Assays were performed blind with respect to information on the subject. Albumin was measured using a colorimetric method (KONE Diagnostics, Espoo, Finland). As a measure of bioavailable hormones, free testosterone and E2 were calculated on the basis of hormone, SHBG and albumin levels, and respective affinity constants according to the method described by Södergård et al. (18) and van den Beld et al. (19).

Genotyping

All participants were genotyped for the PvuII (also known as c.454-397T>C, IVS1-397 T/C, and rs2234693) and XbaI (also known as c.454-351A>G SNPs, IVS1-351 A/G, and rs9340799) polymorphisms, located 397 and 351 bp respectively upstream from the start of exon 2 in the ESR1 gene. Genomic DNA was isolated from peripheral leukocytes by standard procedures. Genotypes were determined using the Taqman allelic discrimination assay. Primer and probe sequences were optimized using the SNP assay-by-design service of
Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands). For details see store.appliedbiosystems.com. Reactions were performed on the Taqman Prism 7900HT 384-well format. We used the genotype data for each of the two polymorphisms to infer frequency of the haplotype alleles present in the population using the program PHASE (20). The alleles were defined as haplotypes such as ‘C-G’ representing the C-allele for the PvuII SNP and the G-allele for the XbaI SNP as described previously (9). The haplotype alleles were coded as haplotype numbers 1 through 4 in order of decreasing frequency in the population (1 = T-A, 2 = C-G, 3 = C-A and 4 = T-G).

Statistical analysis
One-way analysis of co-variance (ANCOVA) and Pearson’s chi-square tests were used to compare baseline characteristics between subjects in our study and the entire Rotterdam Study cohort.

The association between PvuII-XbaI haplotypes, sex hormone levels and possible confounders was evaluated by stratifying subjects by allele copy number (0, 1 or 2) for the haplotype of interest and using ANOVA, linear regression or Pearson’s chi-square tests. Based on previous analyses we chose haplotype 1 as the risk allele (9, 11, 16, 21, 22). To account for possible confounding, we adjusted the analysis of sex hormone levels for age, BMI, years since menopause and the direct precursor for each hormone when available.

Explained variance estimates were calculated by a stepwise linear regression model including age, BMI, smoking, years since menopause and ESR1 haplotype 1 as independent variables. One hundred times the correlation coefficient squared was interpreted as the percentage variability in E2 explained by the polymorphisms of interest.

All statistical analyses were performed using SPSS version 11.0.1 (SPSS, Inc., Chicago, IL, USA).

Results
Baseline characteristics for the approximately 13% of the Rotterdam Study cohort that was included in our study, as compared with the entire cohort, are shown in Table 1. Fewer women (6.7%) were included compared with the entire Rotterdam Study cohort and participants in our study were 0.6 years younger. Participants of our study also tended to smoke more.

We observed the four possible PvuII-XbaI haplotype alleles in the following frequencies: haplotype 1 (T-A) 52.5%, haplotype 2 (C-G) 35.5%, haplotype 3 (C-A) 11.9% and haplotype 4 (T-G) in only one allele of 2318 chromosomes. Genotype distributions were in Hardy–Weinberg equilibrium and similar between genders and across age categories.

As observed previously (16), in the 631 postmenopausal women, a trend was observed for a later age at menopause per allele copy of haplotype 1 (Table 2). PvuII-XbaI haplotype 1 was associated with decreased plasma E2 levels in an allele-dose-dependent manner; per copy of haplotype 1 E2 levels were 1.68 pmol/l lower (P = 0.05). After adjusting for age, BMI, years since menopause and smoking, genotype-dependent differences increased slightly and plasma E2 levels decreased by 1.90 pmol/l per allele copy of haplotype 1 (P < 0.05, Table 2). Extreme genotypes differed by 3.93 pmol/l (P < 0.05). In this group, ESR1 haplotype 1 explained an additional 1% above the effect of age, BMI, smoking and age at menopause in the variation of E2 level (R^2 change 0.01, P < 0.05).

ESR1 haplotype 3 was not

Table 1 Characteristics of 1159 study participants compared with the entire Rotterdam Study cohort. Values are expressed as mean (S.E.).

<table>
<thead>
<tr>
<th></th>
<th>Study</th>
<th>Rotterdam Study</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>1159 (12.7%)</td>
<td>7983</td>
<td>—</td>
</tr>
<tr>
<td>Women</td>
<td>631 (54.4%)</td>
<td>4878 (61.1%)</td>
<td>&lt;0.001^1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>69.7 (0.3)</td>
<td>70.3 (0.1)</td>
<td>0.05^2</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>26.2 (0.1)</td>
<td>26.2 (0.04)</td>
<td>0.9^2</td>
</tr>
<tr>
<td>Age at menopause (years)</td>
<td>48.8 (0.2)</td>
<td>48.8 (0.08)</td>
<td>0.9^2</td>
</tr>
<tr>
<td>Years since menopause</td>
<td>21.7 (0.2)</td>
<td>21.7 (0.08)</td>
<td>0.9^2</td>
</tr>
<tr>
<td>Smoking</td>
<td>—</td>
<td>—</td>
<td>0.07^1</td>
</tr>
<tr>
<td>Current</td>
<td>24.4%</td>
<td>22.6%</td>
<td>—</td>
</tr>
<tr>
<td>Past</td>
<td>42.4%</td>
<td>40.7%</td>
<td>—</td>
</tr>
</tbody>
</table>

^1 Chi-square.
^2 ANCOVA adjusted for gender.
^3 ANCOVA adjusted for age and gender.
associated with plasma $E_2$ levels (results not shown). None of the $ESR1$ haplotypes was associated with plasma testosterone (Table 2).

Since plasma hormone levels were slightly skewed in women we also transformed our data by taking the square root of each hormone value for each participant, thereby lowering the skewness statistic for all hormones to well below 1. We repeated the analyses and our results did not essentially change.

In the 528 men in our study, for $E_2$ levels a modest trend in the opposite direction was observed; however, this did not reach significance (Table 2 for haplotype 1). Within a subset of 446 postmenopausal women and 362 men, plasma levels of other sex hormones were also determined. As observed in the larger sample, in this subset PvuII-XbaI haplotypes were not significantly associated with age, BMI, age at menopause, years since menopause or smoking. Furthermore the larger

![Figure 1](image-url) Estradiol levels in 631 female and 528 male participants plotted against age, including linear regression line, correlation coefficient and $P$ values.

### Table 2

Characteristics of 631 postmenopausal women and 528 men from the Rotterdam Study by genotype for $ESR1$ haplotype 1 (T-A). Values are expressed as mean (s.e.).

<table>
<thead>
<tr>
<th>Number of allele copies of $ESR1$ haplotype 1</th>
<th>Women</th>
<th></th>
<th>Men</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (%)</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>$P$</td>
</tr>
<tr>
<td>Number (%)$^1$</td>
<td>145 (23.0%)</td>
<td>319 (50.6%)</td>
<td>167 (26.5%)</td>
<td>—</td>
</tr>
<tr>
<td>Age (years)</td>
<td>71.4 (0.7)</td>
<td>70.6 (0.5)</td>
<td>70.3 (0.7)</td>
<td>0.5$^5$</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>26.8 (0.3)</td>
<td>26.8 (0.2)</td>
<td>26.7 (0.3)</td>
<td>0.9$^2$</td>
</tr>
<tr>
<td>Age at menopause (years)</td>
<td>48.5 (0.4)</td>
<td>48.7 (0.3)</td>
<td>49.4 (0.4)</td>
<td>0.1$^3$</td>
</tr>
<tr>
<td>Years since menopause</td>
<td>22.9 (0.8)</td>
<td>21.9 (0.6)</td>
<td>20.9 (0.8)</td>
<td>0.07$^3$</td>
</tr>
<tr>
<td>Smoking</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.9$^4$</td>
</tr>
<tr>
<td>Current</td>
<td>19.4%</td>
<td>19.8%</td>
<td>21.3%</td>
<td>—</td>
</tr>
<tr>
<td>Past</td>
<td>27.8%</td>
<td>27.4%</td>
<td>23.8%</td>
<td>—</td>
</tr>
<tr>
<td>Time of blood draw (h min)</td>
<td>11.31 (0.11)</td>
<td>11.21 (0.08)</td>
<td>11.50 (0.10)</td>
<td>0.1</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Crude</td>
<td>1.32 (0.06)</td>
<td>1.38 (0.04)</td>
<td>1.36 (0.06)</td>
<td>0.7$^2$</td>
</tr>
<tr>
<td>Adjusted$^6$</td>
<td>1.35 (0.06)</td>
<td>1.40 (0.04)</td>
<td>1.38 (0.06)</td>
<td>0.8$^8$</td>
</tr>
<tr>
<td>$E_2$ (pmol/l)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Crude</td>
<td>17.45 (1.24)</td>
<td>15.17 (0.83)</td>
<td>14.05 (1.15)</td>
<td>0.05$^3$</td>
</tr>
<tr>
<td>Adjusted$^6$</td>
<td>17.80 (1.23)</td>
<td>15.25 (0.85)</td>
<td>13.87 (1.16)</td>
<td>0.02$^3$</td>
</tr>
<tr>
<td>Adjusted$^6$</td>
<td>17.89 (1.14)</td>
<td>14.97 (0.79)</td>
<td>13.73 (1.08)</td>
<td>0.008$^3$</td>
</tr>
</tbody>
</table>

$^1$ P value for Hardy–Weinberg equilibrium is 0.8 for women and 0.7 for men.

$^2$ ANOVA.

$^3$ Linear regression.

$^4$ Chi-square.

$^5$ Adjusted for age, BMI, smoking (and years since menopause in women).

$^6$ Adjusted for age, BMI, smoking, testosterone (and years since menopause in women).
Discussion

The present population-based association study demonstrates that in postmenopausal women ESR1 PvuII-XbaI haplotype 1 is associated with decreased plasma E2 levels in an allele-dose-dependent manner. Homozygous carriers of haplotype 1 constitute approximately one quarter of the population and have 3.93 pmol/l lower E2 levels as compared with non-carriers. This is a 22% reduction in E2 level.

Our a priori hypothesis for studying the association between ESR1 gene polymorphisms and E2 levels was driven by recent evidence that ESR1 can modulate CYP19 expression in breast cancer cells (5). The authors of that experimental study found that in human breast cancer cells, E2 up-regulates aromatase gene expression via ESR1. We hypothesized that ESR1 gene polymorphisms may modulate the effect of E2 on CYP19 expression. Indeed, the ESR1 gene polymorphisms we studied were associated with E2 levels. However, aromatase not only catalyzes the conversion of testosterone to E2, but also the conversion of androstenedione to E1. If CYP19 expression is modulated by ESR1 polymorphisms then we would expect plasma E1 levels to be influenced in parallel with levels of E2. However, carriers of haplotype 1 did not have lower E1 levels. In fact, we observed a non-significant trend in the opposite direction; carriers of haplotype 1 had higher E1 levels. This led us to hypothesize that it is not CYP19 that is influenced by these polymorphisms, but one of the 17β-hydroxysteroid dehydrogenase (17β-HSD) subtypes. 17β-HSD subtypes 1 and 7, which have been detected in a number of human non-gonadal tissues, selectively catalyze the transformation of E1 into E2 (23). Although regulation of 17β-HSD by E2 or ESR1 has not been shown so far, our results do suggest a role for ESR1 in 17β-HSD expression or activity. Future research will be necessary to show if this hypothesis is true.

How do these specific polymorphisms influence ESR1 gene expression and consequently plasma E2 levels? The PvuII and XbaI polymorphisms have been an important area of research in diseases such as osteoporosis (7–9), cardiovascular disease (11) and cancer (13). A number of hypotheses for the functional significance of these polymorphisms have been reported in the literature. Given their location, 397 and 351 bp upstream from the start of exon 2, possible functional mechanisms include changed ESR1 expression via altered binding of transcription factors and influence on alternative splicing of the ESR1 gene. Both these mechanisms can be a direct result of either of these polymorphisms or through linkage disequilibrium with a truly functional, but so far unknown, sequence variation elsewhere in the ESR1 gene. The first mechanism was recently supported by findings of Herrington et al. (24) and was

Table 3 Characteristics of 446 postmenopausal women and 362 men from the Rotterdam Study by genotype for ESR1 haplotype 1 (T-A). Values are expressed as mean (S.E.).

<table>
<thead>
<tr>
<th>Number of allele copies of ESR1 haplotype</th>
<th>Women</th>
<th></th>
<th></th>
<th></th>
<th>Men</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>P</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Number (%)1</td>
<td>105 (23.5%)</td>
<td>225 (50.4%)</td>
<td>116 (26.0%)</td>
<td>—</td>
<td>81 (22.4%)</td>
<td>181 (50.0%)</td>
<td>100 (27.6%)</td>
</tr>
<tr>
<td>DHEAs (μmol/l)2</td>
<td>3.17 (0.20)</td>
<td>2.78 (0.14)</td>
<td>2.94 (0.19)</td>
<td>0.5</td>
<td>4.76 (0.34)</td>
<td>4.76 (0.26)</td>
<td>5.05 (0.32)</td>
</tr>
<tr>
<td>Androstenedione (nmol/l)3</td>
<td>3.64 (0.17)</td>
<td>3.57 (0.12)</td>
<td>3.14 (0.16)</td>
<td>0.3</td>
<td>3.85 (0.20)</td>
<td>4.05 (0.15)</td>
<td>4.21 (0.18)</td>
</tr>
<tr>
<td>E1 (pmol/l)4</td>
<td>39.01 (3.19)</td>
<td>42.62 (2.18)</td>
<td>42.34 (3.04)</td>
<td>0.5</td>
<td>89.35 (4.95)</td>
<td>85.83 (3.31)</td>
<td>89.71 (4.46)</td>
</tr>
<tr>
<td>Adjusted5</td>
<td>41.02 (3.17)</td>
<td>43.30 (2.19)</td>
<td>44.15 (3.03)</td>
<td>0.5</td>
<td>87.43 (5.18)</td>
<td>83.37 (3.94)</td>
<td>87.54 (4.80)</td>
</tr>
<tr>
<td>Testosterone (nmol/l)6</td>
<td>1.25 (0.06)</td>
<td>1.34 (0.04)</td>
<td>1.26 (0.06)</td>
<td>0.9</td>
<td>11.62 (0.44)</td>
<td>11.00 (0.34)</td>
<td>11.49 (0.41)</td>
</tr>
<tr>
<td>Free testosterone (nmol/l)4</td>
<td>0.025 (0.001)</td>
<td>0.026 (0.001)</td>
<td>0.025 (0.001)</td>
<td>0.8</td>
<td>0.264 (0.010)</td>
<td>0.251 (0.008)</td>
<td>0.258 (0.009)</td>
</tr>
<tr>
<td>E2 (pmol/l)5</td>
<td>17.67 (1.39)</td>
<td>15.20 (0.95)</td>
<td>14.87 (1.32)</td>
<td>0.1</td>
<td>45.08 (2.77)</td>
<td>47.76 (1.85)</td>
<td>50.38 (2.49)</td>
</tr>
<tr>
<td>Adjusted6</td>
<td>17.94 (1.38)</td>
<td>15.12 (0.95)</td>
<td>14.51 (1.32)</td>
<td>0.07</td>
<td>47.22 (2.94)</td>
<td>49.63 (2.24)</td>
<td>52.41 (2.73)</td>
</tr>
<tr>
<td>Free E2 (pmol/l)5</td>
<td>0.46 (0.03)</td>
<td>0.36 (0.02)</td>
<td>0.36 (0.03)</td>
<td>0.02</td>
<td>1.28 (0.07)</td>
<td>1.40 (0.05)</td>
<td>1.43 (0.07)</td>
</tr>
</tbody>
</table>

1 P value for Hardy–Weinberg equilibrium is 0.8 for women and 1.0 for men.
2 Linear regression.
3 Adjusted for age, BMI, smoking (and years since menopause in women).
4 Adjusted for age, BMI, smoking, androstenedione levels (and years since menopause in women).
5 Adjusted for age, BMI, smoking, estrone levels (and years since menopause in women).

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confirmed in our own laboratory (L Stolk, SCE Schuit, HAP Pols, JPTM van Leeuwen & AG Uitterlinden, unpublished observations). Herrington et al. showed that the T-allele of the PvuII Restriction Fragment Length Polymorphism (RFLP) eliminates a functional binding site for the transcription factor B-myb. This implies that the presence of this allele may result in lower ESR1 transcription. The present study reports that the PvuII T-allele, represented in haplotype 1, is associated with decreased plasma E2 levels in an allele-dose-dependent manner in postmenopausal women. This suggests that the potentially lower ESR1 expression caused by the PvuII T-allele leads to a lower expression of an enzyme in the estrogen synthesis pathway, such as 17β-HSD, and, subsequently, reduced E2 synthesis. These findings are further supported by the observation in our study population, as well as in others, that this T-allele of the PvuII polymorphism is associated with increased risk of osteoporosis (9, 10) and myocardial infarction (11), decreased risk of osteoarthritis (22) and hysterectomy (16), lower BMI (25), shorter stature (21), and later age at menopause (16). These phenotypes are known to be related to decreased E2 effects. The fact that the XbaI polymorphism A-allele is also associated with E2 levels may be due to linkage disequilibrium with the PvuII SNP or another functional polymorphism, or to functional significance of the XbaI polymorphism itself.

We observed the association between ESR1 polymorphisms and E2 levels only in postmenopausal women and not in men. A number of explanations arise. First, in contrast to women, men do not experience a cessation of gonadal function similar to menopause. Elderly men still have a largely intact hypothalamic–pituitary–gonadal axis and E2 still plays a role in the regulation of gonadotropin release. Therefore, in men the influence of ESR1 polymorphisms on enzymes involved in the biosynthesis of E2 may be less important. Secondly, in men E2 levels are three times higher than in postmenopausal women; therefore, levels of ligand for the ESR1 may be sufficiently high so that differences in ESR1 expression may not influence feed-back to other genes. Thirdly, the plasma levels of the direct precursors to E2 in the biosynthesis pathway are a great deal higher in men than in postmenopausal women of the same age, as shown in Table 3. Perhaps, in the presence of larger amounts of E1 precursors, genotype-dependent differences in 17β-HSD expression may not lead to changes in E2 levels. Finally, in men testosterone is the main precursor to E2 and the conversion of androstenedione to testosterone is catalyzed by 17β-HSD type 5, which differs from subtypes 1 and 7 that catalyze the conversion of E1 to E2.

In interpreting the clinical implications of these results we must consider two important aspects. First, plasma levels of E2 do not necessarily reflect local tissue levels. The circulating E2 level in postmenopausal women originates in extragonadal sites where it also acts locally. If this peripherally produced E2 escapes local metabolism, it enters the circulation. However, plasma levels of E2 are important in the pathology of a number of diseases. Individual variation in circulation levels of E2 has been shown to influence the risk of diseases such as osteoporosis (2, 3) and breast cancer (4).

Secondly, the variance in E2 levels explained by these ESR1 polymorphisms is 1%. Clearly the impact of this polymorphism on absolute E2 levels is small. However, it is expected that a large number, perhaps hundreds, of genes and ‘low penetrance’ polymorphisms will contribute to individual variation in E2 levels. Each of these polymorphisms will thus explain only a small fraction. For example, polymorphisms in genes encoding other enzymes in the E2 synthesis pathway will probably contribute to individual E2 levels. Recently, Dunning et al. showed that a polymorphism in the CYP19 gene encoding aromatase also influences circulating E2 levels in postmenopausal women (26). Furthermore, interactions between these polymorphisms and environmental factors are also likely to be important. Although the contribution of these ESR1 polymorphisms to the variance in E2 levels may be low, its impact may be much greater. Average E2 levels for the extreme genotypes are above and below 15.5 pmol/l. Our research group has recently shown that individuals with E2 below this ‘cut-off’ value have an increased risk of osteoporosis (3). These findings suggest that the genotype-dependent differences in E2 levels created by these polymorphisms may be clinically significant.

The only other study showing an association between ESR1 gene polymorphisms and sex hormone levels is that of Zofkova et al. (27), who found an association with increased androstenedione levels. These authors found that the PvuII and XbaI polymorphism were not significantly associated with E2 levels in a study of 114 postmenopausal women of Czech origin; however, the PvuII T-allele and XbaI A-allele did show a trend for lower E2 levels. Perhaps the power of their study was insufficient to show a significant association. We were not able to replicate their results on increased androstenedione levels in our larger study.

There are limitations to genetic association studies. First, they can be influenced by population stratification or heterogeneity. This is especially true for case-control studies in a population of mixed racial origin. However, for our study a population-based cohort study design was chosen and all subjects were of Dutch Caucasian origin and had similar social backgrounds. Furthermore, the PvuII-XbaI genotypes were in Hardy–Weinberg equilibrium and haplotype frequencies were similar to those found in studies of other Caucasian subjects (28). Therefore, our study population may be considered ethnically homogeneous and representative of the Dutch population. However, to be included in our
study, participants had to be mobile enough to visit the research center to donate a blood sample. This will have led to a health selection bias, as observed in the finding that our subjects are somewhat younger than the entire Rotterdam Study cohort. Such a healthy responder bias will presumably not be genotype-dependent and we believe it will not have led to bias in our results. Another limitation of association studies is the definition of the phenotype of interest. We used an E\textsubscript{2} assay with an very low detection limit (4.8 pmol/l), and we excluded all participants with medication, such as HRT and corticosteroids, that could have influenced the hormone levels measured. However, due to the small volumes of plasma available, we were not able to run the assay in duplicate. Although the single-sample measurement will have led to less precise estimations of plasma levels, this will only have led us to underestimate the strength of the association.

In conclusion, this population-based study shows a significant reduction in circulating E\textsubscript{2} levels in carriers of ESR1 PvuII-XbaI haplotype 1 (‘T-A’) in an allele-dose-dependent manner in postmenopausal women. The association was not explained or influenced by a number of known confounders such as age, years since menopause, BMI, smoking or levels of the precursor testosterone. From our findings that E\textsubscript{2} levels are, while E\textsubscript{1} levels are not, associated with these ESR1 polymorphisms, we hypothesize that it is likely that these common sequence variants alter 17\beta-HSD expression or activity.

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