Genetics of the Estrogen Signaling Pathway

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Genetic Aspects of the Estrogen Signaling Pathway

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Genetic Aspects of the Estrogen Signaling Pathway

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Contents

PUBLICATIONS AND MANUSCRIPTS BASED ON THE STUDIES DESCRIBED IN THIS THESIS

Chapter 2.1
S.C.E. Schuit, M. van der Klift, A.E.A.M. Weel, C.E.D.H. de Laet, H. Burger, E. Seeman, A. Hofman, A.G. Uitterlinden, J.P.T.M. van Leeuwen, H.A.P. Pols. Fracture incidence and association with bone mineral density in elderly men and women: The Rotterdam Study. *Bone.* 2004;34: 195-202.

Chapter 2.2
J.B.J. van Meurs, S.C.E. Schuit, A.E. Weel, M. van der Klift, A.P. Bergink, P.P. Arp, E.M. Colin, Y. Fang, A. Hofman, C.M. van Duijn, J.P. van Leeuwen, H.A. Pols, A.G. Uitterlinden. Association of 5' estrogen receptor alpha gene polymorphisms with bone mineral density, vertebral bone area and fracture risk. *Human Molecular Genetics* 2003;12:1745-54.

Chapter 2.3
S.C.E. Schuit, J.B.J. van Meurs, A.P. Bergink, M. van der Klift, Y. Fang, G. Leusink, A. Hofman, J.P.T.M. van Leeuwen, A.G. Uitterlinden, H.A.P. Pols. Height in pre- and postmenopausal women is influenced by estrogen receptor alpha gene polymorphisms. *Journal of Clinical Endocrinology & Metabolism.* 2004;89:303-309.

Chapter 3.1
S.C.E. Schuit, H.S. Oei, C.H. J.C.M. Witteman, Geurts van Kessel, J.B.J. van Meurs, R.L. Nijhuis, J.P.T.M. van Leeuwen, F.H. de Jong, M.C. Zillikens, A. Hofman, A.G. Uitterlinden, H.A.P. Pols. Estrogen receptor alpha gene polymorphisms predict myo-cardial infarction risk in Women. *Submitted.*

Chapter 3.2
S.C.E. Schuit, E.J. van Dijk, N.D. Prins, J.B.J. van Meurs, J.P.T.M. van Leeuwen, F.H. de Jong, A. Hofman, H.A.P. Pols, M.M.B. Breteler, A.G. Uitterlinden. Estrogen receptor alpha gene polymorphisms associated with stroke and white matter lesions. *Submitted.*

Chapter 4.1
S.C.E. Schuit, F.H. de Jong, L. Stolk, W.N.H. Koek, J.B.J. van Meurs, M.W.C.J. Schoofs, M.C. Zillikens, A. Hofman, J.P.T.M. van Leeuwen, H.A.P. Pols, A.G. Uitterlinden. Estrogen receptor alpha gene polymorphisms are associated with estradiol levels in postmenopausal women. *Submitted.*

Chapter 4.2
S.C.E. Schuit, A.G. Uitterlinden, W.N.H. Koek, L. Stolk, J.B.J. van Meurs, M.W.C.J. Schoofs, M.C. Zillikens, A. Hofman, J.P.T.M. van Leeuwen, H.A.P. Pols, F.H. de Jong. Aromatase (*CYP19*) gene exon 10 polymorphism and sex-hormone levels. *Submitted.*

Chapter 5.1
S.C.E. Schuit, L. Stolk, H. Jahr, M. Eijken, J.B.J. van Meurs, J.P.T.M. van Leeuwen, H.A.P. Pols, and A.G. Uitterlinden. Functionality of the estrogen receptor alpha gene *Pvu*II polymorphism. *Submitted.*

1 General Introduction

Estradiol, one of the sex hormones responsible for gender dimorphism and reproduction, is a pleitropic hormone with widespread biological actions far beyond human reproduction alone. For example, withdrawal of the effects of estradiol at menopause from non-reproductive tissues like the skeleton, the cardiovascular system, and the brain, is a major risk factor for the development of osteoporosis, coronary artery disease, stroke, and perhaps neurodegenerative diseases like Alzheimer's disease. On the other hand, continuous exposure of reproductive tissues to estrogen during the post reproductive part of life is a risk factor for the development of breast, and uterine cancer.

The naturally occurring estrogens 17β-estradiol (E_2), estrone (E_1), and estriol (E_3) are derived from cholesterol. Different steroids are formed by the reduction of the number of carbon atoms from 27 to 18 by different cytochrome P450 enzymes. Although estradiol also has local autocrine or paracrine actions the majority of estradiol is released into the circulation where 98% is reversibly bound to sex-hormone-binding globulin and with less affinity to albumin. Estradiol can freely diffuse across the plasma and nuclear membranes of all cells, but it is sequestered only within cells that contain estrogen receptors (ER). As free estradiol diffuses into the cell, it binds to the ligand-binding domain of the receptor; the complex of estradiol and ER then diffuses into the cell nucleus. This complex, in association with other coactivators or repressor proteins, can alter the subsequent expression of a variety of relevant target genes. In particular, the estradiol-ER complexes bind to specific sequences of DNA called estrogen-response elements (ERE) as homo- or heterodimers. These ERE DNA sequences function as enhancers, conferring estradiol-inducibility on the genes (figure 1).

There are five broad classes of human proteins relevant to estradiol production and action. These include proteins involved in the neuroendocrine regulation of sex-hormone production (i.e., GnRH, LH and FSH); proteins involved in the synthesis of estradiol (i.e., the P450 enzymes); the estrogen receptors α and β; proteins involved in the downstream pathways of estrogen action (i.e., COMT); and proteins involved in drug absorption, transport, and metabolism. In this thesis, we have chosen two candidate genes in this pathway to study in relation to disease risk;
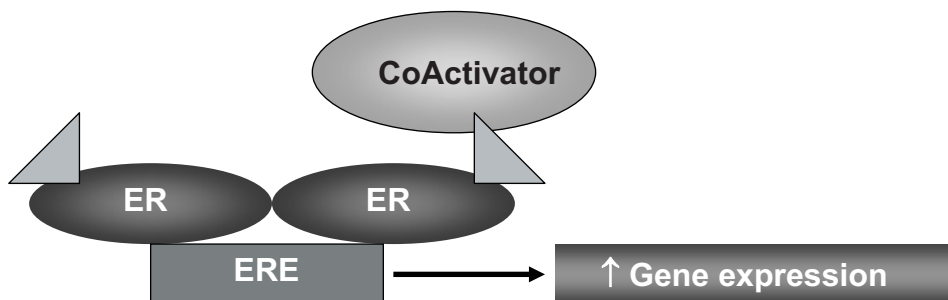
Figure 1.
Estrogen receptor (ER) activation and estrogen-response (ERE) element binding

the estrogen receptor α gene (ERα, also known as *ESR1*) and the gene encoding the P450 enzyme aromatase (*CYP19*).

Current generation of estradiol deficiency models in mice with targeted disruption of the aromatase or the estrogen receptor (ER) gene provides insight into the role of estradiol in female and male reproductive and non-reproductive physiology. The majority of studies of estradiol deficiency have focused on mice lacking the classical ERα, the ERα knockout mice (αERKO). Recently, a second estrogen receptor, ERβ, has been identified.[1,2] The importance of ERβ in humans is still largely unknown. The development of ERβ knockout mouse models (βERKO) is leading to a better understanding of the different effects these two receptors have in a number of tissues. Years of research using the ERKO mice as a research tool to investigate specific pathophysiological consequences of estradiol deficiency has led to a large body of literature (for an extensive review see reference 3).

The main findings of the ERα and ERβ knockout mouse models are summarized in table 1. It appears that ERα and ERβ are both very important in the central nervous system, bone, lung, urogenital tract, cardiovascular system, ovary, testis, kidney and colon. The physiological function of ERβ is under intense study but some results indicate that ERα and ERβ have different or even opposite biological actions.

Through an experiment of nature, medical science has discovered just how widespread the effects of estradiol are in diverse human functions. In the last decade nine men and women have been reported with mutations in the aromatase (*CYP19*) gene leading to estradiol deficiency and one man has been reported with complete estradiol resistance due to a recessive mutation in the ERα gene.[4] These observations stimulated research ranging from integrative physiology to cell and molecular biology within and outside the reproductive system. Human and mouse estradiol deficiency models have many similarities, indicating once again that mouse knockout models are an excellent tool to investigate the role of certain genes in human diseases. In young females, estradiol deficiency gives rise to ambiguous genitalia and a failure of secondary sexual characteristics to develop, while in males a normal male sexual differentiation and pubertal development is seen. The involvement of estradiol in non-reproductive tissues such as bone and the cardiovascular system has led to the basis of this thesis. The absence of estradiol signaling in bone has striking consequences in both men and women, resulting in osteoporosis as well as continued linear bone growth due to non-closure of the epiphyses. Furthermore, estradiol deficiency or resistance also leads to premature atherosclerotic coronary artery disease, endothelial dysfunction, and an abnormally low HDL/LDL cholesterol ratio. Given the striking consequences of complete estradiol deficiency or resistance on the human body, one can also envision more subtle and frequent mutations (i.e., polymorphisms) in genes involved in the estradiol signaling pathway potentially leading to increased risk of age-related diseases such as osteoporosis and cardiovascular disease in the general population.

Table 1. Summary of reported phenotypes in the αERKO and βERKO mouse models (Partially adapted from Couse et al.[3])

		References
General	Neither αERKO nor βERKO is lethal.	8-10
Fertility		
αERKO	Both sexes are infertile.	9
βERKO	Female is subfertile (reduced litter size); male is fertile.	8
Female reproductive tract		
αERKO	Tract undergoes normal pre- and neonatal development, but is insensitive to estradiol, DES, and hydroxy tamoxifen during adulthood.	9, 11, 12
	Presence of non-ERα or -ERβ receptor-mediated estrogenic pathway for 4-OH-estradiol and methoxychlor.	13, 14
	Loss of mitogenic actions of epidermal growth factor (EGF).	15
	Responsive to mitogenic actions of androgens.	16
	Responsive to progesterone and able to undergo artificially induced decidualization.	17
	Ovaries undergo normal pre- and neonatal development, but are anovulatory during adulthood, exhibit multiple hemorrhagic cysts, and no corpora lutea.	9, 18
	30–40% incidence of ovarian tumors by 18 months of age.	3
βERKO	Tract undergoes normal pre- and neonatal development and appears sensitive to ovarian estrogen cycling during adulthood.	8
	Ovaries undergo normal pre- and neonatal development, but do not exhibit normal frequency of spontaneous ovulations during adulthood, exhibit a severely attenuated response to superovulation treatment with reduced numbers of oocytes, follicular arrest and anovulation.	8
Mammary gland		
αERKO	Undergoes normal prenatal development but is insensitive to E_2-induced development during puberty and adulthood.	19
	Responsive to exogenous progesterone and prolactin.	20
	Susceptible to proto-oncogene (Wnt-1) induced ductal hyperplasia and lobuloalveolar adenocarcinoma but tumors exhibit a delayed growth rate compared to those in wild-type.	21

Mammary gland cont.

| βERKO | Undergoes normal prenatal and pubertal development, virgin gland is grossly indistinguishable from that of age-matched wild-type. | 8 |
| | Undergoes normal differentiation and lactation during pregnancy and motherhood. | 8 |

Male reproductive tract

αERKO	Tract undergoes normal pre- and neonatal development.	22, 23
	Age-related phenotype of attenuated fluid resorption in efferent ducts leads to dilation of rete testis, atrophy of the seminiferous epithelium, and decreasing sperm counts.	22, 24
	Disrupted sperm function illustrated by an inability to fertilize.	22
	Age-related decrease in testis weight.	3
	Age-related increase in seminal vesicle weight.	3
βERKO	Undergoes normal pre- and neonatal development without apparent defects in spermatogenesis that impede fertility.	8

Neuroendocrine system: females

αERKO	Anterior pituitary possesses all of the expected cell types, but exhibits elevated transcript levels for the gonadotropin subunits (gonadotropin α subunit, LH-β, FSH-β).	25
	Elevated serum levels of estradiol, testosterone, and LH, but normal serum levels of progesterone and FSH.	11, 26
	Normal lactotroph differentiation and number in the anterior pituitary, but exhibits significant deficits in transcription of the PRL gene and serum PRL levels.	3, 25
	Medial preoptic region of the hypothalamus exhibits elevated levels of PR transcripts which decreased after ovariectomy and return after estradiol treatment.	27, 28
	Rapid actions of estradiol on hippocampal neurons are preserved.	29
βERKO	Normal serum levels of estradiol.	3

Neuroendocrine system: males

| αERKO | Anterior pituitary possesses all the expected cell types, but exhibits elevated levels of LH-β transcripts. | 25, 30 |
| | Elevated serum levels of estradiol, testosterone, and LH, but normal serum levels of progesterone and FSH. | 3, 30 |

Behavior: females

αERKO	Exhibit a lack of estradiol and progesterone-induced sexual behavior, increased aggression, and infanticide.	31-33
βERKO	Exhibit no defects in sexual behavior that impede fertility.	8

Behavior: males

αERKO	Exhibit normal mounting and attraction toward wild-type females but a complete lack of intromission and ejaculation; display reduced aggression.	34-36
βERKO	Exhibit no defects in sexual behavior that impede fertility.	8

Bone

αERKO	Decrease in femoral bone length and diameter.	37
	Decreased femur bone mineral density (BMD).	37-39
βERKO	Males: no major bone abnormalities.	40
	Females: slightly elevated BMD in adults.	40

Cognition

αERKO	Impaired activity in cognitive function tests.	41
βERKO	Normal cognition	41

Cardiovascular

αERKO	Exhibit reduced estradiol-induced angiogenesis and reduced basal levels of vascular nitric oxide	42
	Exhibit wild-type response to estradiol in the carotid artery injury model	43
	Exhibit increased expression of L-type Ca^{2+} channels	44
	Exhibit reduced response to estrogen-induced increases in serum apolipoprotein E	45
	The protective actions of estradiol in preventing stroke are abolished in αERKO mice, but not in βERKO mice.	46

Adipose tissue

αERKO	Significant accumulation of white adipose tissue, which is characterized by an increase in adipocyte size and number.	47, 48
βERKO	Normal body weight and adipose tissue	47

Other

αERKO	Impaired glucose tolerance and insulin resistance	48, 49
	No estradiol induced reduction of B lymphopoietic cells.	50
βERKO	No estradiol induced reduction of B lymphopoietic cells.	50

1.1 Dissection of Complex Traits

To find disease-causing polymorphisms in genes involved in complex diseases a number of approaches can be used. Broadly defined these include genome searches by linkage analysis and candidate gene studies by association analysis. The first method, the so-called "top-down" approach, uses linkage disequilibrium to identify areas of the genome that may carry a gene that influences a phenotype of interest. For such a genome search sibling pairs, a set of family pedigrees, or an isolated population are studied since areas of linkage disequilibrium in the genome are much larger in genetically more closely related individuals than in an out-bred population. This makes it easier to identify regions in the genome that are associated with the phenotype of interest. Once linkage studies, such as the one for height discussed in chapter 2.2 of this thesis, have identified a region of potential linkage, the next step is fine-mapping of the region through the evaluation of candidate gene loci. Hundreds of genes may lie within the area identified by linkage analysis. In association analysis one or more candidate genes are chosen that are potentially the most interesting. Within the candidate gene, polymorphisms are tested for association with the phenotype of interest.

The above described "top-down" method is time-consuming and often not feasible due to the high costs of such genome searches and the necessity of finding and testing sibling pairs, family pedigrees or an isolated population. Furthermore, genome searches often yield false-positive and false-negative results mainly due to the large amount of statistical power such a linkage study needs to identify subtle disease-causing loci. Therefore, the so-called "bottom-up" approach has become the preferred method of research in a growing number of studies of complex diseases. This method builds on known biology and entails choosing candidate genes based on the current knowledge about the pathophysiology of the disease or phenotype of interest. Given the phenotypes described in human and mouse estradiol knock-out models, the genes within the estradiol endocrine pathway are potentially very interesting in bone and cardiovascular research and this thesis focuses on candidate genes within this pathway using the "bottom-up" approach.

1.2 Candidate Genes Approach

Within a candidate gene, potentially interesting polymorphisms are identified and tested for association with the phenotype of interest, preferentially in a large population-based cohort study such as the Rotterdam Study.[5] By sequencing the entire candidate gene, including promoters, introns and other untranslated regions in for example one-hundred individuals, a complete inventory can be made of the polymorphisms present in that gene. From direct sequencing of the vitamin D receptor (VDR) gene performed in our laboratory we have calculated that approximately one in 300 base pairs is polymorphic (Y. Fang, personal communication). That means that in a gene spanning almost 300 thousand base pairs (300 kb), such as the ERα gene, approximately 1000 polymorphisms will be present. From these

polymorphisms the most interesting ones are chosen based on their potential functional significance to the encoded protein. The most promising polymorphisms are those that lead to a change of an amino acid in the encoded protein, i.e., the non-synonymous polymorphisms. Other potentially interesting polymorphisms are located in the promoter regions and the 3-prime untranslated region (3'UTR), since these may modify mRNA expression or stability. In addition, polymorphisms that change potential transcription factor binding sites are also interesting since they may increase or decrease the rate of gene transcription. Although the list of polymorphisms identified by sequencing of the entire gene usually leads to an exhaustive list of polymorphisms, the method is costly and very time-consuming. By consulting polymorphism databases such as Celera (www.celera.com) and NCBI (www.ncbi.nlm.nih.gov), and literature databases such as PubMed (www.ncbi.nlm.nih.gov), an inventory of polymorphisms in a candidate gene can be made within a day. The latter method was used to identify polymorphisms in this thesis.

1.3 Candidate Genes in the Estrogen Endocrine Pathway

The estrogen receptor is essential in de mediation of estradiol's effects in diverse tissues. The ERα gene is the most well studied of the two estrogen receptors identified. The ERα gene, located on chromosome 6q25, is the first candidate gene discussed in this thesis. It is a large gene, spanning almost 300 kb of which only 1791 base pairs are transcribed to the actual ERα protein. The rest of the gene is made up of an extensive promoter area of 150 kb, other regulatory regions such as the 3' UTR (untranslated region) and introns (Figure 2).

Using the above mentioned polymorphism and literature databases we were able to identify a large number of single nucleotide polymorphisms (SNPs) and variable number tandem repeats (VNTR) in the ERα gene (Figure 3). We preferentially chose polymorphisms with frequency data available, either from the literature or from the polymorphism databases, and with a frequency of 5% or more in Caucasians. We focused on non-synonymous polymorphisms and polymorphisms located in the promoter regions and the 3'UTR. An exception was made for two intronic polymorphisms known from the osteoporosis literature.[6] In total thirteen polymorphisms in the ERα gene were chosen to genotype for our studies (Figure 4). Three polymorphisms were identified via public literature databases and were
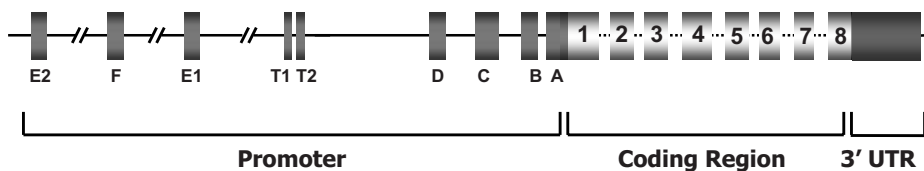
Figure 2.
Estrogen receptor α (ERα) gene (6q25)

chosen based on previously reported associations in Caucasians. These were the TA-repeat VNTR at -1355 from the start of transcription and two intronic SNPs, identified by the restriction enzymes *Pvu*II and *Xba*I, located at -397 and -351 from the start of exon 2. Ten polymorphisms were chosen from the large number of polymorphisms identified by consulting polymorphism databases. One of these polymorphisms was non-synonymous. None of the SNPs reported in the 3'UTR
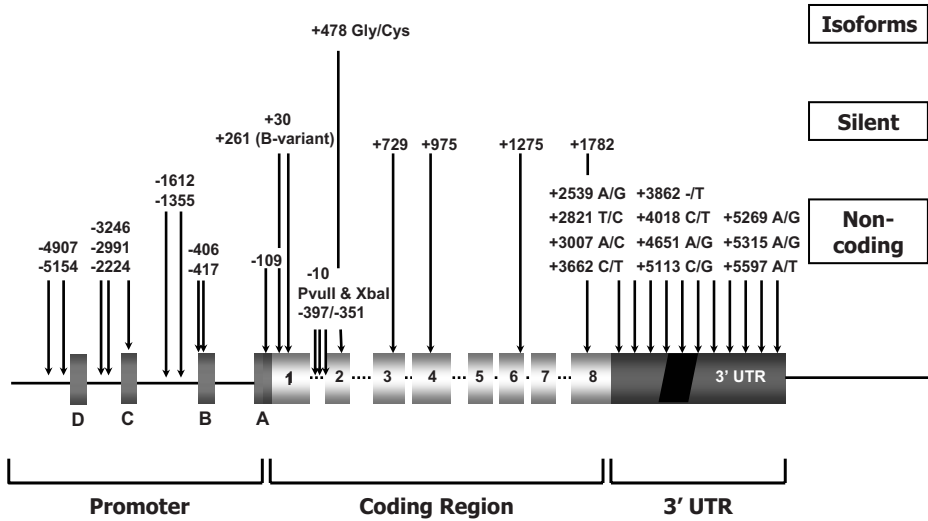
Figure 3.
Polymorphisms identified in the ERα gene
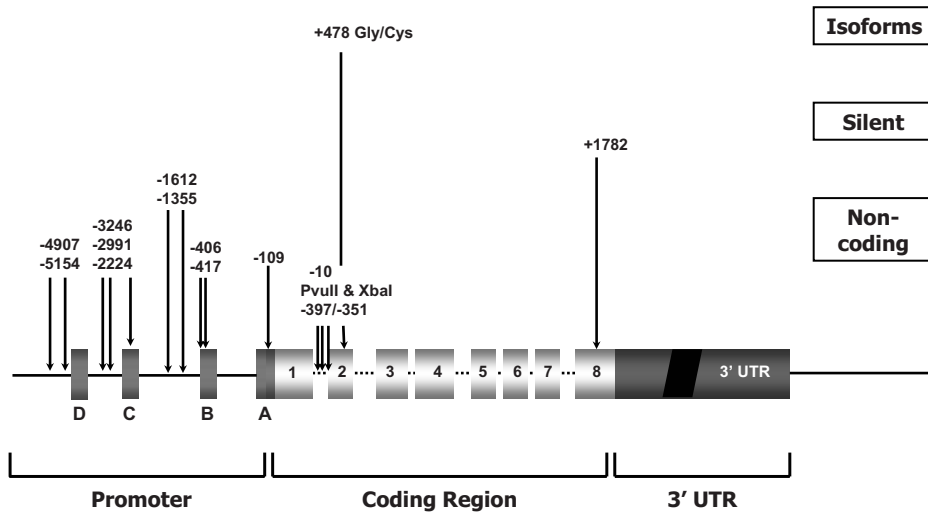
Figure 4.
Polymorphisms chosen for analysis in the ERα gene

none had frequency data available. A synonymous polymorphism in exon 8 was proven polymorphic in Caucasians and since it is very likely that this polymorphism is in linkage disequilibrium with polymorphisms in the 3'UTR we chose to genotype this polymorphism instead. The remaining eight polymorphisms were chosen because of their location in the promoter regions or their ability to change a potential transcription factor binding site. Of the polymorphisms genotyped in a pilot sample of 300 participants, five polymorphisms had frequencies above 5%. These were the three previously known polymorphisms (TA-repeat, *Pvu*II and *Xba*I), one of the promoter polymorphisms (-2224) and the exon 8 polymorphism (+1782). These five polymorphisms were genotyped in a set of 3000 randomly chosen individuals from the Rotterdam Study (38% of total cohort) and association analyses were performed. If an association was observed in the random set of 3000 that polymorphism was genotyped. Of the five polymorphisms genotyped in the random set, the promoter (-2224) and the exon 8 (+1782) SNPs were not associated with the phenotypes we studied. Therefore, the studies reported in this thesis are based on the TA-repeat (-1355), the *Pvu*II (-397) and the *Xba*I (-351) polymorphisms.

The second candidate gene chosen for this thesis is the aromatase (*CYP19*) gene. Aromatase belongs to the cytochrome 450 enzyme super-family and is responsible for the conversion of androstenedione and testosterone to estrone and estradiol, respectively (Figure 5). This enzyme catalyzes the rate-limiting step in the peripheral conversion of estradiol and is therefore essential to circulating estradiol levels. The *CYP19* gene is located on chromosome 15q21.1 and spans 123 kb including a coding region of 9 exons (exons II-X). Upstream of exon II, a number of alternative first exons are differentially spliced into distinct 5'UTRs (Figure 6). To date, nine different transcriptional start sites are known with individual promoters permitting tissue-specific regulation of expression. Even though each tissue expresses a unique 5'UTR, the coding region and translated product (and therefore also the protein expressed) are identical.
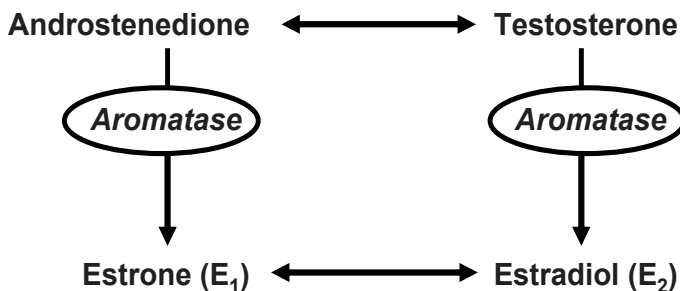
Figure 5.
Conversion of androstenedione and testosterone to estrone and estradiol by aromatase
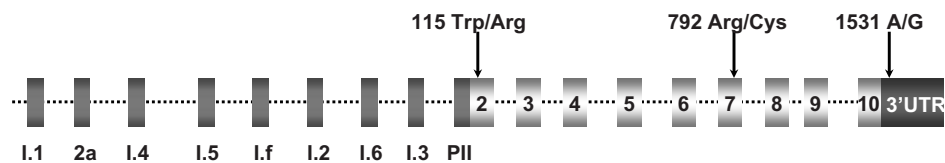
Figure 6.
CYP19 gene encoding aromatase (15q21.1-2) and the polymorphisms chosen for analysis

Similar to our findings for the ERα gene, using literature and polymorphism databases we were able to identify a large number of polymorphisms in the aromatase gene. Three polymorphisms were chosen to test in the random set of 3000 individuals from the Rotterdam Study: two non-synonymous SNPs in exons 1 (+115) and 7 (+792) and one SNP in the 3'UTR (+1531), see figure 6. The exon 1 variation was not found to be polymorphic in our population and we were unable to design primers to genotype the exon 7 SNP. The association with the *CYP19* described in this thesis is based on the polymorphic 3'UTR SNP located at +1531.

1.4 Study Population

The results of the studies presented in this thesis were based on the Rotterdam Study, a prospective population-based cohort study, which was initiated to assess prevalence, incidence, and determinants of diseases in the elderly.[5] The main focus of this study was on cardiovascular disease, neurogeriatric disease, ophthalmologic disease, and locomotor disease. The Rotterdam Study was approved by the medical ethics committee of the Erasmus Medical Center and written informed consent was obtained from all participants. At baseline, between 1990 and 1993, all inhabitants aged 55 years and over (n=10,275) of the district of Ommoord in Rotterdam were invited to take part in the study. A total of 7,983 subjects (response rate 78%), 4878 of whom were women, entered the study. At baseline, and again at the first, second and third follow-up visits (1994-1995, 1997-1999 and 2002-2004, respectively), information was gathered concerning amongst others lifestyle habits, socio-economic status, medical history and pharmacotherapeutic history. In addition to an interview, all subjects were invited to visit our research center for physical examination and blood drawing.

For the entire cohort, information on vital status is obtained continuously from the municipal authorities in Rotterdam. For subjects who moved outside the research area, mortality data are obtained from general practitioners (GPs). GPs in the research area (covering 80% of the cohort) reported all relevant fatal and non-fatal events, such as fractures and myocardial infarctions, through a computerized system. Research physicians verified follow-up information by checking GPs' patients' records. This is possible because in The Netherlands the GP has a gate-

keeper function, which means that the only way to access specialist and hospital care is by consulting a GP. The GP retains all medical information of his patients. For the remaining 20% of the cohort, research physicians regularly visited the GPs and collected data from their records. For hospitalized patients, discharge letters were additionally used for verification. All events were coded independently by two research physicians according to the International Classification of Diseases, 10th revision (ICD-10).[7] If there was disagreement, consensus was reached in a separate session. A medical expert in the field reviewed all coded events for final classification.

1.5 Aim and Description of Chapters

The objective of the studies described in this thesis was to determine the role of polymorphisms in candidate genes within the estrogen signaling pathway in common diseases of the elderly. Chapter 2 focuses on the consequences of ERα polymorphisms on bone pathophysiology. The first article of chapter 2.1 discusses the incidence of fractures in the Rotterdam Study cohort. In chapter 2.2, the association of ERα polymorphisms with BMD and vertebral fractures is discussed. The role of ERα polymorphisms in determining pre- and postmenopausal body height in women is reported in the last article of chapter 2. In chapter 3, the role of ERα polymorphisms in determining the risk of cardiovascular diseases, including myocardial infarction, stroke and cerebral white matter lesions, is reported. The role of ERα and *CYP19* polymorphisms in determining circulating estradiol levels is explored in chapters 4.1 and 4.2. In chapter 5.1, the possible functional significance of the ERα *Pvu*II polymorphisms is reported. Finally, in the general discussion, chapter 6, the main findings of this thesis are placed in perspective.

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2 | Bone

Chapter 2.1

Fracture Incidence and Association with Bone Mineral Density in Elderly Men and Women: The Rotterdam Study

ABSTRACT

The incidence of all non-vertebral fractures, as well as the relation to bone mineral density (BMD), was quantified in 7806 men and women from the Rotterdam Study, a prospective, population-based cohort study of men and women age 55 years and older. In addition, the sensitivity of using a T-score at or below –2.5 for identifying subjects at risk for fractures was assessed.

At baseline, between 1990 and 1993, femoral neck BMD was measurement by dual energy X-ray absorptiometry (DXA). Subsequently, gender-specific T-scores were calculated using the NHANES reference population. During a mean follow-up of 6.8 years, information on incident non-vertebral fractures was gathered.

In general, hip, wrist and upper humerus fractures are the most frequent fractures in both men and women. Femoral neck BMD appears to be an equally important risk factor in both genders, and is especially related to hip fractures. For all non-vertebral fractures, the age adjusted hazard ratio (95% confidence interval) per standard deviation decrease in femoral neck BMD was 1.5 (1.4-1.6) for women and 1.4 (1.2-1.6) for men. For hip fractures the hazard ratios were 2.1 (1.7-2.5) for women and 2.3 (1.6-3.3) for men.

Only 34% of all non-vertebral fractures occurred in women with a T-score below -2.5, in men this percentage was even lower (21%). Thus, there is a clear need for the development of more sensitive risk assessment tools, using not only BMD, but also other clinical predictors of fractures.

Introduction

Osteoporosis is a condition characterized by low bone mineral density (BMD) and micro architectural deterioration of bone, resulting in a loss of bone strength and therefore increased fracture risk. Osteoporotic fractures, the clinical endpoint of osteoporosis, are associated with increased morbidity, mortality and high socio-economic costs.[1,2] Due to increased life expectancy the incidence of fractures is increasing over time, hereby increasing the population burden of fractures.[2]

As early as 1842, Antley Cooper noticed that the incidence of fractures increased with thinning of bone in the elderly.[3] In the 20th century several studies have shown that, independent of age, the likelihood of a fracture increases with decreasing BMD.[4] However, the majority of these studies focused on women only. Therefore, the relationship between BMD and fractures in men is largely unknown. Furthermore, due to limited power most studies focus on the most common osteoporotic fractures such as hip fractures. Therefore, information on the occurrence as well as the relationship to BMD of less common osteoporotic fractures in men and women is scarce.

In clinical practice the definition of osteoporosis is based on the WHO-based T-score of BMD, which expresses BMD as the number of standard deviations (SD) below the average BMD in young adult men and women. In this definition subjects with a T-score at or below –2.5 are considered to have osteoporosis. Similarly, osteopenia is defined as a T-score between -1.0 and -2.5, whereas a T-score above -1.0 is considered normal.[5] These cut-off values were originally intended to assess the prevalence of osteoporosis, and not, as is common practice nowadays, to be used as a treatment threshold. Currently, there is ongoing debate about the strengths and limitations of bone densiometry in clinical practice.

The aim of our study was to investigate the incidence of the common osteoporotic fractures as well as less common fractures in both men and women in the Rotterdam Study, a large population based study of diseases in the elderly. Furthermore, the association between femoral neck BMD and these fractures is compared between men and women. And finally, the sensitivity of using a T-score at or below -2.5 in order to identify subjects who will eventually sustain a fracture is studied.

Methods

Study population

The Rotterdam Study is a prospective population-based cohort study of men and women aged 55 and over. The objective is to investigate the incidence of and risk factors for chronic disabling diseases. Both the rationale and the study design have been described previously.[6] The focus of the Rotterdam Study is on neurological, cardiovascular, ophthalmologic and locomotor diseases. The Medical Ethics

Committee of the Erasmus Medical Centre has approved the Rotterdam Study. All 10,275 inhabitants of Ommoord, a district in Rotterdam, the Netherlands, were invited to participate. Of these, 7,983 (4,878 women) participated in the Rotterdam Study (resulting in a response rate of 78%). For various reasons informed consent for follow-up registration could not be obtained for 177 (2.2%) participants and these individuals were therefore excluded from the analyses, leaving 7806 men and women who were included in our analyses. For femoral neck BMD, analyses were restricted to the 5794 (74.2%) participants who were able to visit the study center for a BMD measurement at baseline.

Clinical examination

Between 1990 and 1993, participants were invited to come to the research centre for clinical examination. BMD measurement of the femoral neck was performed by dual energy X-ray absorptiometry (DXA) (Lunar DPX-L densitometer, Madison, Wisconsin, USA) as described previously.[7]

Follow-up procedures

The present analysis is based on follow-up data collected from baseline (1990-1993) until December 31st 1999. Follow-up time was calculated as time from baseline to first fracture, death or end of the follow-up period, which ever occurred first. The average follow-up time was 6.8 (SD 2.3) years. All events were reported either by general practitioners (GPs) in the research area by means of a computerized system (80% of the cohort) or through hospital records. Information from GPs 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 events were reviewed by a medical expert for final classification. When studying the association between BMD and incident fractures, only non-vertebral fractures were considered.

Statistics

All analyses were performed for the total group and for men and women separately. Fracture incidence rates were calculated according to fracture site and subdivided in three main categories: upper extremity, lower extremity and other fractures. The incidence rates were expressed as numbers of fractures per 1,000 person-years and the 95 percent confidence intervals were calculated using the exact Poisson formula. The most frequent fractures were also studied in 5-year age groups. To determine which specific types of fracture were associated with low BMD, we estimated the relative risk for a first fracture associated with one standard deviation (SD) decrease in femoral neck BMD using a Cox proportional hazards model. For these analyses we used separate femoral neck BMD standard deviation for men and women (0.134 and 0.132 g/cm², respectively). Since multiple

fractures do not contribute to independent observations these analyses were based on the first fracture in each individuals. To account for confounding by age, gender-specific age adjustments were made by including age as a continuous variable in all models.

Gender-specific T-scores were calculated from the femoral neck BMD using the NHANES reference population of Caucasian males and females aged 20 to 29 years.[8] Peak bone mass, as converted to the corresponding Lunar value, was 1.04 g/cm^2 (SD 0.14) for women and 1.13 g/cm^2 (SD 0.16) for men. The absolute BMD cut-off values for osteoporosis (T-score below -2.5) and osteopenia (T-score between -1.0 and -2.5) were 0.69 g/cm^2 and 0.90 g/cm^2 in women and 0.72 g/cm^2 and 0.96 g/cm^2 in men, respectively. Hazard ratios for the risk of incident non-vertebral fractures in subjects with osteoporosis and osteopenia were calculated using a Cox' proportional hazards model. Subjects with a normal BMD (T-score ≥ -1.0) were defined as the reference group.

SPSS 11.0 was used for all analyses.

Results

Follow-up was completed for 7806 individuals (3075 men) after a mean follow-up time of 6.8 years (SD 2.3 years, range 1 day to 10.5 years). BMD measurements were available for 5794 individuals (2437 men). Women were on average 2.4 years older and had 0.07 g/cm^2 lower mean femoral neck BMD.

Incidence of non-vertebral fractures

During follow-up 939 (12.0%) participants sustained at least one incident non-vertebral fracture. Table 1 shows the fracture incidence rates by site and gender. Overall, the incidence of non-vertebral fractures was 9.6 (95% confidence interval 8.3-11.0) per 1,000 person-years in men and 25.0 (95% confidence interval 23.3-26.9) per 1,000 person-years in women. For all fracture sites, women had a higher incidence rate and overall the age-adjusted incidence was 2.3 (95% confidence interval 2.0-2.7) times higher in women than in men. In both men and women, the predominant fracture sites were the wrist, upper humerus and hip.

Figure 1 shows the age-related incidence of all non-vertebral, wrist, upper humerus and hip fractures in men and women. In both men and women, the incidence of hip fracture increased exponentially with age. In women, a moderate increase with age was observed for upper humerus fractures, whereas the incidence of wrist fractures increased markedly from the age of 55 years onward and stabilized after 70 years of age. In men, upper humerus fractures appeared to show only a slight increase with age, while wrist fracture incidence showed the largest increase after 75 years of age.

Table 1. Gender and site specific incidence rates of first non-vertebral fractures per 1000 person years in The Rotterdam Study

Type of Fracture	Men			Women			Total		
	Number	Incidence Rate	95% CI	Number	Incidence Rate	95% CI	Number	Incidence Rate	95% CI
Upper extremities									
Upper humerus	17	0.8	0.5-1.3	83	2.6	2.1-3.2	100	1.9	1.6-2.3
Wrist	24	1.2	0.8-1.8	232	7.3	6.4-8.3	256	4.9	4.4-5.6
Hand	23	1.1	0.8-1.7	57	1.8	1.4-2.3	80	1.5	1.2-1.9
Other upper arm [a]	15	0.7	0.4-1.2	36	1.1	0.8-1.5	51	1.0	0.7-1.3
Other lower arm [b]	3	0.1	0.0-0.5	24	0.7	0.5-1.1	27	0.5	0.4-0.7
Lower extremities									
Pelvis	8	0.4	0.2-0.8	40	1.2	0.9-1.7	48	0.9	0.7-1.2
Hip	61	3.0	2.3-3.9	221	6.9	6.1-7.9	282	5.4	4.8-6.1
Ankle	7	0.3	0.2-0.7	35	1.1	0.8-1.5	42	0.8	0.6-1.1
Metatarsal	7	0.3	0.2-0.7	35	1.1	0.8-1.5	42	0.8	0.6-1.1
Other foot [c]	10	0.5	0.3-0.9	35	1.1	0.8-1.5	45	0.9	0.6-1.1
Other upper leg [d]	1	0.0	0.0-0.3	9	0.3	0.1-0.5	10	0.2	0.1-0.4
Other lower leg [e]	5	0.2	0.1-0.6	42	1.3	1.0-1.8	47	0.9	0.7-1.2
Other [f]	29	1.4	1.0-2.1	49	1.5	1.1-2.0	78	1.5	1.2-1.8
All non-vertebral	190	9.6	8.3-11.0	749	25.0	23.3-26.9	939	18.9	17.7-20.1

a   Other upper arm fractures include fractures of the scapula, clavicle and non-proximal fractures of the humerus

b   Other lower arm fractures include fractures of other parts of the radius and/or ulna

c   Other foot fractures include non-metatarsal foot fractures

d   Other upper leg fractures include non-hip fractures of the femur and patella fractures

e   Other lower leg fractures include non-ankle fractures of the tibia and/or fibula

f   Other fractures include skull, rib and sternum

Figure 1. Fracture incidence per 1000 person years by 5 year age categories
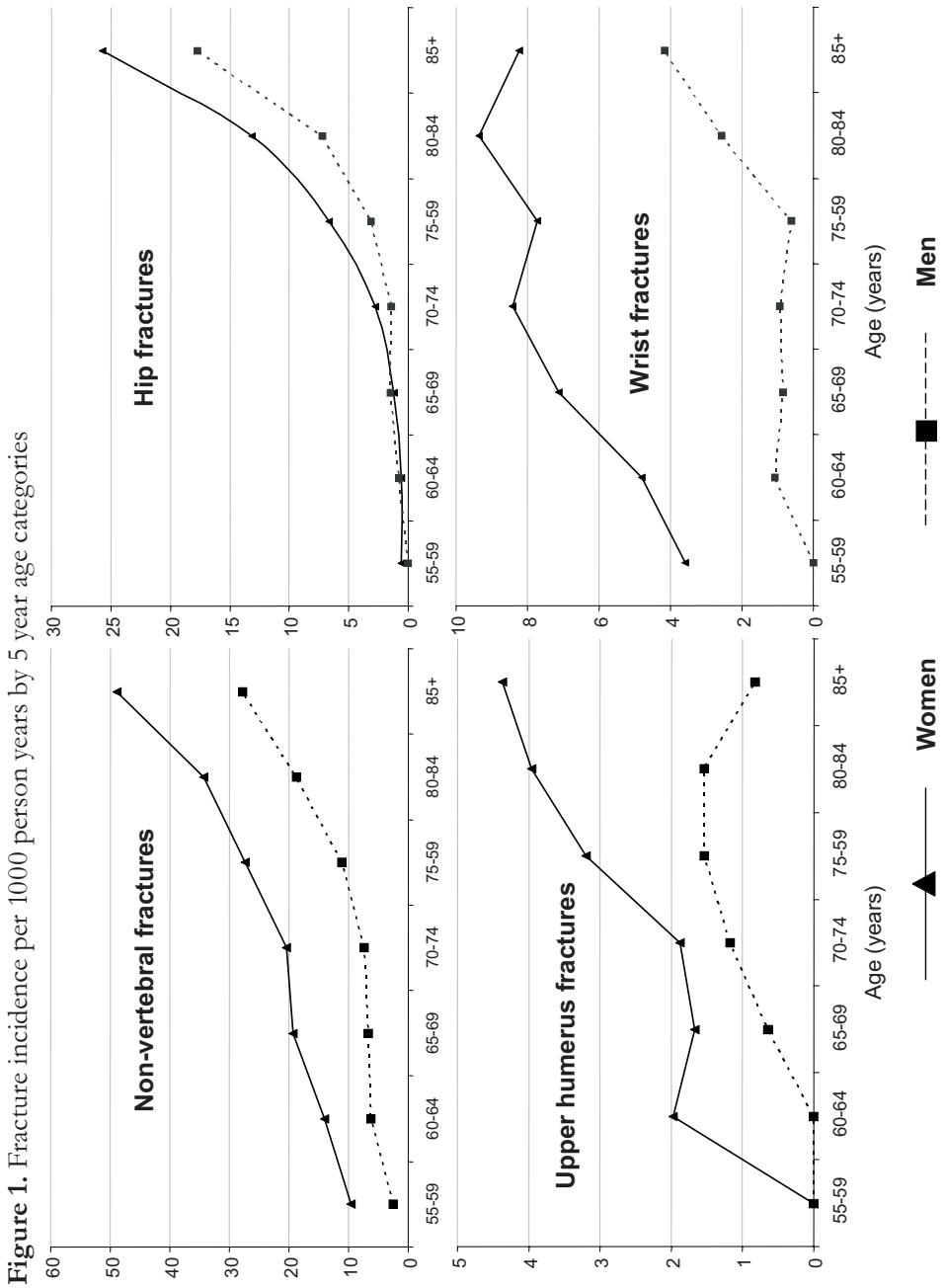
Relation of bone mineral density with non-vertebral fractures

In the subgroup of subjects with BMD data available, 644 (11.1%) individuals suffered at least one non-vertebral fracture. Compared to the total population we observed a lower incidence of non-vertebral fractures (16.4 vs. 18.9 per 1,000 person-years) and hip fractures (3.7 vs. 5.4 per 1,000 person-years) in these younger (mean age 67.9 vs. 70.3 years) individuals.

The relative risk for site-specific non-vertebral fractures per standard deviation decrease in femoral neck BMD is shown in table 2. For all non-vertebral fractures combined, we observed a relative risk of 1.4 (95% confidence interval 1.2-1.6) per gender-specific SD decrease in BMD in men and 1.5 (95% confidence interval 1.4-1.6) in women. The overall relation with BMD and fracture risk was similar in men and women. Specifically, fractures of the hip and wrist were strongly associated with low BMD, in both men and women, while for upper humerus fractures this was only significant in women.

Figure 2 shows the prevalence of gender-specific T-score defined osteoporosis and osteopenia for men and women. Overall, the age-adjusted prevalences of oteoporosis in the Rotterdam Study were 12.1% for men (mean age 67.5 years) and 19.2% for women (mean age 68.3 years). The prevalence of osteoporosis increased with age, reaching 36.4% and 45.2% in men and women aged 85 years or over, respectively.

Figure 3 shows percentages of all non-vertebral and hip fractures that occurred in men and women with osteoporosis, osteopenia and normal bone. Only 34% of all non-vertebral fractures occurred in women with a T-score below -2.5, in men this percentage was even lower (21%). In women, approximately half (53%) of all hip fractures occurred in subjects with a T-score below -2.5. In men, only a little over a third (39%) of all hip fractures occurred in subjects with osteoporosis as defined by T-score. In addition, approximately one third of wrist and upper humerus fractures occurred in women with a T-score below –2.5 (30% and 36%, respectively), while for men this was 19% for wrist fractures and 36% for upper humerus fractures.

In Table 3 numbers of non-vertebral fractures are shown for men and women in 10 year age strata for individuals with a T-score > -1, as well as for osteoporotic and osteopenic subjects. In addition, incidence rates and relative fracture risks as compared to subjects with a T-score above – 1.0 are shown. Importantly, even though osteoporotic subjects both have a higher incidence rate and a higher relative fracture risk as compared to "normal" subjects, the higher number of osteopenic subjects in the population results in an equal absolute number of fractures in participants with osteopenia as compared to those with osteoporosis for women and a three times higher number for men.

Table 2. Age adjusted hazard ratios (95% CI) for the predictive value of femoral neck BMD (g/cm^2) per gender-specific standard deviation (SD) decrease in The Rotterdam Study

Type of Fracture	Men		Women		Total	
Upper extremities						
Upper humerus	11	1.7 (0.9-3.4)	59	1.5 (1.1-2.0)	70	1.7 (1.3-2.2)
Wrist	21	1.6 (1.0-2.6)	164	1.5 (1.3-1.8)	185	1.7 (1.5-2.0)
Hand	21	1.5 (0.9-2.4)	46	1.2 (0.9-1.6)	67	1.3 (1.0-1.7)
Other upper arm [a]	9	0.9 (0.5-1.7)	21	1.6 (1.0-2.6)	30	1.4 (0.9-2.0)
Other lower arm [b]	3	0.9 (0.5-1.7)	15	1.6 (1.0-2.6)	18	1.4 (0.9-2.0)
Lower extremities						
Pelvis	7	1.3 (0.6-3.0)	17	1.4 (0.9-2.4)	24	1.4 (0.9-2.1)
Hip	36	2.3 (1.6-3.3)	116	2.1 (1.7-2.5)	152	2.1 (1.8-2.5)
Ankle	7	1.0 (0.5-2.2)	28	1.1 (0.7-1.6)	35	1.2 (0.8-1.7)
Metatarsal	7	1.4 (0.6-3.1)	28	1.2 (0.8-1.8)	35	1.4 (1.0-2.0)
Other foot [c]	8	2.0 (0.9-4.3)	30	1.2 (0.8-1.8)	38	1.5 (1.1.-2.1)
Other upper leg [d]	0	-	5	3.1 (1.2-7.8)	5	-
Other lower leg [e]	5	1.5 (0.6-3.8)	28	2.1 (1.4-3.1)	33	2.2 (1.5-3.1)
Other [f]	25	1.0 (0.7-1.4)	34	1.3 (0.9-1.9)	59	1.1 (0.9-1.4)
All non-vertebral	145	1.4 (1.2-1.6)	499	1.5 (1.4-1.6)	644	1.6 (1.4-1.7)

a   Other upper arm fractures include fractures of the scapula, clavicle and non-proximal fractures of the humerus

b   Other lower arm fractures include fractures of other parts of the radius and/or ulna

c   Other foot fractures include non-metatarsal foot fractures

d   Other upper leg fractures include non-hip fractures of the femur and patella fractures

e   Other lower leg fractures include non-ankle fractures of the tibia and/or fibula

f   Other fractures include skull, rib and sternum
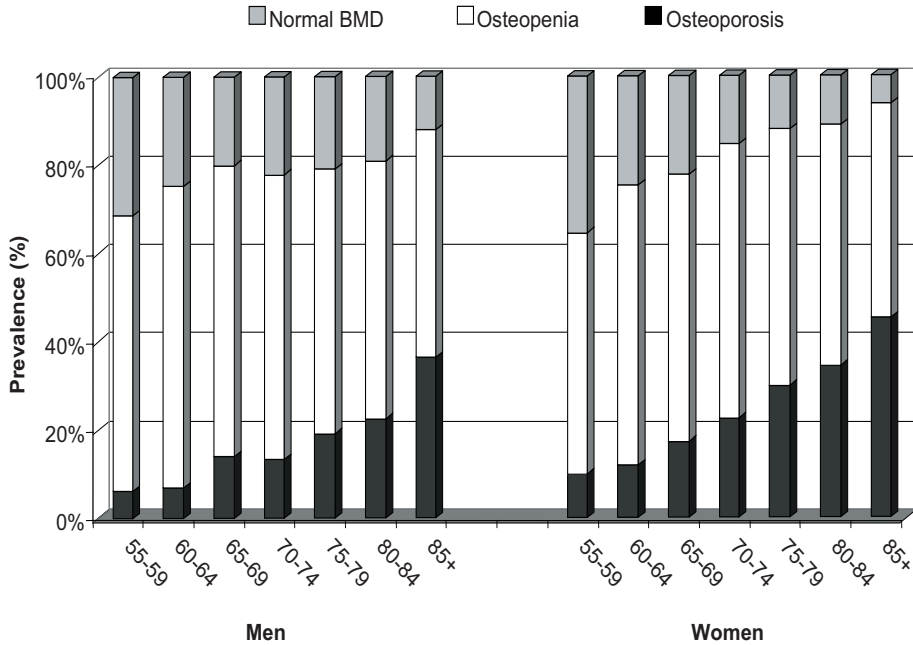
Figure 2. Prevalence of osteoporosis and osteopenia in men and women by gender specific T-scores
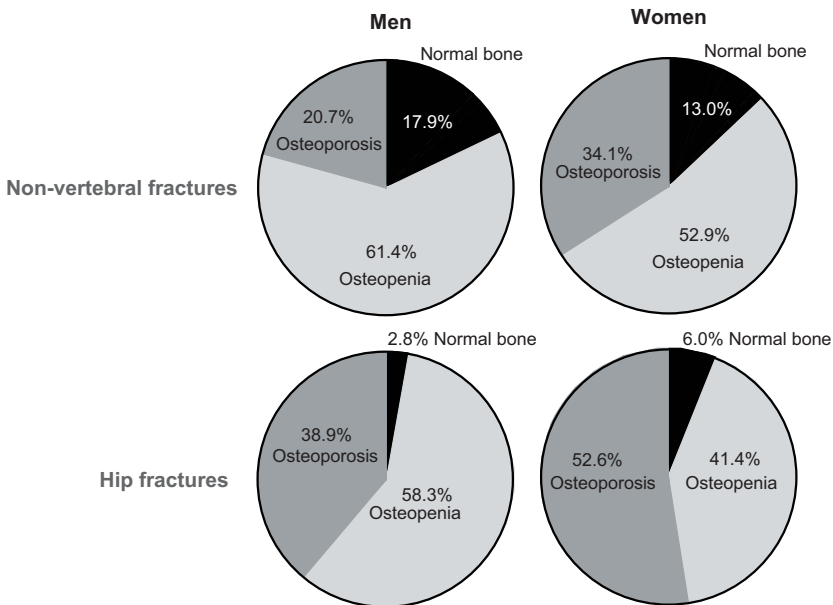
Figure 3. Percentage of non-vertebral, hip, upper humerus and wrist fractures that occurred in men and women with osteoporosis, osteopenia or normal BMD using gender specific T-scores

Table 3. Numbers of non-vertebral fractures in the population by T-score of FN BMD

Age categories	Subjects with normal bone			Osteopenic subjects			Osteoporotic subjects		
	Nr of fractures	Incidence rate [a]	Relative fracture risk (± 95% CI) [b]	Nr of fractures	Incidence rate [a]	Relative fracture risk (± 95% CI) [b]	Nr of fractures	Incidence rate [a]	Relative fracture risk (± 95% CI) [b]
Men									
55-64	7	3.5	1 (Ref)	26	5.3	1.5 (0.7-3.5)	7	16.3	4.7 (1.6-13.4)
65-74	11	7.8	1	34	7.9	1.0 (0.5-2.0)	10	11.7	1.5 (0.6-3.6)
>= 75	8	15.8	1	29	18.9	1.2 (0.5-2.6)	13	28.6	1.9 (0.8-4.6)
Total	26	6.6	1	89	8.3	1.3 (0.8-2.0)	30	17.3	2.7 (1.6-4.5)
Women									
55-64	34	11.8	1	84	14.6	1.2 (0.8-1.8)	23	22.8	1.9 (1.1-3.3)
65-74	23	13.5	1	115	21.9	1.6 (1.0-2.6)	67	42.2	3.2 (2.0-5.1)
>= 75	8	13.6	1	65	24.5	1.8 (0.9-3.8)	80	56.9	4.2 (2.0-8.7)
Total	65	12.6	1	264	19.3	1.5 (1.2-2.0)	170	42.5	3.4 (2.5-4.5)

a   per 1000 person years

b   As compared to subjects with normal bone, estimated as Hazard Ratio by Cox's proportional hazards regression analysis, together with 95% Confidence Interval

DISCUSSION

In this population-based prospective cohort study of fractures, the most frequent non-vertebral fractures in elderly men and women are those of the wrist, upper humerus and hip. The risk of these fractures particularly increases with decreasing femoral neck BMD, an association that is similar in men and women. However, in absolute numbers, most non-vertebral fractures occur in individuals without osteoporosis (T-score above -2.5), suggesting that although low BMD is a strong risk factor for fractures, using only a T-score of BMD below –2.5 as a treatment threshold may not sufficiently relieve the total burden of fractures in the whole population.

Our study has a number of advantages. Only one other study has demonstrated a comparison of the relation between BMD and fractures in men and women from the same population. However, the prospective Hawaii Osteoporosis Study was limited to vertebral fractures only.[9] To our knowledge, this study is the first prospective study that includes all non-vertebral fractures in both men and women. Furthermore, we did not exclude fractures that resulted from high-energy trauma, thereby providing a more valid estimate of the incidence of both osteoporotic and non-osteoporotic fractures. In addition, in the Netherlands the only way to access specialist and hospital care is consulting a general practitioner. Therefore by checking the general practitioners' medical records of all participants should have resulted in a near complete follow-up.

There are, however, disadvantages to this study design. Selective non-response of subjects with impaired mobility, or otherwise at an increased risk of fractures, may have occurred at baseline. Such non-response bias may have decreased the overall incidence rates. Furthermore, only about one third of all vertebral fractures spontaneously come to clinical attention.[10] This leads to underestimation of the incidence and the inability to accurately compare cohorts and associations. Therefore, when studying the association between BMD and incident fractures, only non-vertebral fractures were considered.

Our study found that the hip fracture incidence in men at any age approximates that of women who are on average 5 years younger. This confirms the estimate from data based on national registration data previously reported by de Laet et al.[11] In women in our study the incidence rate of wrist fractures increased until approximately 70 years of age and then leveled off. These results are confirmed by a number of other population based studies.[12-15] Impaired neuromuscular coordination and slower gait in elderly people predisposing them to fall on their side instead of their out-stretched hands may explain this phenomenon.[16] This is supported by a strong increase in incidence of hip and upper humerus fractures in women over the age of 70. It is not clear why this pattern would apply only to women, although, in this study, the power for this comparison was low due to the limited number of wrist fracture in men. However, debate about this explanation is still ongoing

given that other recent European studies have not shown this pattern for wrist fractures.[17-20] In these studies in women the incidence of wrist fractures increased progressively with age also including the older age ranges studied.

Only few previous population-based studies have assessed incidence rates of fractures at all skeletal sites in both genders.[12,15,21] With regard to the overall incidence of fractures we confirm the estimates observed by Singer et al. and Donaldson et al., whereas we observed a lower incidence compared to Melton et al. Although comparison of absolute incidence rates of fractures is complicated due to differences in age, gender, general health and fracture ascertainment, the observed age related incidence rates of the most common types of non-vertebral fractures, i.e. hip, upper humerus and wrist fractures approximated those observed in both studies mentioned.[22-25] Overall the incidence rates for mainly trauma-related fractures, such as fractures of the hand, ankle, foot, rib and pelvis, were lower in our study as compared to the North American study.[15]

The finding that the incidence of all non-vertebral fractures is inversely related to femoral neck BMD is concordant with previous studies.[4,26-32] For women, we showed that fractures of the upper humerus, wrist and hip, as separate groups, have the strongest association with BMD, again in agreement with data reported previously.[27] The fact that we observed the highest risk estimates for hip fractures is probably due to the site (femoral neck) where BMD was measured. Cummings et al. previously suggested that site-specific BMD best predicts the fracture risk for that specific site.[26] Our data show that in men as well as in women, the risk of hip, upper humerus and wrist fractures increases with decreasing BMD in a similar way.

Since osteoporosis is a common condition amongst the elderly and the associated (socio-economic) costs are high, there is a strong need for adequate fracture prevention. However, the results of this study show that if a BMD screening program would be introduced with T-score of -2.5 as treatment threshold, the majority of individuals who will fracture would not be offered treatment. In fact, in women, two thirds of all non-vertebral fractures and approximately half of all hip fractures during the next seven years would be missed. In men these percentages are even larger. However, simply lowering the T-score threshold is not the answer either since treatment may not be cost-effective in the lower risk population. The numbers needed to treat with an intervention are directly influenced by the fracture incidence in those in whom an intervention is undertaken. If a lower T-score cut-off value were chosen more individuals would reach the required fracture risk threshold for intervention and the fracture incidence in this group would also be lower. Therefore, when lowering the T-score threshold the numbers needed to treat will also increase thereby decreasing the cost-effectiveness of the intervention. Thus, from a public health point of view, a screening strategy based on BMD alone is unlikely to be sufficient for prevention of fractures in the population. This problem is not unique for bone disease. Rose previously spoke about the prevention paradox: a large number of people at a small risk may give rise to more cases than the small

number of people who are at high risk.[33] Using other clinical predictors of fractures, such as age, weight, prevalent fractures, medication use and co-morbidity, along side BMD screening, we may be able to more accurately identify those subjects who are at increased risk for fractures.[34] Nevertheless, this prevention paradox does not diminish the value of the T-score in identifying subjects at high risk for fractures. This 'case finding' approach offers these individuals the opportunity to reduce their fracture risk substantially. However, as the high-risk group of individuals with osteoporosis constitutes only approximately 16% of the population over 55 years, reducing the incidence of fractures in that group alone will not be sufficient to adequately relieve the public health burden of fractures.

In conclusion, of all non-vertebral fractures, wrist, upper humerus and hip fractures are the most frequent fractures in elderly men and women. In addition, BMD is an equally important risk factor in men and women. Finally, this study shows that using only a T-score at or below -2.5 SD as a criterion for interventions will not resolve the population burden of fractures. There is a clear need for the development of more sensitive risk assessment tools, using not only BMD, but also other clinical predictors of fractures. Using such an approach, we may be able to more accurately identify those subjects who are at increased risk for fractures.

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Chapter 2.2

Association of 5' Estrogen Receptor Alpha Gene Polymorphisms with Bone Mineral Density, Vertebral bone Area and Fracture Risk

ABSTRACT

This study investigates the influence of genetic variation of the estrogen receptor alpha (ERα) gene locus on several bone parameters in 2042 individuals of The Rotterdam Study, a prospective population-based cohort study of elderly subjects. We analyzed three polymorphic sites in the 5' region of the ERα 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 ERα 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 ERα 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 ERα 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 (ERα) 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 increased risk for osteoporosis. The serum level of estradiol has been shown to be an important predictor of subsequent bone mass and risk for osteoporotic fractures.[4-6] 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 ERα and the pivotal role of ERα in the regulation of bone mass was suggested by a report of a young adult male with a loss-of-function mutation of the ERα gene resulting in a phenotype with low BMD.[10] Consistent with this, an ERα knockout mouse model showed that both female and male mice had decreased bone mass.[11]

Several genetic variations in the ERα 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 ERα 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) in intron 1, about 400 bp upstream of exon 2.[22] Since no functional effect of these sequence variations on expression or function of the ERα 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 ERα 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 ERα polymorphism on BMD, vertebral bone area and fractures and compared our results with those obtained in other association studies.

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.[23] 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 cytostatic 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.[24] 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.[25]

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, as described previously.[26,27] 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'-GATATCCAGGGGTTATGTGGCA-3') and a reverse primer (ER-R: 5'-AGGT GTTGCCTATTATATTAACCTTGA-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 0.5xTBE (1x 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 *Pvu*II–*Xba*I 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 was possible when the combined genotype per individual was known.[12-15,17] We used these data to infer the frequency of the haplotypes present in the population using the program 3LOCUS.pas.[28]

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 for determining the frequencies of the TA–*Pvu*II–*Xba*I haplotypes in different subpopulations.[29]

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 separately 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).

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.

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.

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 (yrs)	67.0 ± 6.9	65.7 ± 6.4	<0.001
Height (cm)	162 ± 6	162 ± 7	0.09
Weight (kg)	68.5 ± 10.3	68.7 ± 10.0	0.75
BMI (kg/m²)	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 (yrs)	48.7 ± 4.9	48.7 ± 5.0	0.97
Age at menarche (yrs)	13.7 ± 1.7	13.7 ± 1.8	0.67
Men			
Number	942	527	
Age (yrs)	67.3 ± 7.1	65.4 ± 6.5	<0.001
Height (cm)	175 ± 7	176 ± 6	0.03
Weight (kg)	78.2 ± 10.6	79.2 ± 10.0	0.09
BMI (kg/m²)	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

Table 2. Genotype distribution according to *Pvu*II–*Xba*I haplotypes. 1=px; 2=PX; 3=Px.

	Men	Women
Genotype	number (frequency)	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)

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
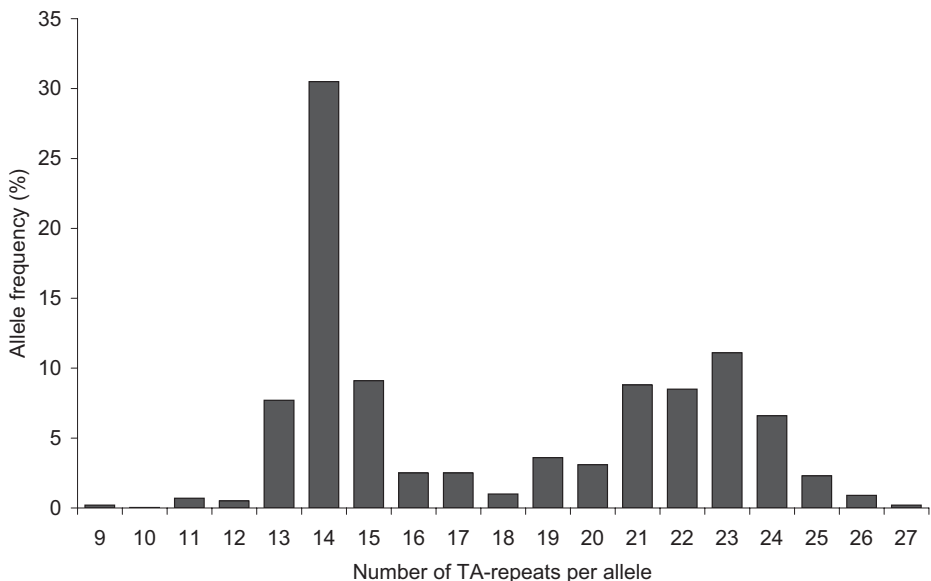
Figure 1.
Allele frequencies of $(TA)_n$ VNTR polymorphism in the total study population.

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 for haplotype reconstruction of each individual (Table 4).[29] 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 ERα polymorphism with bone characteristics for the *Pvu*II–*Xba*I haplotypes and the (TA)$_n$ VNTR separately and for the combined LRHs.

Table 4. Estimated frequencies of long range haplotypes from *Pvu*II–*Xba*I haplotype alleles and (TA)$_n$ alleles in 1100 women. The (TA)$_n$ alleles are defined as L (n<18) and H (n≥18).

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)

*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.[25] 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.
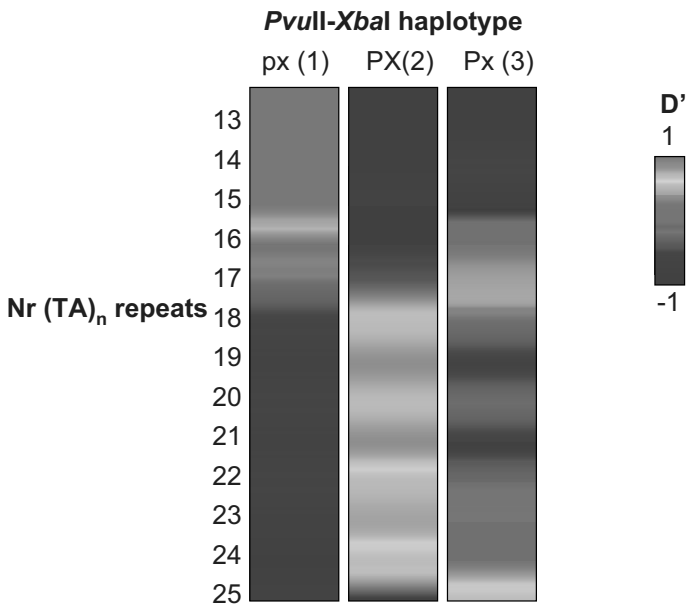
Figure 2.
Pairwise disequilibrium coefficients (D') between the three *Pvu*II-*Xba*I halpotype alleles and the 13 most common (TA)$_n$ repeat alleles. The coefficient is positive when the alleles are linked, and is negative when the alleles exclude each other. When D' is 1, the two alleles are completely linked, when D' is –1, the two alleles exclude each other completely.

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

Bone measures	Number of copies of *Pvu*II–*Xba*I haplotype 1			P-value [c]
	0	1	2	
Women	n=240	n=548	n=311	
Femoral neck BMD (g/cm²)				
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/cm²)				
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 (cm²)				
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=472	n=274		
Femoral neck (g/cm²)				
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/cm²)				
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 (cm²)				
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.

c P-values were calculated using linear regression analysis.

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

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 2		
	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).

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 ERα polymorphism was still present (data not shown). This strongly suggests that the effect of ERα 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. 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 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≥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.

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]	Δ LS BMD[b]	Δ LS bone area[c]
13	153	+9.6	-0.02	-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.

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]	Δ LS BMD (g/cm^2)[b]	Δ LS 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≥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 ERA 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]	Δ LS BMD[c] (g/cm^2) per copy of the allele	P value[b]	Δ LS 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.

DISCUSSION

This study shows an association between the *Pvu*II–*Xba*I haplotype and the $(TA)_n$ repeat in the ERα 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 ERα 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.[12,14,20,30] 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.[16,18,31] 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 ERα locus among different populations. This should be accom-

panied with differences in genotype distributions, which we indeed found when we studied the allelic frequencies among different ethnic populations. The two Asian populations studied 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.[15,18] 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 ERα variations and bone characteristics. Our findings of the association with vertebral fractures are in line with those of Langdahl et al.and Becherini et al.[12,14] 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 ERα 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 ERα-dependent, and as such indirectly influences the risk of fractures, the BMD-independent relation of ERα genotype with fractures suggests that other underlying biological mechanisms than those reflected in BMD explain the increased fracture risk. This same phenomenon was also observed for the relation between the collagen-type I alpha 1 Sp1 polymorphism, BMD and fracture risk, and for VDR polymorphisms.[32,33] 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 ERα 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 ERα polymorphism and bone size. Therefore, we hypothesize that ERα 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 ERα polymorphism with stature.[34] Together with our previous observation of the genetic effect of ERα polymorphism on menopausal status, and associations with cardiovascular disease and cancer, this illustrates the pleiotropic nature of the ERα protein.[25,35,36] 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 ERα 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 I alpha 1 gene, which is known to change the mRNA production of the gene which eventually leads to decreased BMD an increased fracture risk.[32,37] The *Pvu*II–*Xba*I polymorphic site in the first intron of the ERα gene could influence gene expression in a similar manner. Recently, a report showed that the *Pvu*II polymorphism is located within a potential B-myb binding site, which was able to regulate transcription efficacy of a reporter gene.[38] Alternatively, the location of a variable length of the $(TA)_n$ VNTR in the promoter of the ERα 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.[39-41] 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 ERα 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.[19,42-44] Probably, the effect of the ERα is more pronounced 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.

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Chapter 2.3

Height in Pre- and Postmenopausal Women is Influenced by Estrogen Receptor Alpha Gene Polymorphisms

ABSTRACT

The estrogen receptor alpha gene (ERα) is known to be involved in metabolic pathways influencing growth. We have performed two population-based association studies using three common polymorphisms within this candidate gene to determine whether these are associated with variation in adult stature.

In 607 women, aged 55 to 80 years from the Rotterdam Study, the ERα *Pvu*II-*Xba*I haplotype 1 (px) and the L-allele of the TA-repeat polymorphism (< 18 TA-repeats) were significantly associated with an allele dose dependent decrease in height. Per allele copy of ERα *Pvu*II-*Xba*I haplotype 1 height was 0.9 cm shorter (p-trend = 0.02) and 1.0 cm per allele copy of the TA-repeat L-allele (p-trend = 0.003,). These results were independent of age, age at menarche and menopause and lumbar spine BMD, and remained significant after participants with vertebral fractures were excluded. In 483 men from the Rotterdam Study we found no association with height. In 1500 pre- and perimenopausal women from the Eindhoven Study a similar association was observed; women were 0.5 cm shorter per allele copy of the ERα haplotype 1 (p-trend = 0.03).

In conclusion, we demonstrate a role for genetic variations in the estrogen receptor alpha gene in determining adult stature in women.

INTRODUCTION

Adult stature has been a topic of genetic research since the beginning of the 20th century. Early studies considered the racial differences in stature proof of heritability.[1] Later, studies of twins and families quantified this heritability, which is generally believed to be over 80%.[2-4] Today, adult stature is commonly recognized as a complex trait that is regulated by multiple genetic and environmental factors.

The importance of genetic research of height lies not only in unraveling the physiological processes involved in growth. In clinical practice, short stature is frequently treated in pediatric endocrine departments. In addition, aspects of skeletal size have been implicated in the risk of many diverse diseases including osteoporotic fractures,[5] cancer,[6] and cardiovascular disease.[7] These associations emphasize the heterogeneity of factors that determine stature; that is variations in genes affecting skeletal size may also determine the risk for certain diseases through direct or indirect pathways (pleiotropy). Thus, unraveling the genetic origins of stature will not only give important information about the physiology of growth, but may also provide new insights into the mechanisms of diseases such as osteoporosis, cancer, and cardiovascular disease.

To identify the individual genetic factors underlying differences in height several approaches can be pursued, including genome searches by linkage analysis followed by candidate gene studies by association analysis. Recently, the first four genome wide linkage studies of stature were published, leading to the identification of several potential regions of linkage.[8-11] The region on chromosome 6 (6q24-25) is of special interest not only because the results were independently replicated, but also because it is centered on a gene which is known to be involved in metabolic pathways influencing growth, the estrogen receptor alpha gene (ERα).[8,11] The estrogen resistant male described in 1994 illustrates the importance of the ERα gene in bone development and growth.[12] A disruptive mutation in the ERα gene, producing a non-functional estrogen receptor in this man, led to absence of the pubertal growth spurt, delayed bone maturation, unfused epiphyses, and continued growth into adulthood. Given the striking effects of complete estrogen resistance on stature, it is conceivable that subtler and more frequent variations (polymorphisms) in the ERα gene, may affect skeletal size in healthy individuals.

The aim of this study was to determine whether polymorphisms in the ERα gene are associated with variation in height in an adult population. We addressed this question in two Dutch population-based studies.

METHODS

The Rotterdam Study

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.[13] All 10,275 inhabitants, age 55 or older, of Ommoord, a district of Rotterdam, The Netherlands, were invited to participate. Base-line examinations, including a home interview and an extensive physical examination, took place between 1990 and 1993. The overall response rate was 77% for the home interview and 6451 participants (71%) were also able to visit the research center, where anthropometrics were measured and blood samples were taken.

Subjects

For the present study we included independently living subjects, age 55 to 80 years and of Northern European decent who were initially part of a large epidemiological study of bone mineral density. Baseline measurements of bone mineral density were available for 5931 independently living subjects from the initial 7983 participants of the Rotterdam Study, 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 individuals, we studied a random sample of 2042 subjects. Information on vertebral fractures was available for 1184 participants of the study. Subjects with one or more prevalent vertebral fractures at baseline (n = 94) were excluded. In the remaining sample of 1090 subjects (607 women and 483 men) the following polymorphisms were determined: the *Pvu*II and *Xba*I RFLP haplotype and the TA-repeat VNTR polymorphism in the ERα gene.

Clinical examination

During the home interview female participants were asked to recall their age at menarche and menopause and responses were validated as described previously.[14] At the research center, height and weight were measured in standing position in indoor clothing without shoes. All height measurements were attained by a research assistant using a standard wall-mounted statiometer. Body mass index (BMI) was computed as weight in kilograms divided by height in meters squared (kg/m^2). At baseline, average bone mineral density (BMD expressed in g/cm^2) was measured over L2 to 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). Spine radiographs were assessed for the presence of vertebral fractures at baseline by the McCloskey-Kanis method.[15,16] Vertebral body area (cm^2) was measured over the second through fourth lumbar vertebra by postero-anterior scanning using dual energy X-ray absorptiometry (DEXA; Lunar DPX-L, Lunar Corp., Madison, WI, U.S.A.).

The Eindhoven Perimenopausal Osteoporosis Study

Study population

The Eindhoven Study is a population-based cohort study of women born between 1941 and 1947, living in the city of Eindhoven, The Netherlands. Rationale and design have been described previously.[17] Of the eligible 8503 women, 6700 (79%) participated. Base-line examinations, including an interview by a trained research assistant and an extensive physical examination, took place between 1994 and 1995. All participants gave their written informed consent and two medical ethical committees approved the study.

Subjects

For the present study we included a random sample of 1500 pre- and perimenopausal women of Northern European decent. Pre- and perimenopausal status was defined as last menses less than one year ago. This cohort was initially part of a large epidemiological study of cholesterol in which subjects were excluded according to the following criteria: use of hormone replacement therapy, oral contraceptives, and cholesterol-lowering therapy.

Clinical examination

During the interview participants were asked about age of menarche and menopause. Height and weight were measured in indoor clothing without shoes at either of two research centers: Diagnostic Center Eindhoven and St. Joseph Hospital in Veldhoven, a suburb of Eindhoven. All height measurements were attained by a research assistant using a standard wall-mounted statiometer. Body mass index (BMI) was computed as weight in kilograms divided by height in meters squared (kg/m^2).

Genotyping

Genomic DNA was isolated from peripheral leukocytes by standard procedures. The *Pvu*II (C to T substitution) and *Xba*I (G to A substitution) restriction fragment length polymorphisms (RFLP) are located in intron 1 of the ERα gene, 397 bp and 351 bp, respectively, upstream of exon 2. To increase genetic resolution, we constructed haplotypes in this area of the ERα gene.[18] As the two RFLPs are separated by only 46 bp, we identified haplotypes by a direct molecular haplotyping method. A 346 bp PCR fragment was generated by a forward primer (ER-F: 5'-GATATCCAGGGTTATGTGGCA-3') and a reverse primer (ER-R:5'-AGGTG TTGCCTATTATATTAACCTTGA-3') in a reaction mixture of 10 μL containing 20 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 sec. 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 0.5xTBE (1xTBE = 89 mM Tris, 89 mM boric acid, 2 mM Na$_2$EDTA) for 80 minutes at 125 Volts. Separation patterns were documented with a digital camera (DC120, Kodak Company, Rochester, NY) under UV illumination (302 nm).

The alleles were defined as haplotypes such as "Px", capitals denoting absence and lower cases letters denoting presence of the restriction site for the *Pvu*II (P/p) and *Xba*I (X/x) enzymes on each of the alleles. The haplotype alleles were coded as haplotype numbers 1 through 4 in order of decreasing frequency in the population (1 = px, 2 = PX, 3 = Px, and 4 = pX). Genotypes are analyzed as combinations of two alleles.

The (TA)$_n$ Variable Number of Tandem Repeat (VNTR) polymorphism is located 1 kb upstream of the first exon at –1118 bp from the transcription start site and –1351 bp from the translation start site. A 160-194 bp PCR fragment containing the TA-repeat VNTR was generated 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 sec. 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 allele length was determined using internal size standards.

Statistical analysis

One-way analysis of variance (ANOVA) and Pearson's chi-square were used to compare age, gender, and age at menarche, in our study population to the entire Rotterdam Study cohort. To account for the possible confounding effects of age and gender, all other baseline characteristics were compared by adjusted analysis of covariance (ANCOVA).

To analyze the relationship between the ERα genotypes, height and potential confounders, we stratified subjects by allele copy number (0,1 or 2) for the *Pvu*II-*Xba*I haplotype in the ERα gene. In view of the large number of individual genotypes, the ERα TA-repeat alleles were grouped according to the number of TA-repeats: group H including alleles with a high number of TA-repeats (TA ≥ 18) and group L including alleles with a low number of TA-repeats (TA < 18). Individuals were then genotyped and analyzed as "LL", "LH" or "HH". The cut off

value of 18 TA-repeats is based on previous findings which indicate that 18 or more TA-repeats are associated with osteoporosis in our population.[18]

We allowed for three possible models to explain differences between genotype 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 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 versus non-carriers while for recessive effects, subjects homozygous for the test allele were compared to heterozygous carriers and non-carriers. First, the crude differences between genotype groups were calculated stratified by gender. Subsequently, the analysis was adjusted for the potential confounding effects of age and age at menarche in women.

We searched for possible intergenic interactions within the ERα gene. We used the genotype data for each of the polymorphisms to infer frequency of the long range haplotype alleles within each gene using the PHASE program.[19]

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

Results

The baseline characteristics of the subjects selected from the Rotterdam Study differ from the total cohort. More men have been included in our study (44% versus 39%) and the participants of our study were on average 5.2 years younger as compared to the entire Rotterdam Study population. There were no significant differences in age at menarche or menopause. After adjustment for age, women in our study population weighed less (68.1 vs. 69.4 kg, $p<0.01$) and had a significantly lower BMI (26.2 vs. 26.7 kg/m^2, $p<0.01$) as compared to the entire Rotterdam Study cohort. In both men and women, after adjustment for age, there no were significant differences in height as compared to the entire Rotterdam Study population.

To study the effects of the ERα *Pvu*II-*Xba*I haplotypes on height in women prior to menopause, we analyzed a random set of 1500 pre- and perimenopausal women from the Eindhoven Perimenopausal Osteoporosis Study. The participants selected for our study were on average 0.8 years younger then the entire Eindhoven Study cohort. There was no significant difference in age at menarche and after adjustment for age and there were no significant differences in any of the anthropometric parameters between our study population and the total cohort.

Table 1 shows the location, number of subjects analyzed and allele frequencies for all polymorphisms. All genotypes and haplotypes were in Hardy-Weinberg equilibrium and frequencies were similar to other studies of Caucasian subjects.[20-22]

Table 1. Polymorphisms analyzed in this study

Polymorphism	Alleles	No. subjects analyzed	Allele frequency (%)
The Rotterdam Study			
*Pvu*II/*Xba*I RFLP (intron 1 -397 C/T and -351 G/A)	Haplotype 1 - px - T-A	1090	/ 1176/2180 (54)
	Haplotype 2 - PX - C-G	1090	/ 754/2180 (35)
	Haplotype 3 - Px - C-A	1090	/ 250/2180 (11)
	Haplotype 4 - pX - T-G	1090	-/2180 (0)
(TA)$_n$ (-1351 from translation start site)	"L"-allele (< 18 repeats)	1090	/ 1173/2180 (54)
The Eindhoven Study			
*Pvu*II/*Xba*I RFLP (intron 1 -397 C/T and -351 G/A)	Haplotype 1 - px - T-A	1500	1620/3000 (54)
	Haplotype 2 - PX - C-G	1500	1077/3000 (36)
	Haplotype 3 - Px - C-A	1500	/ 303/3000 (10)
	Haplotype 4 - pX - T-G	1500	-/3000 (0)

In the 607 female subjects remaining after exclusion of participants with prevalent vertebral fractures at baseline (n=94), an association was seen between height and ERα *Pvu*II-*Xba*I haplotypes. In women, a significant allele dose effect was observed for the ERα haplotype 1 (the most frequent allele), corresponding to a 0.9 cm decrease in height per allele copy (p-trend 0.02, Table 2), extreme genotypes varied 1.8 cm. We observed a trend for association of ERα haplotype 1 with age at menopause (p-trend 0.06), in that homozygous carriers of the ERα haplotype 1 had a 1.1 year later age at menopause. However, age at menarche was not associated with ERα haplotype 1. Lumbar spine BMD and lumbar vertebral area were both significantly associated with ERα haplotype 1 (p-trend 0.05 and 0.01, respectively). A significant variation in weight was also observed, however investigation of the relationship with BMI revealed this association to be driven by height differences. Adjustment for age, age at menarche, age at menopause and lumbar spine BMD did not significantly change the observed association between ERα haplotype 1 and height. In women, the ERα haplotype 2 showed a similar, but opposite allele dose effect on height corresponding to a 0.7 cm increase in height per haplotype 2-allele copy (p-trend = 0.06), extreme genotypes differed 1.6 cm (results not shown). No association with height was observed for the ERα haplotype 3 (results not shown).

When these same analyses were performed on the 483 male subjects (mean age 65.2 standard deviation 6.6), no relationship with height was seen; non-carriers of haplotype 1 were 176.5 cm, heterozygous carriers 175.7 cm and homozygous carriers 176.0 cm (p-value 0.6, Table 2). We also analyzed ERα haplotype 1 dependent

Table 2. Characteristics of 607 women and 483 men by ERα PvuII-XbaI haplotype 1, The Rotterdam Study

Number of allele-copies ERα haplotype 1	Women				Men			
	0	1	2	P-value	0	1	2	P-value
Number (%)	142 (23.4)	294 (48.4)	171 (28.2)	0.5 [a]	95 (19.7)	236 (48.9)	152 (31.5)	0.8 [a]
Age ± SE (years)	65.8 ± 0.5	65.2 ± 0.4	66.0 ± 0.5	0.4 [b]	65.0 ± 0.7	65.3 ± 0.4	65.2 ± 0.5	0.9 [b]
Age at menarche ± SE (yrs)	13.8 ± 0.1	13.7 ± 0.1	13.6 ± 0.1	0.5 [c]				
Age at menopause ± SE (yrs)	48.0 ± 0.4	48.9 ± 0.3	49.1 ± 0.4	0.06 [c]				
Weight ± SE (kg)	70.1 ± 0.8	68.9 ± 0.6	67.6 ± 0.8	0.03 [c]	77.6 ± 1.0	79.6 ± 0.7	79.8 ± 0.8	0.1 [c]
BMI ± SE (kg/m²)	26.3 ± 0.3	26.1 ± 0.2	26.0 ± 0.3	0.5 [c]	24.9 ± 0.3	25.8 ± 0.2	25.8 ± 0.2	0.04 [c]
Lumbar Spine BMD (g/cm²)	1.034 ± 0.014	1.032 ± 0.010	0.998 ± 0.013	0.05 [c]	1.158 ± 0.019	1.172 ± 0.012	1.140 ± 0.015	0.3 [c]
Height ± SE (cm)	163.4 ± 0.6	162.6 ± 0.4	161.6 ± 0.5	0.02 [c]	176.5 ± 0.6	175.7 ± 0.4	176.0 ± 0.5	0.6 [c]
Height ± SE (cm) [d]	163.4 ± 0.5	162.5 ± 0.4	161.6 ± 0.5	0.02 [c]	176.5 ± 0.6	175.7 ± 0.4	176.0 ± 0.5	0.6 [c]
Height ± SE (cm) [e]	163.3 ± 0.5	162.5 ± 0.4	161.8 ± 0.5	0.04 [c]	176.5 ± 0.6	175.6 ± 0.4	176.0 ± 0.5	0.7 [c]
Lumbar apparent vertebral area (cm²)	14.5 ± 0.1	14.3 ± 0.1	14.1 ± 0.1	0.01 [c]	17.4 ± 0.2	17.5 ± 0.1	17.6 ± 0.1	0.4 [c]

a P-value for Hardy-Weinberg equilibrium

b ANOVA

c Linear regression

d Adjusted for age (and age at menarche and menopause in women)

e Adjusted for age and lumbar spine BMD (and age at menarche and menopause in women)

height differences in men in age categories, above and below the median age of 65 years. However, in both age categories no association was observed; the p-value for trend was 0.4 in youngest age category and 0.7 in the oldest age category.

We analyzed the association between the ERα TA-repeat VNTR polymorphism, located in the promoter region, and height in The Rotterdam Study. Nineteen different alleles were identified. The bimodal distribution pattern of the TA-repeat alleles in our population (Figure 1) shows two peaks at 13-15 and 21-23 TA-repeats. A low frequency was seen for the intermediate 16-20 TA-repeats and the extremes of the spectrum (9-12 and 24-33 TA-repeats).

In women, the L-allele (fewer than 18 TA-repeats) of the TA-repeat VNTR polymorphism showed a similar significant association with decreased height as the ERα haplotype 1. A 1.1 cm decrease in height per allele copy of the L-allele (p-trend < 0.01), independent of age, age at menarche and age at menopause was observed (results not shown); extreme genotypes differed 2.2 cm in mean height. Apparent lumbar vertebral area was also significantly associated with the L-allele, extreme genotypes differed 0.4 cm^2 (p-trend 0.02). While in men, as for the *Pvu*II-*Xba*I haplotypes, no association with height or apparent lumbar vertebral area was seen. We observed strong linkage disequilibrium between the polymorphic sites at the 5' region of the ERα gene. In order to determine which of the polymorphic sites is driving the association with height and vertebral bone area, we constructed long range haplotypes (LRH) using the PHASE program.[19] Table 3 shows frequencies
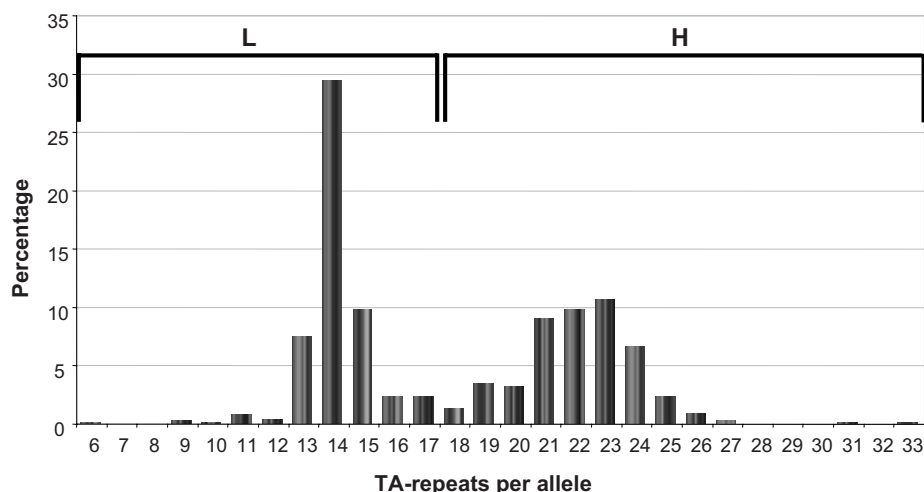

Figure 1.
Frequency distribution of the ERα dinucleotide (TA) repeat polymorphism in 1090 subjects (2180 chromosomes). Group "H" includes alleles with a high number of TA-repeats (TA ≥ 18) and group "L" includes alleles with a low number of TA-

Table 3. Association of the 6 ERα *Pvu*II-*Xba*I-TA-repeat long range haplotype (LRH) alleles in women (n=607), The Rotterdam Study

LRH allele	TA-repeat	Haplotype alleles *Pvu*II-*Xba*I haplotype	Nr. of carriers	Δ height (cm)
A	L	1	441	- 1.30
B	L	2	30	- 1.28
C	L	3	28	+ 0.90
D	H	1	54	+ 0.75
E	H	2	339	+ 0.77
F	H	3	101	+ 1.10

L = Low number of TA-repeats (< 18), H = High number of TA-repeats (≥ 18)

Δ height = mean height of allele carriers minus mean height of non-carriers

of each of the 6 observed haplotypes. Due to the strong linkage disequilibrium between the *Pvu*II-*Xba*I haplotype and the TA-repeat VNTR, we observed two frequent LRH alleles (allele A and E) among the 6 possible haplotypes. Table 3 also shows the difference in mean height between carriers and non-carriers of each particular LRH allele. The largest effect on height was observed for the LRH allele A, which is the combination of a low number of TA repeats and *Pvu*II-*Xba*I haplotype 1.

To study the effects of the ERα *Pvu*II-*Xba*I haplotype 1 on height in women prior to menopause, we analyzed a random set of 1500 pre- and perimenopausal women from the Eindhoven Perimenopausal Osteoporosis Study. The ERα haplotypes were also associated with variation in height in these pre- and perimenopausal women. A significant allele dose effect was observed for ERα haplotype 1, corresponding to a 0.5 cm decrease in height per allele copy (p-trend = 0.03, Table 4).

DISCUSSION

The first genome wide linkage studies of stature identified a region of potential linkage to adult stature on chromosome 6 (6q24-25).[8-11] This region is centered on a gene, which is known to be involved in metabolic pathways influencing growth, the estrogen receptor alpha gene (ERα). Following these results three polymorphic sites in the estrogen receptor alpha gene were investigated for associations with height. We found these common polymorphisms in the 5' region of the ERα gene to be significantly associated with height in postmenopausal women, as well as pre- and perimenopausal women. Difference in height between extreme genotypes between 1.0 and 2.2 cm were observed, dependent upon the group of women studied and

Table 4. Characteristics of Women by ERα *Pvu*II-*Xba*I haplotype 1 (n=1500), The Eindhoven Study

	Copy number of ERα haplotype 1 allele			P-value
	0	1	2	
Number (%) [a]	331 (22.13)	718 (47.9)	451 (30.1)	0.2 [a]
Age ± SE (years)	49.8 ± 0.1	50.1 ± 0.1	50.0 ± 0.1	0.3 [b]
Age at menarche ± SE (yrs)	14.0 ± 0.4	14.2 ± 0.3	14.2 ± 0.4	0.7 [b]
Weight ± SE (kg) [c]	68.8 ± 0.7	69.3 ± 0.4	68.7 ± 0.6	0.8 [b]
BMI ± SE (kg/m²) [c]	25.2 ± 0.3	25.7 ± 0.2	25.5 ± 0.2	0.5 [b]
Height ± SE (cm)	165.3 ± 0.4	164.7 ± 0.2	164.3 ± 0.3	0.03 [b]
Height ± SE (cm) [d]	165.3 ± 0.4	164.7 ± 0.2	164.3 ± 0.3	0.04 [b]

a P-value for Hardy-Weinberg equilibrium

b Linear regression

c Adjusted for age and age at menarche

d Adjusted for age

the polymorphism analyzed.

In the Rotterdam Study, a population-based study of adults aged 55 years and older, the ERα *Pvu*II-*Xba*I haplotype 1 (-397 T and -351 A) and the L-allele of the TA-repeat VNTR polymorphism (fewer than 18 TA-repeats) were significantly associated with a decrease in height per allele copy (0.9 cm and 1.0 cm per allele copy, respectively). Homozygous carriers of *Pvu*II-*Xba*I haplotype 1 were 1.8 cm shorter than subjects who did not carry this haplotype; homozygous carriers of the TA-repeat L-allele were 2.2 cm shorter than non-carriers. Furthermore, these polymorphisms were also associated with a smaller apparent lumbar vertebral area (0.4 cm²). These associations were not found in the male participants of our study.

We considered that the postmenopausal drop in estrogen levels may be instrumental in the association we found for the ERα gene with height. This would indicate that it is not attained adult height that is influenced by ERα gene polymorphisms, but the age-related decrease in stature. To address this hypothesis we excluded participants with radiologically confirmed vertebral fractures at baseline and adjusted for age at menopause and found that each did not change our results. However, vertebral fractures that do not meet the McCloskey-Kanis criteria, that is vertebral deformities with a reduction in vertebral height that do not meet the cut-off level, may still be present in the Rotterdam population. We have tried to address this by adjusting for lumbar spine BMD. Women most susceptible to these less severe vertebral fractures will on average have a lower lumbar spine BMD. Furthermore, as has been reported previously, ERα haplotype 1 is associated with lumbar spine BMD.[18] Therefore, by adjusting our analysis for lumbar spine BMD we have adjusted for

a surrogate for vertebral fractures and have shown that these fractures most likely do not play an essential role in the observed association. Furthermore, we analyzed the association between the ERα *Pvu*II-*Xba*I haplotypes and height in 1500 pre-and perimenopausal women from The Eindhoven Study, a population-based cohort of women aged 46 to 57 years. In this study the *Pvu*II-*Xba*I haplotype 1 was also significantly associated with decreased height, where haplotype 1 was associated with a 0.5 cm shorter stature per allele copy. Thus, in pre- and perimenopausal women, where vertebral fractures are not likely to be an important confounder, the results were similar to the association found in postmenopausal women. Given these findings we expect the confounding by less severe vertebral fractures that do not meet the McCloskey-Kanis cut-off level will not have influenced our results. Furthermore, we hypothesize that ERα polymorphisms influence attained height earlier in life, rather than after menopause. However, the effect does seem to be greater in postmenopausal women than in pre- and perimenopausal women (1.8 cm vs. 1.0 cm). Yet, we can not be sure whether this is a significant difference or falls within the 95% confidence intervals for both populations.

As seen in other populations, in The Rotterdam Study strong linkage disequilibrium exists between the intron 1 *Pvu*II and *Xba*I polymorphic sites and the TA-repeat VNTR located approximately 21 kb upstream of these polymorphisms.[18,21,23] It is still unclear which of these polymorphisms is driving the association we observed with stature. It is difficult to distinguish the different effects of the two polymorphic sites by association analyses because of the strong linkage between them. However, the association studies with the long range haplotypes suggest that both sites are contributing since the long range haplotype defined by carrier status for both the *Pvu*II-*Xba*I as well as the TA-repeat VNTR risk alleles (LRH A) shows the strongest effects on stature, especially when we compare LRH alleles A and B versus C and D and E versus F. However, larger studies are necessary to prove this hypothesis.

In this study we observed that the genetic associations between ERα polymorphisms and height were found only in women. This is unexpected because the only known clinical example of the consequences of a non-functional estrogen receptor alpha on height was observed in a male. Two explanations arise. First, the physiology of pubertal growth in males differs from females in that it begins and finishes later in life and has a higher peak velocity. Furthermore, men are taller than women mostly as a result of longer legs rather than longer torsos.[24] In addition, there are structural and physiological differences between vertebral and long shafted bone growth. This raises the possibility that the ERα gene polymorphisms particularly influence mechanisms controlling vertebral bone growth. To investigate this theory we measured average vertebral area from lumbar spine DEXA scans. Indeed, ERα polymorphisms were significantly associated with apparent vertebral body area which supports the hypothesis that ERα gene polymorphisms influence vertebral bone growth.

Another explanation for the fact that the association with stature was only seen in women relates to the predominant role of androgens in males. Perhaps, while in the predominantly estrogenic puberty of females, polymorphisms in the ERα gene lead to height differences, in males variances in androgenic response (perhaps through polymorphisms) may override these variances.

The ERα *Pvu*II and *Xba*I polymorphisms have been an important area of research in diseases such as osteoporosis, cardiovascular disease, and cancer.[21,25,26] Until recently neither of the *Pvu*II or *Xba*I RFLPs was known to have functional consequences. It was thought that the *Pvu*II and *Xba*I polymorphisms acted as a marker through linkage disequilibrium for a truly functional sequence variation elsewhere in the gene. Therefore, to increase genetic resolution, we combined the *Pvu*II and *Xba*I polymorphisms in multi-allelic haplotype makers. However, recently Herrington et al. have shown that the C-allele of the *Pvu*II RFLP produces a functional binding site for the transcription factor B-myb, suggesting that presence of this allele may result in augmented estrogen receptor alpha transcription or produce estrogen receptor alpha isoforms that have different properties than the full-length gene product.[27]

The TA-repeat VNTR polymorphism is located in the promoter region of the ERα gene. The location of the TA-repeat VNTR polymorphism in the promoter region, 700 bp upstream of promoter "B" and 600 bp downstream of promoter "C", indicates it may have functional significance.[28] Studies have shown that variable number of tandem repeats (VNTR) polymorphisms in proximity to gene promoters, such as the TA-repeat, can have a significant influence on transcriptional regulation.[29] It is, therefore, plausible that the number of TA repeats could be important for ERα gene transcription, but molecular biological functional studies are necessary to address this issue.

How do these possibly functional polymorphisms in the ERα gene influence attained height in women? Perhaps early age at menarche leads to premature closure of the epiphyseal growth plates and subsequent shorter stature.[30] Stavrou et al. have shown that age at menarche is influenced by ERα polymorphisms.[31] However, adjustment for age at menarche did not change the association, making this a less likely mechanism. Therefore, we hypothesize that the ERα polymorphisms lead to genotype-dependent differences in ERα expression and consequently variable estrogen "sensitivity" directly at the site of linear bone growth, the epiphyseal growth plates. A prerequisite for a direct estrogen action at the level of the growth plate is the presence of ERα on chondrocytes in the growth plate. Indeed, the ERα has been localized in all zones of the growth plate, i.e. resting, proliferating, and hypertrophic chondrocytes.[32]

Two previous studies have shown an association between ERα polymorphisms and adult stature. Lehrer et al. showed that a rare synonymous polymorphism in codon 87 in exon 2 of the ERα gene ("B-variant") was associated with height in American women of multiracial descent.[33] However, given the synonymous nature

of this polymorphism it is unlikely that the "B-variant" allele will lead to a functionally different estrogen receptor alpha. This "B-variant" polymorphism has previously been shown to be in strong linkage disequilibrium with the *Pvu*II and *Xba*I polymorphisms,[34] raising the possibility that it is the *Pvu*II or *Xba*I polymorphism which is driving this observed association. In a study of adolescent boys Lorentzon et al. found an association between shorter stature and the *Pvu*II T-allele and the *Xba*I A-allele, which corresponds to the ERα haplotype 1.[35] Although this study was in young boys, it is in line with our findings in adult women.

Our study has limitations. Within the Rotterdam Study vertebral fracture data was only collected in individuals who survived the follow-up period of approximately 7 years. This resulted in a healthy responder bias (as illustrated by differences in mean age with the entire Rotterdam Study cohort). However, this bias is not likely to be genotype-dependent, since we did not find an influence of the ERα polymorphisms we studied on survival (data not shown). Genetic association studies can be influenced by population heterogeneity. This is especially true for case-control studies in a population of mixed racial origin. However, our study groups are drawn from two population based cohort studies and all subjects were Dutch Caucasians and have similar social background. Therefore, our study populations may be considered ethnically homogeneous and representative of the Dutch population.

In conclusion, in the present population-based association studies of stature, we have confirmed the role of the estrogen receptor alpha gene in determining adult stature. The effects were present in pre-, peri- and postmenopausal women, but not seen in males. However, confirmation of the role of this candidate gene in determining height does not exclude that other genes also located in the region defined by recently published genome scans do not also play a role in between-person variation in height.

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3 | Cardiovascular Disease

Chapter 3.1

Estrogen Receptor Alpha Gene Polymorphisms
predict Myocardial Infarction Risk in Women

ABSTRACT

Context: The role of estrogens in ischemic heart disease (IHD) is uncertain. Evidence suggests that genetic variations in the estrogen receptor alpha (ERα) gene may influence IHD risk, but the role of common sequence variations in the ERα gene is unclear.

Objective: To determine if the ERα haplotype created by the *Pvu*II (c.454-397T>C) and *Xba*I (c.454-351A>G) polymorphisms is associated with myocardial infarction and IHD risk.

Design, Setting, and Participants: In 6408 men and postmenopausal women from The Rotterdam Study, a population-based, prospective cohort study of participants aged 55 years and over, ERα *Pvu*II-*Xba*I haplotypes were determined. The average follow-up was 7.0 years.

Main Outcome Measures: The primary outcome was incident myocardial infarction and IHD defined as myocardial infarctions, revascularization procedures and IHD mortality. Detailed interviews, physical examinations, and blood samples were taken, including assessment of cardiovascular risk factors.

Results: In women, the ERα haplotype 1 (*Pvu*II T and *Xba*I A) allele was associated with an increased myocardial infarction risk in an allele-dose dependent manner. Approximately 29% of women were homozygous carriers of haplotype 1, 49% were heterozygous carriers and 22% were non-carriers. After adjusting for known cardiovascular risk factors such as age, previous myocardial infarction, BMI, age at menopause, use of HRT, blood pressure, smoking, diabetes mellitus and cholesterol, heterozygous carriers of haplotype 1 were at a 2.23 times increased risk of myocardial infarction (95% confidence interval (CI) 1.13-4.43) as compared to non-carriers, while homozygous carriers were at 2.48 times increased risk (95% CI 1.22-5.03). Myocardial infarction event rates for homozygous carriers, heterozygous carriers and non-carriers of haplotype 1 were 3.2%, 2.8% and 1.3%, respectively. For IHD events we observed a similar association. In women, the effect of haplotype 1 on fatal IHD was larger than on non-fatal IHD. Although the association was not significant, we observed that carriers of haplotype 1 died more often as a result of IHD. In men, the ERα haplotypes were not associated with myocardial infarctions or IHD.

Conclusions: This population-based, prospective cohort study shows a significant two-fold increased risk of incident myocardial infarction and IHD, in postmenopausal women who carry ERα haplotype 1 (*Pvu*II T-allele and *Xba*I A-allele). This association is independent of known cardiovascular risk factors.

INTRODUCTION

Ischemic heart disease (IHD) has a strong genetic component, but the identity of the genetic risk factors is unknown. There is a large ongoing effort to find genes involved in cardiovascular disease. Recently, a large case-control study was published in which 112 polymorphisms in 71 candidate genes for myocardial infarction were examined and three associated gene variants were identified.[1] However, genes involved in the pathways of sex steroids were not considered. Several lines of evidence implicate sex hormones in cardiovascular disease risk, such as the difference in disease risk between men and women. The risk of IHD in women between puberty and menopause is lower than that in age-matched men. However, this gender difference diminishes when postmenopausal women and men of similar age are compared.[2,3] These observations have led to the suggestion that it is the lowering of endogenous estrogen following menopause that may be the critical factor in removing the relative protection against IHD women have in their pre-menopausal years.

Estrogen exerts its effects by binding to the estrogen receptors alpha and beta that, once activated, regulate the expression of multiple genes. A large body of data implicates the estrogen receptor alpha gene (ERα, also known as *ESR1*) in cardiovascular disease. First, in 1994 Smith et al. described a man with a null mutation in the ERα leading to unresponsiveness to estrogen. This 31-year-old man was reported to have premature atherosclerotic coronary artery disease and endothelial dysfunction despite the presence of high levels of circulating estrogen.[4] Furthermore, ERα has been identified in most cardiovascular tissues such as the coronary arterial wall in smooth muscle cells,[5,6] endothelial cells[7] and myocardial cells.[8] In addition, in premenopausal women, fewer estrogen receptors were found in women with atherosclerotic coronary arteries than in those with normal coronary arteries.[5] Finally, variant ERα transcripts are extensively expressed in human vascular tissues.[9]

It is conceivable that common sequence variations (polymorphisms) in the ERα gene may affect cardiovascular disease risk in the general population. Several single nucleotide polymorphisms (SNP) and variable number tandem repeats (VNTR) polymorphisms have been identified in the ERα gene (http://www.ncbi.nlm.nih.gov). Cross-sectional studies have reported associations between a number of these polymorphisms in the ERα gene and cardiovascular risk factors and phenotypes, including body mass index,[10] hypertension,[11] coronary flow reserve[12] and coronary atherosclerosis.[13,14] Of the polymorphisms identified in the ERα gene, the *Pvu*II (also known as c.454-397T>C, IVS1-397 T/C and rs2234693) and *Xba*I (also known as c.454-351A>G, IVS1-351 A/G and rs9340799) SNPs are the most widely studied so far. These polymorphisms are located in the first intron of the ERα gene, 397 and 351 basepairs upstream of exon 2. Herrington and colleagues have recently shown a potential functional significance for the *Pvu*II polymorphism.[15] Therefore,

the aim of this study was to determine whether these polymorphisms in the ERα gene are associated with incident IHD and myocardial infarction in particular in a population of men and postmenopausal women aged 55 years and older. We have addressed this in a prospective study design by analyzing 6408 subjects from a population-based cohort study including the assessment of IHD and its clinically most relevant manifestation, myocardial infarction.

METHODS
Study population
The Rotterdam Study is a population-based, prospective cohort study of men and women aged 55 years and over. The study was initiated to assess prevalence, incidence, and determinants of diseases in the elderly.[16] The main focus of the Rotterdam Study was on cardiovascular disease, neurogeriatric disease, ophthalmologic disease, and locomotor disease. Between July 1st, 1989 and May 17th, 1993 all residents age 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 and 7085 participants were able to visit the research center. Baseline examinations took place between July 5th, 1989 and September 21st, 1993 and included an initial home visit and interview by a trained research assistant and an extensive physical examination at the research center. The Rotterdam Study was approved by the medical ethics committee of the Erasmus Medical Center. Each eligible person received written and oral information about the goals and research methodology of the study together with a description of the examinations involved. Written informed consent was obtained from all participants.

Our study population of 6408 participants (3791 women) included men and postmenopausal women of Caucasian origin who were able to visit the research center and who completed all parts of the baseline examination including a blood sample.

Cardiovascular risk factors
Cardiovascular risk factors were obtained by interview and physical examination at baseline. Interview information, including smoking habits, age at menopause and use of hormone replacement therapy (HRT), was obtained by a trained research assistant. HRT was defined as current or former user and never user. Smoking was categorized as current, past or never smoker. Anthropometric measurements were obtained at the research center. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Two standardized blood pressure measurements were taken with the subject in sitting position using a random zero sphygmomanometer and averaged. Hypertension was defined as a systolic pressure $\geq$ 160 mmHg and/or a diastolic pressure $\geq$ 100 mmHg and/or use of antihypertensive medication, encompassing grade 2 and grade 3 hypertension according to the World Health Organization (WHO) criteria.[17] After an overnight fast, blood

samples were obtained. Serum total cholesterol was determined by an enzymatic procedure.[18] High-density lipoprotein (HDL) was measured similarly, after precipitation of the non-HDL fraction. Diabetes mellitus was considered present with current use of antidiabetic medication or a non-fasting or postload glucose level above 11 mmol/L according to the WHO.[19] A history of myocardial infarction was based on self-reported information checked with the general practitioner's or hospital records which included written information on diagnosis and treatment and/or ECG evidence. Infarctions detected by the Modular ECG analysis system (MEANS) without evidence of symptoms (silent myocardial infarctions), were verified by an experienced cardiologist.[20,21]

Incident Ischemic Heart Disease

Follow-up started at the time of inclusion into the study and ended either on the 1st of January 2000 or, when earlier, at the participant's death. Research assistants collected follow-up data on cardiovascular disease morbidity and mortality data from the general practitioners and in case of treatment by a specialist hospital records were retrieved. With respect to vital status of the participants, information was also obtained regularly from the municipal health authorities in Rotterdam.

All collected events were verified by reviewing hospital discharge reports and letters from medical specialists. Two research physicians independently coded events according to the International Classification of Diseases, 10th revision (ICD-10).[22] In case of discrepancy, consensus was attained in a separate session. Finally, a medical expert in cardiovascular disease reviewed all coded events for final classification. In the analyses we used the following outcome measurements: incident myocardial infarction (I21) and IHD (defined as myocardial infarction (I21), percutaneous transluminal coronary angioplasty (PTCA; Z95.5), coronary artery bypass graft (CABG; Z95.1) and death from IHD (I20-I25)). In identifying myocardial infarctions, general practitioner and hospital records were consulted and all available information, which included ECG, cardiac enzymes and the clinical judgment of the treating specialist, were used to code the events. Silent myocardial infarctions were not included in the analysis. Revascularization procedures were identified by consulting hospital discharge letters from the medical specialist. For further analyses myocardial infarction and IHD were also classified as fatal and non-fatal. In addition, mortality due to all causes was also documented during follow up.

Genotyping

All participants were genotyped for the *Pvu*II and *Xba*I polymorphisms. The variations were 397 and 351 nucleotides upstream in the intron. These polymorphisms have also been described at http://www.ncbi.nlm.nih.gov/SNP under identification numbers rs2234693 (c.454-397T>C) and rs9340799 (c.454-351A>G).

DNA was extracted using proteinase K and sodium-dodecyl-sulphate digestion

at 37°C overnight and purified using phenol–chloroform extractions. The extracted DNA was then precipitated using 4 mol/l NaCl and two volumes of cold absolute ethanol. DNA was solubilized in double-distilled water and stored at -20°C until used for DNA amplification. Genotypes were determined in 5 ng genomic DNA using the Taqman allelic discrimination assay. Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems. For details see http://store.appliedbiosystems.com. Reactions were performed on the Taqman Prism 7900HT 384 wells format. We used the genotype data for each of the two polymorphisms to infer the haplotype alleles present in the population using the program PHASE which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data.[23] The alleles were defined as haplotypes such as "T-A" representing a thymidine (T) nucleotide at the *Pvu*II polymorphic site and an adenosine (A) nucleotide at the *Xba*I polymorphic site. 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).[24,25]

Statistical analysis

To compare possible confounders between subjects grouped by the ERα haplotype of interest one-way analysis of variance (ANOVA) was used for continuous variables and Pearson's chi-square for dichotomous.

The association between the ERα haplotypes and first incident IHD events was evaluated by stratifying subjects by gender and allele copy number (0,1 or 2) for the haplotype of interest and using a standard age-adjusted Cox proportional hazards model (Model 1). The proportional hazards assumption was tested and met for the Cox proportional hazards models. Based on previous analyses we chose haplotype 1 as the risk allele.[24-27] Hazard ratios of events were computed as estimates of relative risk. To account for possible confounding we excluded all subjects with previous myocardial infarctions at baseline (Model 2) and computed relative risks in a multivariate model containing the following predictors of coronary heart disease: age, BMI, age at menopause, use of HRT, systolic blood pressure, smoking, diabetes mellitus, total and HDL-cholesterol (Model 3).[28] For the analysis of fatal and non-fatal IHD, participants who did not have an IHD event during follow-up were categorized as the reference group. To study the risk of suffering a fatal IHD event between carriers and non-carriers of the ERα haplotypes a logistic regression model was used with the above mentioned cardiovascular risk factors as covariates.

For missing data on categorical covariates, we used a missing value indicator, whereas for missing data on continuous covariates, we used the median value of the respective value as calculated from the total sample. Missing values did not exceed 3.5% for any covariate. For all statistical analysis p-values below 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 11.0.1 (SPSS Inc., Chicago, USA).

Results

We observed the four possible *Pvu*II-*Xba*I haplotype alleles in the following frequencies: haplotype 1 (T-A) 53.5%, haplotype 2 (C-G) 34.7%, haplotype 3 (C-A) 11.8% and haplotype 4 (T-G) was present in 1 allele in 12,816 chromosomes. Genotype distributions were in Hardy-Weinberg equilibrium (HWE).

The baseline characteristics of the study population are shown in Table 1. ERα haplotype 1 (*Pvu*II T-allele and *Xba*I A-allele) was associated with diastolic blood pressure in women (p = 0.03).

Follow-up started at the time of inclusion into the study (between July 5th, 1989 and September 21st, 1993) and ended either on the 1st of January 2000 or, when earlier, at the participant's death. The mean follow-up duration was 7.0 years (standard deviation 2.0 years; range 18 days to 10.5 years). Of the 6408 participants, 1474 (23.0%) died of various causes during follow-up and 167 were lost to follow-up (2.6%). Two-hundred and eighty-five (4.4%) participants had an incident myocardial infarction during follow up, of which 53 (18.6%) were fatal and 232 (81.4%) non-fatal. Four-hundred and forty (6.9%) participants had an incident IHD event, of which 97 (22.0%) were fatal and 343 (78.0%) non-fatal.

In the 3791 postmenopausal women in our study, ERα haplotype 1 was significantly associated with increased risk of myocardial infarction as well as IHD (Table 2, Model 1). Exclusion of 303 women with prevalent myocardial infarctions at baseline and adjustment for age (Table 2, Model 2) and subsequently for BMI, age at menopause, use of HRT, diastolic blood pressure, smoking, diabetes mellitus, and total and HDL-cholesterol did not essentially change the results (Table 2, Model 3). As compared to non-carriers, heterozygous carriers of haplotype 1 were at a 2.23 times increased risk of myocardial infarction (95% confidence interval (CI) 1.13-4.43), while homozygous carriers were at 2.48 times increased risk (95% CI 1.22-5.03). For IHD the risk for heterozygous carriers was 2.04 times increased (95% CI 1.16-3.58), for homozygous carriers the risk was 2.41 times higher (95% CI 1.35-4.31). Adjustment for current use of HRT, as opposed to any prior HRT use, did not influence the estimates. In women, ERα haplotype 2 showed an opposite, but non-significant effect on IHD risk compared to haplotype 1: for incident myocardial infarctions the hazard ratio was 0.76 (95% CI 0.55-1.05) per copy of the "C-G" allele and 0.78 (95% CI 0.59-1.01) per allele copy for IHD. No association with myocardial infarction or IHD was observed for the ERα haplotype 3 (S.C.E. Schuit, unpublished data, November 2003).

The two polymorphisms were also analyzed separately. Given the strong linkage disequilibrium between the *Pvu*II and *Xba*I polymorphisms and the virtual non-existence of haplotype 4, haplotype 1 fully represents the *Pvu*II polymorphism. Therefore, the results for haplotype 1 presented here are exactly the same as for the *Pvu*II polymorphism alone. The TT-genotype group is represented by the absence of haplotype 1, and the TC- and CC-genotypes by the presence of one or two copies

Table 1. Baseline characteristics and ERα haplotype 1 (T-A) in 3791 women and 2617 men from the Rotterdam Study

Number of allele-copies ER haplotype 1	Women				Men			
	0 [a]	1	2	P-value	0	1	2	P-value
Numbers	832	1854	1105	0.3 [b]	560	1320	737	0.5 [b]
(%)	(21.9%)	(48.9%)	(29.1%)		(21.4%)	(50.4%)	(28.2%)	
Age (years)	70.2±9.6	70.3±9.4	70.5±9.7	0.7 [c]	67.8±7.9	68.5±8.3	68.0±8.0	0.2 [c]
BMI (kg/m²)	26.7±4.2	26.8±4.0	26.7±4.1	0.8 [c]	25.6±2.8	25.7±3.0	25.7±3.0	0.9 [c]
Age at menopause (years)	48.6±4.9	48.7±5.2	49.0±4.8	0.07 [c]				
Ever use of HRT (%)	13.0%	13.8%	12.8%	0.7 [d]				
Total cholesterol (mmol/L)	6.80±1.17	6.82±1.22	6.82±1.24	0.9 [c]	6.31±1.13	6.30±1.19	6.28±1.17	0.9 [c]
HDL-cholesterol (mmol/L)	1.44±0.36	1.43±0.39	1.43±0.36	0.8 [c]	1.22±0.30	1.22±0.32	1.21±0.36	0.7 [c]
Diastolic blood pressure (mmHg)	73.0±11.6	72.7±11.4	73.9±11.4	0.03 [c]	74.4±11.4	74.4±11.3	74.7±12.0	0.9 [c]
Systolic blood pressure (mmHg)	140.0±23.5	139.4±22.6	140.2±22.1	0.6 [c]	138.2±21.2	139.2±21.8	138.1±22.3	0.5 [c]
Smokers (%)								
Current	19.1%	17.5%	18.2%	0.7 [d]	30.2%	29.2%	30.3%	0.6 [d]
Past	28.2%	27.5%	26.5%		60.3%	62.7%	62.5%	
Hypertension (%)	36.4%	38.0%	37.8%	0.7 [d]	29.5%	30.5%	30.6%	0.9 [d]
Diabetes Mellitus (%)	11.3%	10.4%	12.1%	0.4 [d]	10.5%	11.0%	8.4%	0.2 [d]
Previous MI (%)	7.9%	9.2%	8.6%	0.5 [d]	17.8%	18.0%	18.6%	0.9 [d]

Values are expressed as mean plus/minus standard deviation

a   0, 1 and 2 denote number of copies of haplotype 1 (T-A)

b   P-value for Hardy-Weinberg Equilibrium

c   ANOVA

d   Chi-square

Table 2. Risk of myocardial infarction and IHD and ERα haplotype 1 (T-A) in women and men from the Rotterdam Study

	n	events	Model 1[c]	n	events	Model 2[d]	Model 3[e]
Women							
Myocardial Infarction							
0[a]	832	15 (1.8%)	1.00 (reference)	771	10 (1.3%)	1.00 (reference)	1.00 (reference)
1	1854	61 (3.3%)	1.85 (1.05-3.25)	1698	47 (2.8%)	2.17 (1.10-4.30)	2.23 (1.13-4.43)
2	1105	39 (3.5%)	1.96 (1.08-3.55)	1019	33 (3.2%)	2.52 (1.24-5.12)	2.48 (1.22-5.03)
Ischemic Heart Disease[b]							
0	832	23 (2.8%)	1.00 (reference)	771	15 (1.9%)	1.00 (reference)	1.00 (reference)
1	1854	88 (4.7%)	1.70 (1.08-2.70)	1698	67 (3.9%)	2.01 (1.15-3.53)	2.04 (1.16-3.58)
2	1105	57 (5.2%)	1.88 (1.16-3.05)	1019	47 (4.6%)	2.41 (1.35-4.31)	2.41 (1.35-4.31)
Men							
Myocardial Infarction							
0	560	40 (7.1%)	1.00 (reference)	465	27 (5.8%)	1.00 (reference)	1.00 (reference)
1	1320	87 (6.6%)	0.90 (0.62-1.30)	1097	63 (5.7%)	0.96 (0.61-1.51)	0.93 (0.59-1.46)
2	737	43 (5.8%)	0.78 (0.51-1.21)	610	31 (5.1%)	0.84 (0.50-1.41)	0.82 (0.82-1.38)
Ischemic Heart Disease[b]							
0	560	64 (11.4%)	1.00 (reference)	465	42 (9.0%)	1.00 (reference)	1.00 (reference)
1	1320	128 (9.7%)	0.82 (0.61-1.11)	1097	85 (7.7%)	0.84 (0.58-1.22)	0.81 (0.56-1.17)
2	737	80 (10.9%)	0.92 (0.66-1.28)	610	55 (9.0%)	0.97 (0.65-1.45)	0.93 (0.62-1.39)

a   0, 1 and 2 denote number of copies of haplotype 1 (T-A)

b   Ischemic Heart Disease is defined as incident myocardial infarction, PTCA, CABG and death from chronic IHD

c   Model 1: Adjusted for age

d   Model 2: Participants with previous myocardial infarction excluded and adjusted for age

e   Model 3: Participants with previous myocardial infarction excluded and adjusted for age, BMI, age at menopause, ever use of HRT, diastolic blood pressure, smoking, diabetes mellitus, total and HDL cholesterol

of haplotype 1, respectively. The *Xba*I A-allele was non-significantly associated with myocardial infarction risk (hazard ratio 1.31 (95% CI 0.96-1.80) per copy of the A-allele) and IHD (hazard ratio 1.29 (95% CI 0.99-1.68) per copy of the A-allele).

In the 2617 men in our study the ERα haplotypes were not significantly associated with incident myocardial infarctions or IHD (Table 2 for haplotype 1).

Figure 1 shows the association between ERα haplotype 1 and the cumulative proportional hazard of incident myocardial infarction during follow-up in women, after exclusion of participants with a prevalent myocardial infarction at baseline and adjustment for cardiovascular risk factors. Table 3 shows that, for postmenopausal women, the effect of haplotype 1 on fatal IHD was larger than on non-fatal IHD; the hazard ratio for fatal IHD was 6.13 (95% CI 1.41-26.68) for extreme genotypes and 1.86 (95% CI 0.97-3.56) for non-fatal IHD. For the effect of haplotype 1 on fatal and non-fatal myocardial infarctions results were similar. In women who do not carry haplotype 1, 13.3% of IHD events were fatal, for heterozygous carriers this was 26.9% and 34.0% for homozygous carriers. This resulted in cardiovascular risk factor adjusted odds ratios of 3.66 (95% CI 0.56-23.80) for heterozygous and 4.39
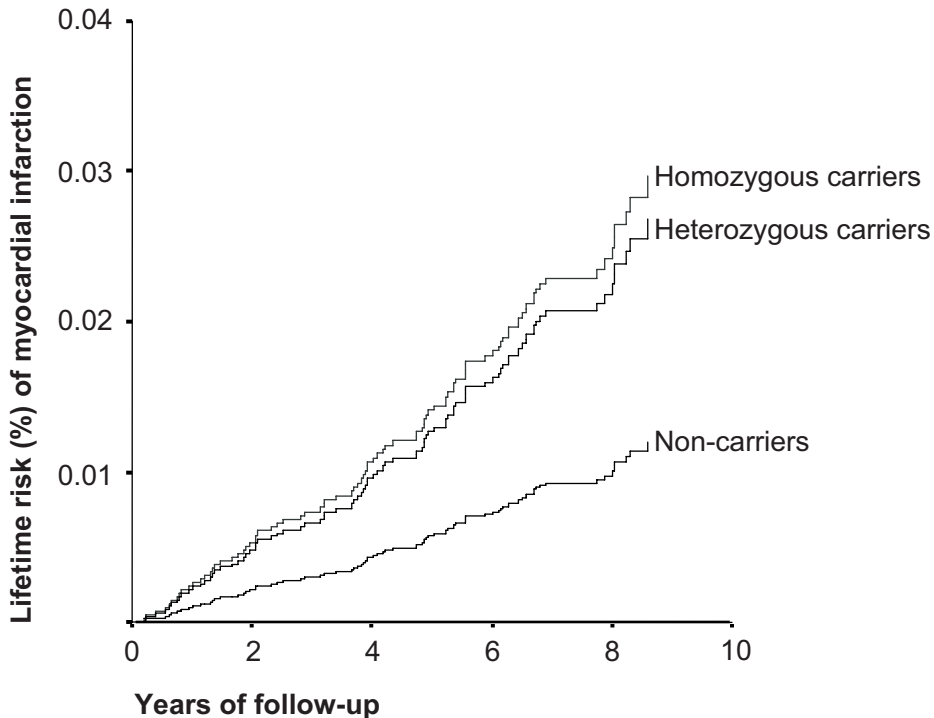

Figure 1.
Myocardial infarction hazard curve for women according to ERα haplotype 1 (T-A) in 3488 women.

Table 3. Risk of fatal and non-fatal IHD and ERα haplotype 1 ('T-A') in women from the Rotterdam Study

	n	events	Model 1[b]	n	events	Model 2[c]	Model 3[d]
Ischemic Heart Disease (IHD)							
Fatal IHD							
0[a]	813	4 (0.5%)	1.00 (reference)	758	2 (0.3%)	1.00 (reference)	1.00 (reference)
1	1789	23 (1.3%)	2.64 (0.91-7.64)	1649	18 (1.1%)	4.22 (0.98-18.17)	4.52 (1.05-19.51)
2	1065	17 (1.6%)	3.20 (1.08-9.52)	988	16 (1.6%)	6.13 (1.41-26.64)	6.13 (1.41-26.68)
Non-fatal IHD							
0	828	19 (2.3%)	1.00 (reference)	769	13 (1.7%)	1.00 (reference)	1.00 (reference)
1	1831	65 (3.5%)	1.52 (0.91-2.54)	1680	49 (2.9%)	1.68 (0.91-3.10)	1.70 (0.92-3.14)
2	1088	40 (3.7%)	1.62 (0.94-2.80)	1003	31 (3.1%)	1.85 (0.97-3.54)	1.86 (0.97-3.56)

a   0, 1 and 2 denote number of copies of ERα haplotype 1 ('T-A')

b   Model 1: Adjusted for age

c   Model 2: Participants with previous myocardial infarction excluded and adjusted for age

d   Model 3: Participants with previous myocardial infarction excluded and adjusted for age, BMI, age at menopause, ever use of HRT, diastolic blood pressure, smoking, diabetes mellitus, total and HDL cholesterol
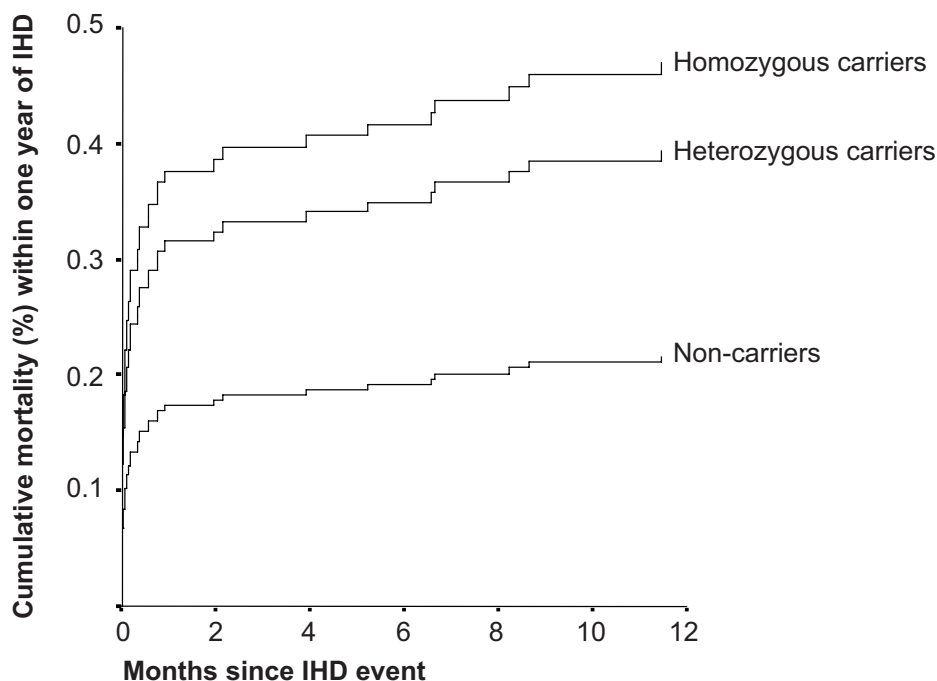
Figure 2.
First year cumulative mortality in 168 women with an IHD event.

(95% CI 0.69-28.06) for homozygous carriers versus non-carriers. For myocardial infarctions the odds ratios were similar.

In addition, we analyzed the first year cumulative mortality in the 168 women who had an IHD event during follow up. Although these results did not reach statistical significance, Figure 2 shows that the first year mortality due to all causes in female homozygous carriers of haplotype 1 is approximately twice that of non-carriers.

Discussion

In this prospective population-based study, we show an increased risk of myocardial infarctions in postmenopausal women who carry of ERα haplotype 1 (*Pvu*II T-allele and *Xba*I A-allele), while in men no association was seen. The risk estimates did not change after adjusting for clinically relevant cardiovascular risk factors, indicating that ERα haplotype 1 is an independent risk factor. Heterozygous carriers of haplotype 1 had a 2.23 times increased risk of myocardial infarction as compared

to non-carriers, while homozygous carriers had a 2.48 times increased risk. We also analyzed the risk of IHD events by taking together myocardial infarctions as well as revascularization procedures (PTCA and CABG) and IHD mortality. Using this approach, we included approximately 50% more events and observed a similar increased risk in female carriers of haplotype 1. In men no association between the ERα haplotypes and myocardial infarction or IHD risk was observed.

In women, the effect of haplotype 1 on fatal IHD was larger than on non-fatal IHD and carriers of haplotype 1 died more often as a result of IHD. Furthermore the first year cumulative mortality after IHD was two times higher in women homozygous for haplotype 1 as compared to non-carriers. This is mainly due to genotype-dependent differences in mortality within the first month after an IHD event. Although the latter two analyses did not reach statistical significance, these results suggest that postmenopausal women who carry ERα haplotype 1 are not only twice as likely to have an IHD event, they are also more likely to die from it.

A number of possible indirect as well as direct ERα-dependent mechanisms via which estrogens may exert their cardioprotective effects have been presented in the literature.[29,30] Some of the protective effects of estrogens could potentially be mediated through systemic effects such as changes in lipid profile, coagulation and fibrinolytic systems.[29] However, in our study the ERα *Pvu*II and *Xba*I genotypes were not associated with differences in a number of cardiovascular risk factors at baseline such as hypertension, hypercholesterolemia and diabetes mellitus. This is in accordance with two previous studies that have shown that the *Pvu*II and *Xba*I genotypes were not associated with baseline cholesterol levels in healthy men and postmenopausal women, as well as in men and women with pre-existing coronary artery disease.[31,32] Furthermore, when our analyses were adjusted for the presence of these and other common cardiovascular risk factors, the observed hazard ratio did not fundamentally change. This suggests that it is not through these patho-physiological pathways that ERα gene polymorphisms influence IHD disease, but that this haplotype is an independent risk factor.

Direct actions of estrogen on blood vessels could contribute substantially to the cardioprotective effects of estrogen.[29,33,34] One of these direct actions on the blood vessel wall that may be essential in IHD pathology is the influence of estrogen on nitric oxide (NO) production. Endothelial cell-derived NO plays a critical role in cardiovascular disease pathology. NO, a primary vascular target of estrogens, not only causes the relaxation of the vascular smooth muscle cells, but also inhibits platelet activation.[35] Estrogen increases NO production in vessels such as the aorta by increasing the expression and enzymatic activity of the enzyme responsible for NO synthesis, endothelial NO synthase (eNOS), as well as by inducing the release of NO. Several studies have shown that ERα is essential to all three of these estrogen effects on vascular NO production.[36-39] This is further supported by the observation that basal production of endothelium-derived NO was significantly lower in the aorta of ERα knock-out mice compared to wild-type mice.[40] Studies

have shown that ERα also mediates two other effects of estrogen in the vessel wall: acceleration of re-endothelialization and inhibition of the vascular injury response.[41,42] Particularly the latter two mechanisms may explain why the ERα polymorphisms are most strongly associated with fatal IHD.

How could these specific polymorphisms in the ERα gene influence myocardial infarction risk in postmenopausal women? The *Pvu*II and *Xba*I polymorphisms have been an important area of research in diseases such as osteoporosis,[24,43,44] cardiovascular disease,[31] and cancer.[45] A number of hypotheses for the functional significance of these polymorphisms have been reported in the literature. Given their location, 397 and 351 basepairs upstream from the start of exon 2, possible functional mechanisms include altering ERα expression via altered binding of transcription factors and influencing alternative splicing of the ERα 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 ERα gene. The first mechanism was recently supported by findings of Herrington et al.[15] which were confirmed in our own laboratory (S.C.E. Schuit, unpublished data, November 2003). Herrington et al. showed that the *Pvu*II T-allele eliminates a functional binding site for the transcription factor B-myb. This suggests that presence of this allele may result in lower ERα transcription. In the presence of a decreased number of alpha estrogen receptors, estrogen signaling may be less effective and, therefore, estrogen actions may be decreased. These findings are further supported by the observation in our study population, as well as in others, that this *Pvu*II T-allele has previously been associated with a number of phenotypes that are known to be related to low estradiol levels and therefore low estrogen activity, such as increased risk of osteoporosis, decreased risk of osteoarthritis, and hysterectomy, lower BMI, shorter stature, and later age at menopause.[10,24-27] In our study, we have found the T-allele to be associated with increased risk of myocardial infarction and IHD events in postmenopausal women. This suggests that the potentially lower ERα expression caused by the presence of the T-allele at the -397 polymorphic site may lead to a higher susceptibility to IHD. The fact that the *Xba*I A-allele is also associated with IHD may be due to linkage disequilibrium with the *Pvu*II SNP or to functional significance of the *Xba*I polymorphism itself.

An intriguing aspect of this study is that ERα haplotype 1 is significantly associated with an increased risk of IHD in women, but not in men. Recently, Shearman et al. also reported an association between the *Pvu*II polymorphism and cardiovascular disease in the Framingham Heart Study.[46] This study found that the *Pvu*II T-allele prevented IHD in men. This is seemingly in contrast to our findings that the T-allele prevents IHD in women in our study. However, there is not a clear conflict with the seemingly opposite results reported by Shearman et al. since the men in our study showed a non-statistical trend opposite to the women. Furthermore, the men in the Rotterdam Study were much older than the

men from Framingham Heart Study and we know that effects of risk factors often change in older cohorts. Furthermore, the Framingham Heart Study had very few cardiovascular events in women, for example only 3 cases of myocardial infarction were documented in the female participants of that study.

An explanation for these opposing results in men and women is not immediately apparent. The most obvious difference between men and postmenopausal women is the cessation of gonadal function in women. Perhaps the presence of an intact hypothalamic-pituitary-gonadal (HPG) axis in men leads to protection of the ERα *Pvu*II T-allele on cardiovascular disease, while in postmenopausal women, who are completely dependent on peripheral conversion to estradiol, an opposing effect occurs. In men, compared to postmenopausal women of the same age, estradiol levels are approximately three times higher (S.C.E. Schuit, unpublished data, November 2003). Apparently in the presence of sufficiently high estradiol levels, differences in ERα expression do not have clinical consequences. However, estrogen deficiency after menopause in combination with lower ERα expression caused by the *Pvu*II T-allele may lead to a higher susceptibility to IHD. This could explain why we did not see an association between ERα gene polymorphisms and IHD in men.

There are limitations to genetic association studies. 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 prospective cohort study-design was chosen and all subjects were of Dutch Caucasian origin. Furthermore, the *Pvu*II and *Xba*I genotypes were in Hardy-Weinberg equilibrium and haplotype frequencies were similar to those found in other studies of Caucasian subjects.[47] Therefore, our study population may be considered ethnically homogeneous and representative of the Dutch population. Another limitation of association studies is the definition of the phenotype of interest and the occurrence of phenotype heterogeneity. We diagnosed incident myocardial infarctions and IHD in strict adherence to the ICD-10 guidelines and consulted hospital discharge letters and reports from treating specialists to identify cardiovascular disease events. Therefore, we feel that phenotype heterogeneity will not have influenced our results. Furthermore, in the Netherlands the only way to access specialist and hospital care is by consulting a general practitioner. Therefore, checking the general practitioners' medical records for all participants should have resulted in a near complete follow-up. As can be said for all association studies, the validity of genetic association studies is greatly strengthened by confirmation of the results. The findings reported in this study are supported by not only functional studies, but also by associations with other phenotypes (pleiotropy) in women such as osteoporosis, osteoarthritis, hysterectomy, BMI, stature and age at menopause. The presented body of data supports the theory that in women the presence of the *Pvu*II T-allele leads to lower estrogen action.

Selective non-response of subjects with impaired health, or otherwise at an increased risk of cardiovascular disease, may have occurred. However, such a

non-response bias will presumably not be genotype dependent and will not lead to overestimation of the hazard ratios.

In interpreting the clinical implications of these results we must take in account that 78% of the population is carrier of the ERα haplotype 1 risk allele and that heterozygous and homozygous carriers of haplotype 1 have a two-fold increased risk of IHD. Perhaps we should view this not as a "risk" allele, but consider the non-carriers as having a protective allele. This implies that non-carriers (22% of the population) have a 50% reduced risk.

In conclusion, this population-based prospective cohort study shows a significant two-fold increased risk of incident myocardial infarction, as well IHD events, in postmenopausal women who carry ERα haplotype 1 (*Pvu*II T-allele and *Xba*I A-allele). The association was not explained by known cardiovascular risk factors such as age, previous myocardial infarction, BMI, age at menopause, use of HRT, blood pressure, smoking, diabetes mellitus and cholesterol. Therefore, haplotype 1 is an independent risk factor for IHD. Furthermore, our results also suggest that postmenopausal women who carry ERα haplotype 1 are not only twice as likely to have an IHD event, they are also more likely to die from it.

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Chapter 3.2

Estrogen Receptor Alpha Gene
Polymorphisms associated with Stroke
and White Matter Lesions

Abstract

Lacunar brain infarcts and cerebral white matter lesions are frequently seen on MRI scans in elderly people. These lesions probably result from cerebral small vessel disease. Since the risk of cerebrovascular disease is different between men and women, estrogens and estrogen receptor genes may play a role. We studied the role of common sequence variations in the ERα gene in relation to infarcts and white matter lesions.

From the Rotterdam Scan Study, a population-based, prospective cohort study of participants aged 60 years and over, 522 non-demented men and postmenopausal women underwent cerebral MRI scanning and ERα *Pvu*II-*Xba*I haplotypes were determined.

In postmenopausal women ERα *Pvu*II-*Xba*I haplotype 1 (allele frequency 54.6%) was associated with a two to three times increased risk of brain infarcts on MRI and increased severity of subcortical white matter lesions, both in an allele-dose dependent manner. In men, opposite effects were observed, male carriers of haplotype 1 had significantly less severe periventricular as well as subcortical white matter lesions. Furthermore, although the association was not significant, male carriers of haplotype 1 tended to have brain infarcts on MRI less frequently. These associations were independent of known cardiovascular risk factors such as age, BMI, smoking, systolic blood pressure, diabetes mellitus, total and HDL-cholesterol, age at menopause and ever use of HRT. The findings in women are in line with a previous report by the Rotterdam Study that ERα haplotype 1 was associated with a two-fold increased risk of ischemic heart disease in postmenopausal women, but not in men.

In conclusion, in postmenopausal women, the ERα *Pvu*II-*Xba*I haplotype 1 allele increases the risk of cerebral small vessel disease related lesions on MRI, while in men an opposite effect is seen.

INTRODUCTION

Brain infarcts and cerebral white matter lesions are frequently observed on magnetic resonance imaging (MRI) scans in elderly people.[1,2] These lesions are associated with an increased risk of stroke and dementia.[3-5] They are considered to be caused by ischemic small vessel disease and have a multifactorial etiology. However, the exact pathogenesis is not fully understood.

Epidemiological and animal-based studies provide strong evidence that genetic factors are important in the pathogenesis of cerebrovascular disease.[6] A number of monogenetic disorders responsible for cerebrovascular disease have been identified, but the prevalence of each is quite low.[6] Therefore, the population-attributable risk of cerebrovascular disease due to these disorders is also very limited. The majority of cases of cerebrovascular disease in the general population are multifactorial in etiology and here genetic factors are expected to play an important role. As in other complex traits, classical patterns of inheritance can not be demonstrated and the genetic etiology is polygenic.

The risk of stroke in women between puberty and menopause is almost half the risk in age-matched men.[7,8] However, this gender difference diminishes within ten years after menopause.[9,10] This observation has led to the suggestion that it is the lowering of endogenous estrogen following menopause that may be the critical factor in removing the relative protection against stroke women have in their pre-menopausal years. Furthermore, lacunar brain infarcts and white matter lesions seem to be more frequent in elderly women than men,[4,11] indicating a role for estrogen in cerebral small vessel disease pathology.

Common variations in the gene encoding estrogen receptor α (ERα, also known as *ESR1*) that alter its expression or function may account for some of the genetic variability in the general population, especially in postmenopausal women. ERα, the most well studied of the two estrogen receptors (the other being ERβ), regulates gene expression resulting in transcription activation of numerous genes. ERα gene expression has been identified in a number of human vascular tissues such as endothelial cells and vascular smooth muscle cells.[12,13] Several single nucleotide polymorphisms (SNP) and variable number tandem repeats (VNTR) polymorphisms have been identified in the ERα gene (http://www.ncbi.nlm.nih.gov). Of the polymorphisms identified, the *Pvu*II and *Xba*I SNPs, located in the first intron 397 and 351 basepairs upstream of exon 2, are the most widely studied so far. The haplotype created by these two SNPs has recently been associated with cardiovascular disease risk in women in our study population.[14]

The aim of this study was to determine whether polymorphisms in the ERα gene are associated with prevalent brain infarcts and the extent of white matter lesions on MRI in men and postmenopausal women aged 60 years and older.

Methods

Study population

This study is based on the Rotterdam Study, a large prospective population-based study in 7983 elderly aged 55 years or older that aims to assess determinants of diseases in later life.[15] The Medical Ethics Committee of Erasmus Medical Center approved the study protocol and participants gave written informed consent. Baseline examinations took place from 1990 to 1993. In 1995 to 1996, a random selection was made of 965 living members of the cohort in strata of age (5-years strata from 60 to 90) and sex for participation in the Rotterdam Scan Study. As part of the eligibility criteria, we excluded persons who had dementia at the time of selection (n=17) and persons who had contraindications to undergo MRI (n=116). This left 832 participants eligible. MRI images were obtained for 563 (68%) of these eligible subjects in 1995 and 1996. Participants of non-Caucasian decent (n=7) were excluded from analyses and all women were postmenopausal. Data on cardiovascular risk factors was not available for 41 individuals leaving 522 participants (258 women).

Cardiovascular risk factors

Cardiovascular risk factors were obtained by interview and physical examination from 1995 to 1996. Interview information, including smoking habits (never, current, former smoker), age at menopause and use of hormone replacement therapy (HRT, ever versus never use), was obtained by a trained research assistant. Participants were asked to bring all prescribed medication to the research center, where a physician checked the indication for use. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Blood pressure was measured twice using a random zero sphygmomanometer with the subject in sitting position and the results were averaged. Hypertension was defined as a systolic pressure $\geq$ 140 mmHg or a diastolic pressure $\geq$ 90 mmHg, or use of antihypertensive medication. Diabetes mellitus was defined as the use of oral anti-diabetics, insulin or a random or post-load glucose level of 11.1 mmol/l or higher. Serum total cholesterol and HDL-cholester were determined by an enzymatic procedure.[16]

Cerebral Infarcts and White Matter Lesions

Axial T1-, T2- and proton density-weighted scans were made on a 1.5-T MRI scanner (MRVISION, Siemens, Germany) as described previously.[5] Slices were 5 mm tick with an interslice distance of 1 mm. Infarcts were defined as $\geq$ 3 mm focal hyperintensities on T2-weighted images. Lesions in the white matter also had to have corresponding prominent hypointensities on T1-weighted images to distinguish infarcts from cerebral white matter lesions. Lacunar infarcts were defined as infarcts 3 to 20 mm in size and located in the subcortical white matter or basal ganglia.

White matter lesions were considered present if visible as hyperintense on proton density and T2 weighted images, without prominent hypointensity on T1 weighted scans. We summed three region specific semiquantitative grades (lesions adjacent to the frontal horns, the lateral walls, and the occipital horns of the lateral ventricle) to get a total periventricular white matter lesions grade (range 0-9). We counted subcortical white matter lesions in three size categories based on their maximal diameter (< 3 mm, 3-10 mm, >10 mm). A total volume was approximated by assuming that these subcortical lesions were spherical with a fixed diameter (volume range 0-29.5 ml). Both intra-rater and inter-rater studies (n=100) showed a good to excellent agreement (κ = 0.79-0.90, r = 0.88-0.95).[17]

Genotyping

Genomic DNA was isolated from peripheral leucocytes 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 http://store.appliedbiosystems.com. Reactions were performed on the Taqman Prism 7900HT 384 wells 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.[18] The alleles were defined as haplotypes such as "C-G" representing the C-allele for the *Pvu*II SNP and the G-allele for the *Xba*I SNP. 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 variance (ANOVA), linear regression and Pearson's chi-square were used to compare baseline characteristics between subjects grouped by the *Pvu*II-*Xba*I haplotype of interest.

The association between *Pvu*II-*Xba*I haplotypes and mean values for size and grade of periventricular and subcortical white matter lesions was evaluated by stratifying subjects by gender and allele copy number (0,1 or 2) for the haplotype of interest and using a multivariate linear regression model. Based on previous analyses we chose haplotype 1 as the risk allele.[14,19-22] To evaluate the association with cerebral infarcts a multivariate logistic regression model was used. In the analyses with lacunar infarcts, people with non-lacunar infarcts were excluded. Since confounding may have influenced the observed associations, the analyses were adjusted for predictors of cerebrovascular disease: age, BMI, age at menopause, use of HRT, systolic blood pressure, smoking, diabetes mellitus, total and HDL-cholesterol.

We allowed for three possible models to explain differences between genotype groups, i.e., an allele dose effect, a dominant effect, or a recessive effect. Allele dose was defined as the number of copies of the haplotype.

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

Results

We observed the four possible ERα *Pvu*II-*Xba*I haplotype alleles in the following frequencies: haplotype 1 (T-A) 54.6%, haplotype 2 (C-G) 35.6%, haplotype 3 (C-A) 9.8% and haplotype 4 (T-G) was not present in our population. Genotype distributions were in Hardy-Weinberg equilibrium (HWE) and similar between genders and across age categories.

The baseline characteristics of the 522 participants in our study population are shown in Table 1. ERα *Pvu*II-*Xba*I haplotype 1 ("T-A") was not associated with any of the baseline characteristics.

MRI diagnosed cerebral infarcts were found in 138 (26.4%) participants. Of these 115 (83.3%) were lacunar infarcts. In the 258 postmenopausal women in our study, *Pvu*II-*Xba*I haplotype 1 was significantly associated with brain infarcts in an allele-dose dependent manner (Table 2). After adjustment for the cardiovascular risk factors age, BMI, systolic blood pressure, smoking, diabetes mellitus, total and HDL-cholesterol, age at menopause and ever use of HRT, homozygous carriers of haplotype 1 were at a 2.95 (95% confidence interval (CI) 1.09-8.04) times increased risk of having one or more brain infarcts visible by MRI as compared to non-carriers, while heterozygotes were at a 2.34 (95% CI 0.91-6.02) times increased risk. In looking specifically at lacunar brain infarcts a similar allele-dose dependent association with ERα haplotype 1 was observed. In women, ERα haplotype 2 showed a significant, opposite allele-dose effect on brain infarcts: the odds ratio for homozygous carriers versus non-carriers was 0.21 (95% CI 0.06-0.81; results not shown). No association between ERα haplotype 3 and brain infarcts was found (results not shown).

Although no significant association was found between ERα haplotypes and brain infarcts in the 264 male participants of our study, brain infarcts on MRI were seen less often in male carriers of haplotype 1 than in non-carriers (Table 2).

The overall mean subcortical white matter lesion volume was 1.8 ml (SD 3.4 ml) and the mean periventricular WML grade was 2.8 (SD 2.2). In both men and women, white matter lesions were associated with ERα haplotype 1 (Table 3). In postmenopausal women, ERα haplotype 1 was associated with an almost two times greater severity of subcortical white matter lesions (p-trend = 0.04). For periventricular white matter lesions similar results were seen, however, statistical significance was not reached. In men, ERα haplotype 1 was significantly associated with a two times lower volume of subcortical (p-trend = 0.05) white matter lesions. For periventricular white matter lesions similar significant results were seen (p-trend = 0.03). Adjustment for cardiovascular risk factors did not change these associations.

Table 1. Baseline characteristics by ERα haplotype 1 (T-A) in 258 women and 264 men from the Rotterdam Scan Study

Number of allele-copies ER haplotype 1	Women				Men			
	0[a]	1	2	P-value	0	1	2	P-value
Numbers (%)	47 (18.2%)	135 (52.3%)	76 (29.5%)	0.3 [b]	59 (22.3%)	127 (48.1%)	78 (29.5%)	0.6 [b]
Age (years)	73.6 (1.2)	73.7 (0.7)	72.7 (0.9)	0.7 [c]	72.8 (1.0)	74.3 (0.7)	72.2 (0.9)	0.1 [c]
BMI (kg/m^2)	26.5 (0.6)	26.6 (0.3)	26.7 (0.5)	0.9 [c]	26.2 (0.4)	25.9 (0.3)	26.5 (0.3)	0.4 [c]
Age at menopause (years)	48.7 (0.8)	48.3 (0.4)	49.3 (0.6)	0.4 [c]				
Ever use of HRT (%)	0%	5.9%	2.6%	0.2 [d]				
Total cholesterol (mmol/L)	6.23 (0.15)	6.02 (0.09)	6.34 (0.12)	0.09 [c]	5.47 (0.13)	5.55 (0.09)	5.61 (0.11)	0.7 [c]
HDL-cholesterol (mmol/L)	1.44 (0.05)	1.41 (0.03)	1.36 (0.04)	0.5 [c]	1.13 (0.04)	1.13 (0.03)	1.14 (0.03)	1.0 [c]
Diastolic blood pressure (mmHg)	75.3 (1.5)	77.1 (0.9)	75.8 (1.2)	0.5 [c]	76.8 (1.6)	76.6 (1.1)	77.1 (1.4)	1.0 [c]
Systolic blood pressure (mmHg)	142.9 (2.8)	147.9 (1.6)	146.7 (2.2)	0.3 [c]	142.9 (2.8)	146.6 (1.9)	145.5 (2.4)	0.5 [c]
Smokers (%)				0.2 [d]				1.0 [d]
Current	14.9%	12.5%	21.1%		20.3%	19.4%	17.9%	
Former	46.8%	34.6%	31.6%		69.5%	72.9%	74.4%	
Hypertension (%)	44.7%	54.4%	52.6%	0.5 [d]	47.5%	55.0%	53.8%	0.6 [d]
Diabetes Mellitus (%)	2.1%	2.2%	5.3%	0.4 [d]	8.5%	9.3%	1.3%	0.07 [d]

Values are unadjusted means (plus/minus standard error) or percentages

a   0, 1 and 2 denote number of copies of haplotype 1 (T-A)

b   P-value for Hardy-Weinberg Equilibrium

c   ANOVA

d   Chi-square

Table 2. Brain infarcts and ERα haplotype 1 (T-A) in 258 women and 264 men from the Rotterdam Scan Study

	Total participants	Participants with infarcts on MRI	Model 1[b]	Model 2[c]
Women				
Brain infarct on MRI				
0[a]	47	7 (14.9%)	1.00 (reference)	1.00 (reference)
1	135	37 (27.4%)	2.16 (0.89-5.24)	2.34 (0.91-6.02)
2	76	25 (32.9%)	2.80 (1.10-7.13)	2.95 (1.09-8.04)
Lacunar infarct on MRI				
0	46	6 (13.0%)	1.00 (reference)	1.00 (reference)
1	131	33 (25.2%)	2.24 (0.87-5.77)	2.22 (0.81-6.07)
2	75	24 (32.0%)	3.13 (1.17-8.40)	3.23 (1.12-9.29)
Men				
Brain infarct on MRI				
0	59	17 (28.8%)	1.00 (reference)	1.00 (reference)
1	127	36 (28.3%)	0.98 (0.49-1.94)	0.83 (0.40-1.73)
2	78	16 (20.5%)	0.64 (0.29-1.40)	0.64 (0.28-1.49)
Lacunar infarct on MRI				
0	54	12 (22.2%)	1.00 (reference)	1.00 (reference)
1	119	28 (23.5%)	1.08 (0.50-2.32)	0.88 (0.39-2.00)
2	74	12 (16.2%)	0.68 (0.28-1.65)	0.67 (0.26-1.74)

a   0, 1 and 2 denote number of copies of haplotype 1 (T-A)

b   Model 1: Unadjusted

c   Model 2: Adjusted for age, BMI, systolic blood pressure, smoking, diabetes mellitus, HDL and total cholesterol (plus age at menopause and ever use of HRT in women)

Table 3. White matter lesions by ERα haplotype 1 (T-A) in 258 women and 264 men from the Rotterdam Scan Study

Number of allele-copies	Women				Men			
ER haplotype 1	0[a]	1	2	P-value[b]	0	1	2	P-value [b]
Numbers (%)	47 (18.2%)	135 (52.3%)	76 (29.5%)		59 (22.3%)	127 (48.1%)	78 (29.5%)	
Subcortical white matter lesions (ml)								
Crude	1.38 (0.49)	1.73 (0.29)	2.58 (0.39)	0.04	2.18 (0.43)	1.85 (0.29)	1.09 (0.37)	0.05
Adjusted[c]	1.41 (0.89)	1.64 (0.77)	2.55 (0.80)	0.03	2.26 (0.56)	1.64 (0.48)	1.21 (0.57)	0.07
Periventricular white matter lesions (grade 0-9)								
Crude	2.81 (0.33)	2.75 (0.19)	2.91 (0.26)	0.7	3.19 (0.28)	2.69 (0.19)	2.38 (0.24)	0.03
Adjusted[c]	2.61 (0.56)	2.49 (0.48)	2.77 (0.50)	0.5	3.43 (0.35)	2.76 (0.30)	2.73 (0.35)	0.05

Values are expressed as mean plus/minus standard error

a   0, 1 and 2 denote number of copies of haplotype 1 (T-A)

b   Linear regression

c   Adjusted for age, BMI, systolic blood pressure, smoking, diabetes mellitus (plus age at menopause and ever use of HRT in women)

Discussion

The present population-based study demonstrates that in postmenopausal women ERα *Pvu*II-*Xba*I haplotype 1 is associated with increased risk of cerebral infarcts on MRI and increased severity of white matter lesions. In men, opposite effects were observed, haplotype 1 was significantly associated with decreased white matter lesion severity. Although statistical significance was not reached, brain infarcts were seen less often in male carriers of haplotype 1 than in non-carriers. These associations were independent of known cardiovascular risk factors such as age, BMI, smoking, systolic blood pressure, diabetes mellitus, total and HDL-cholesterol age at menopause and ever use of HRT.

Brain infarcts and white matter lesions were frequent in our population and are associated with an increased risk of stroke and dementia.[3-5] Several lines of evidence support the idea that estrogen and the ERα are important in the pathogenesis in cerebrovascular disease. Schmidt et al. reported a lower incidence and less extensive white matter hyperintensities, and smaller total white matter hyperintensity areas on MRI scans in women on hormone replacement therapy.[23] This was interpreted as reflecting less silent ischemic brain damage in estrogen users. In addition, the total volume of white matter hyperintensity areas was inversely related to the duration of estrogen therapy. Evidence supporting a mediatory role for ERα in estrogen related neuroprotection has been reported by Dubal et al. They demonstrated that following middle cerebral artery occlusion in rats, ERα expression was highly upregulated in the injured cerebral cortex.[24] Furthermore, they also established that ERα is a critical mechanistic link in mediating the protective effects of estradiol in brain injury.[25] Finally, Sawada et al. reported that administration of a potent ER antagonist dramatically increased infarct size in female rats following middle cerebral artery occlusion.[26]

A number of possible indirect as well as direct ERα-dependent mechanisms via which estrogens may exert their cardioprotective effects have been presented in the literature.[27,28] Some of the protective effects of estrogens could potentially be mediated through systemic effects such as changes in lipid profile, coagulation and fibrinolytic systems.[27] In our study, ERα *Pvu*II-*Xba*I genotypes were not associated with differences in a number of cardiovascular risk factors at baseline such as hypertension, hypercholesterolemia and diabetes mellitus. Furthermore, when our analyses were adjusted for the presence of these and other common cardiovascular risk factors, the observed hazard ratio did not fundamentally change. These findings suggest that it is not through these known risk factors that ERα gene polymorphisms influence cerebrovascular disease. However, we can not exclude a role for the coagulation and fibrinolytic systems.

Direct actions of estrogen on blood vessels could contribute substantially to the vascular protective effects of estrogen.[27,29,30] One of these direct actions on the blood vessel wall that may be essential in ischemic cerebrovascular disease

pathology, is the influence of estrogen on nitric oxide (NO) production. NO, a primary vascular target of estrogens, not only causes the relaxation of the vascular smooth muscle cells; it also inhibits platelet activation.[31] Estrogen increases NO production in vessels such as the cerebral microvessels by increasing the expression and activity of the enzyme responsible for endothelial NO synthesis (eNOS), as well as by inducing the release of NO. Several studies have shown that ERα is essential to all three of these effects of estrogen on vascular NO production.[32-35] This hypothesis is further supported by the observation that basal production of endothelium-derived NO was significantly lower in the aortas of ERα knock-out mice compared to wild-type mice.[36]

The large majority (84%) of strokes detected by MRI are silent, that is there are no clinical manifestations, and most silent strokes (89%) are lacunar infarcts.[4,37] Cerebral brain infarcts, in particular lacunar brain infarcts, and white matter lesions are considered to be caused by ischemic small vessel disease.[38] Perhaps augmented auto-regulation in small cerebral arteries due to changed NO action causes to the genotype-dependent differences in the prevalence of infarcts and white matter lesions.

How do these specific polymorphisms in the ERα gene influence cerebral infarction risk in postmenopausal women? The *Pvu*II and *Xba*I polymorphisms have been an important area of research in diseases such as osteoporosis,[22,39,40] cardiovascular disease,[41] and cancer.[42] A number of hypotheses for the functional significance of these polymorphisms have been reported in the literature. Given their location, 397 and 351 basepairs upstream from the start of exon 2, possible functional mechanisms include changed ERα expression via altered binding of transcription factors and influence on alternative splicing of the ERα 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 ERα gene. The first mechanism was recently supported by findings of Herrington et al.[43] and which were confirmed in our own laboratory (chapter 5.1). Herrington et al. showed that the T-allele of the *Pvu*II RFLP eliminates a functional binding site for the transcription factor B-myb. This implies that the presence of this allele may result in lower ERα transcription. This finding is supported by the observation in our study population, as well as in others, that the T-allele of the *Pvu*II polymorphism is associated with increased risk of phenotypes that are known to be related to decreased estradiol-effectiveness such as osteoporosis,[22] decreased risk of osteoarthritis and hysterectomy,[21,44] shorter stature,[19] and later age at menopause.[21] In our study, we have found the T-allele to be associated with increased risk of cerebral infarcts and white matter lesions in women in an allele dose-dependent manner. Lower ERα expression caused by the *Pvu*II T-allele may be the functional mechanism behind this finding in females. In line with this hypothesis the fact that the *Xba*I polymorphism A-allele is also associated with estradiol levels may be due to linkage disequilibrium with the *Pvu*II SNP or another functional polymorphism,

or due to functional significance of the *Xba*I polymorphism itself.

An intriguing aspect of this study as that opposing effects are observed in men and women. This is not the first time opposite effects of ERα polymorphism have been reported in men and women. Recently, two studies have reported associations between these SNPs and cardiovascular disease risk: the Rotterdam Study and the Framingham Study.[14,45] In the Rotterdam Study, ERα *Pvu*II-*Xba*I haplotype 1 is an independent risk factor for myocardial infarction and other forms of ischemic heart disease in postmenopausal women.[14] In men, no significant association was observed with ischemic heart disease. In the Framingham study, Schearman et al. reported that the T-allele of the *Pvu*II polymorphism prevented cardiovascular disease, including stroke in men.[45] The association in women could not be examined due to lack of power. These findings may be compared to our findings for haplotype 1 since the *Pvu*II polymorphism is fully represented by haplotype 1 given the strong linkage disequilibrium between the *Pvu*II and *Xba*I SNPs,. Rather than concluding the results of Rotterdam and Framingham studies are contradictory, one may venture to theorize that ERα polymorphisms have opposing effects in men and women. Especially in light of the findings of the present study where opposite effects of ERα haplotype 1were also seen in men and women.

A paradoxical relationship seems to exist between ERα gene polymorphisms and gender. The Rotterdam Study recently reported an association between haplotype 1 and lower estradiol levels in postmenopausal women, a finding which may be inline with the increased risk cerebral infarcts and white matter lesions in women reported here.[20] This study also reported a non-significant trend in the opposite direction in men, male carriers of haplotype 1 tended to have higher estradiol levels then their non-carrier counterparts. It is hypothesized that the reason for such an opposite effect was that elderly men, in contrast to postmenopausal women, still have a largely intact hypothalamic-pituitary-gonadal (HPG) axis and estradiol still plays an important role in the regulation of gonadotropin release. These findings fit with our observation that these polymorphisms seem to play a protective role against cerebrovascular disease in men, but are a risk factor in women.

The overall picture presented by our results for the role of ERα gene polymorphisms in cerebral small vessel disease is that in women haplotype 1 is an independent risk factor, while in men haplotype 1 is an independent protective factor. However, not all the associations were significant in men and women. In women, cerebral infarcts and subcortical white matter lesions were significantly increased. For periventricular lesions the same trend was observed in women, however statistical significance was not reached. In men, both subcortical and periventricular lesions were significantly decreased. Although a similar reduction of cerebral infarcts was observed in men, statistical significance not reached. From our results we can not determine whether the non-significant results for periventricular white matter lesions in women and cerebral infarcts in men are false-negative results or that different pathophysiological mechanisms are the basis. Differences in the

vascularization and susceptibility to hypoperfusion of the different brain regions might underlie the differences in associations.[46] Further studies will be necessary to answer this question in the future.

The strengths of this study are the population-based design and the large number of elderly non-demented participants in whom both brain imaging and ERα genotyping are available. However, the possibility of population stratification and related limitations, as cautioned for in all genetic association studies, can not be excluded. For the present study a population-based cohort study-design was chosen and all subjects were of Dutch Caucasian origin. Furthermore, the *Pvu*II-*Xba*I genotypes were in Hardy-Weinberg equilibrium and haplotype frequencies were similar to those found in other studies of Caucasian subjects.[47] Therefore, our study population may be considered ethnically homogeneous and representative of the Dutch population. We can not exclude that selection bias played a role in our findings since the response rate in our study was 54%. However, such a non-response bias will presumably not be genotype dependent and will not lead to overestimation of the hazard ratios.

In conclusion, this population-based study showed an increased risk of cerebrovascular disease in postmenopausal women who carry the ERα *Pvu*II-*Xba*I haplotype 1 allele. In men, this same allele protected against cerebrovascular disease. These associations were not explained or influenced by known cardiovascular risk factors such as age, previous myocardial infarction, BMI, age at menopause, use of HRT, blood pressure, smoking, diabetes mellitus and total and HDL-cholesterol. These findings are in line with a previous report by the Rotterdam Study that ERα haplotype 1 is associated with a two-fold increased risk of ischemic heart disease in postmenopausal women[14] and a report from the Framingham Study that haplotype 1 is a protective factor against cardiovascular disease in men.[45]

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4 Sex-hormone Levels

Chapter 4.1

Estrogen Receptor Alpha Gene Polymorphisms
are Associated with Estradiol levels in
Postmenopausal Women

ABSTRACT

Individual variation in postmenopausal estradiol (E_2) level is an important determinant of diseases such as osteoporosis. It has been suggested that the estrogen receptor alpha (ERα) gene may influence serum E_2 levels, but the role of common sequence variations in the ERα gene is unclear.

In 631 postmenopausal women and 528 men from the Rotterdam Study, a population-based, prospective cohort study of individuals aged 55 years and over, ERα *Pvu*II-*Xba*I haplotypes were determined. In women, haplotype 1 (T-A) was significantly associated with an allele-dose dependent decrease in E_2. After adjusting for age, BMI, years since menopause and testosterone levels, serum E_2 levels decreased by 1.90 pmol/L per allele copy of this haplotype (p-trend 0.02). Extreme genotypes, representing 23% and 27% of the population, varied 3.93 pmol/L. No association with serum testosterone was observed. In a subset of 446 women, no association with dehydroepiandrosterone sulfate, androstenedione or estrone was seen. In men, none of the sex-hormone levels were associated with the ERα *Pvu*II-*Xba*I haplotypes.

In conclusion, we have demonstrated a role for genetic variations in the ERα gene in determining postmenopausal E_2 levels in women. From our finding that E_2 levels are, while estrone (E_1) levels are not, associated with these ERα polymorphisms, we hypothesize that it is likely that these common sequences variants alter expression or activity of 17β-HSD subtype 1 or 7.

INTRODUCTION

Estradiol (E_2) has a general metabolic role that reaches far beyond reproductive processes. E_2 levels play an important role in a number of diseases. For example, withdrawal of the effects of E_2 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 E_2 during the postreproductive 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 E_2 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 serum E_2 levels is the estrogen receptor alpha (ERα, also known as estrogen receptor 1, *ESR1*) gene. At first glance, the importance of the most common of the two estrogen receptor genes (the other being the estrogen receptor beta) in E_2 biosynthesis may not be obvious. However, E_2 exerts its effects by binding to estrogen receptors that, once activated, regulate the expression of multiple genes. One of the genes E_2 and ERα may regulate is the aromatase (also known as *CYP19*) gene, as recently found by Kinoshita et al.[5] Aromatase catalyzes the conversion of C19-steroids to estrogens and is essential for E_2 biosynthesis. Kinoshita et al. found that E_2, through ERα, can modulate *CYP19* gene expression in human breast cancer cells. Modulation of the *CYP19* expression by E_2 has also been shown in other vertebrates.[6]

Several single nucleotide polymorphisms (SNP) and variable number tandem repeats (VNTR) polymorphisms have been identified in the ERα gene (http://www.ncbi.nlm.nih.gov). Of the polymorphisms identified so far, the *Pvu*II and *Xba*I SNPs, located in the first intron 397 and 351 basepairs upstream of exon 2, are the most widely studied. These polymorphisms have previously been associated with disease phenotypes such as osteoporosis, cardiovascular disease and cancer.[7-11] Recently a potential functional significance of the *Pvu*II polymorphisms was reported.[12] The aim of the present study was to determine if these common ERα polymorphisms are associated with serum E_2 levels in a population of both men and postmenopausal women aged 55 years and older.

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.[13] 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. Base-line 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 serum 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. Serum E_2 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, past or never smoker. Age at menopause responses were validated as described previously.[14] 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 venapuncture at the baseline examination in the research center between 08.30 and 16.00 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 x g at 4°C and then for 30 min at 7000 x 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 E_2, testosterone, androstenedione, estrone (E_1), dehydroepiandrosterone sulfate (DHEAS), and sex hormone binding globulin (SHBG) were estimated in 12 separate batches of samples using coated tube or double antibody radioimmunoassays purchased from Diagnostic Systems Laboratories, Inc. (Webster, TX). The mean minimum detection limit for E_2 was 4.8 pmol/l, which enabled us to study the association with ERα polymorphism at very low levels. Non-detectable estradiol 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) serum pools with 3 different levels of each analyte, were below 15% for all assays, with the exception of E_2 (18%) and E_1 (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. 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, non-SHBG-bound testosterone and E_2 were calculated on the basis of hormone, SHBG and albumin levels, and respective affinity constants according to the method described by Södergård et al.[15] and van den Beld et al.[16]

Genotyping

All participants were genotyped for the *Pvu*II and *Xba*I polymorphisms, located 397 and 351 basepairs, respectively, upstream from the start of exon 2 in the ERα gene. Genomic DNA was isolated from peripheral leucocytes 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 wells 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.[17] The alleles were defined as haplotypes such as "C-G" representing the C-allele for the *Pvu*II SNP and the G-allele for the *Xba*I 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 *Pvu*II-*Xba*I 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.[9,14,18,19] 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, testosterone and ERα haplotype 1 as independent variables. One hundred times the correlation coefficient squared was interpreted as the percentage variability in E_2 explained by the polymorphisms of interest.

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

RESULTS

Baseline characteristics for the approximate 13% of the Rotterdam Study cohort that was included in our study, as compared to the entire cohort are shown in Table 1. Fewer women (6.7%) were included compared to the entire Rotterdam Study cohort and participants in our study were 0.6 years younger. Participants of our study also tended to smoke more. Figure 1 shows the distribution of E_2 values for men and women in our study as plotted against age. There was a significant association between E_2 levels and age in both men and women.

We observed the four possible *Pvu*II-*Xba*I 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 1 allele in 2,318 chromosomes. Genotype distributions were in Hardy-Weinberg equilibrium (HWE) and similar between genders and across age categories.
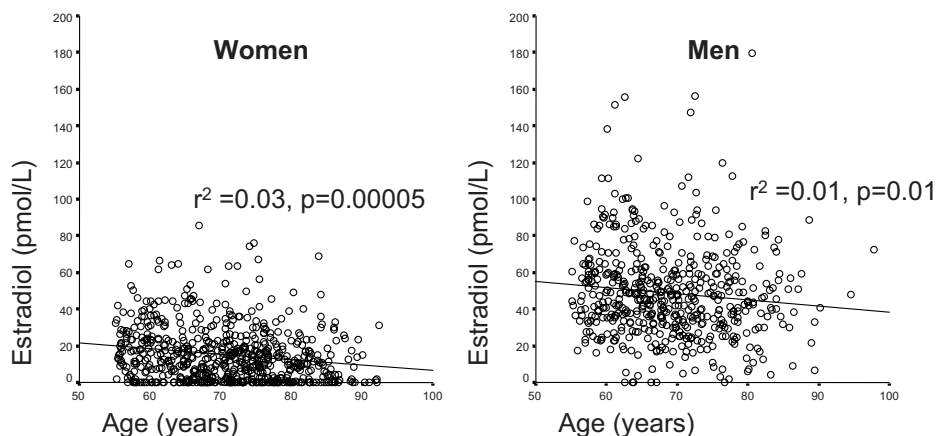

Figure 1.
Measured estradiol levels in 631 female and 528 male participants, including linear regression line, correlation coefficient and p-values

As observed previously,[14] in the 631 postmenopausal women, a trend was observed for a later age at menopause per allele copy of haplotype 1 (Table 2). *Pvu*II-*Xba*I haplotype 1 was associated with decreased serum E_2 levels in an allele-dose dependent manner; per copy of haplotype 1 E_2 levels were 1.68 pmol/L lower (p-trend 0.05). After adjusting for age, BMI, years since menopause, and smoking, genotype dependent differences increased slightly and serum E_2 levels decreased by 1.90 pmol/L per allele copy of haplotype 1 (p-trend 0.02, Table 2). Extreme genotypes differed 3.93 pmol/L (p=0.02). In this group ERα haplotype 1 explained an additional 1% above the effect of age, BMI, smoking, and age at menopause in the variation of E_2 level (R square change 0.01, p=0.02). After excluding the 187 women with E_2 levels below the mean detection limit of 4.8 pmol/L, haplotype 1 was still significantly associated, per copy of haplotype 1 the serum E_2 was 1.94 pmol/L lower (p-trend=0.04). ERα haplotype 2 showed a similar significant, but opposite allele-dose effect on serum E_2 levels in women: per copy of haplotype 2 E_2 levels were 1.74 pmol/L higher (p-trend 0.05). The results remained similar after adjustment for age, BMI, years since menopause, smoking and testosterone levels. ERα haplotype 3 was not associated with serum E_2 levels (results not shown). None of the ERα haplotypes was associated with serum testosterone (Table 2).

In the 528 men in our study *Pvu*II-*Xba*I haplotypes were not associated with serum E_2 or testosterone levels (Table 2 for haplotype 1).

Within a subset of 446 postmenopausal women and 362 men, serum levels of other sex-hormones were also determined. As observed in the larger sample, in this

Table 1. Characteristics of 1159 study participants compared to the entire Rotterdam Study cohort

	Study	Rotterdam Study	P-value
Numbers (%)	1159 (12.7%)	7983	
Women (%)	631 (54.4%)	4878 (61.1%)	< 0.001[a]
Age (years)	69.7 (0.3)	70.3 (0.1)	0.05[b]
BMI (kg/m²)	26.2 (0.1)	26.2 (0.04)	0.9[c]
Age at menopause (yrs)	48.8 (0.2)	48.8 (0.08)	0.9[b]
Years since menopause (yrs)	21.7 (0.2)	21.7 (0.08)	0.9[b]
Smoking (%)			0.07[a]
Current	24.4%	22.6%	
Past	42.4%	40.7%	

Values are expressed as mean (standard error or percentage)

a   Chi-square

b   ANCOVA adjusted for gender

c   ANCOVA adjusted for age and gender

Table 2. Characteristics of 631 postmenopausal women and 528 men from the Rotterdam Study by genotype for ERα haplotype 1 (T-A)

Number of allele-copies	Women				Men			
ER haplotype 1	0	1	2	P-value	0	1	2	P-value
Numbers (%)[a]	145 (23.0 %)	319 (50.6%)	167 (26.5%)		116 (22.0%)	259 (49.1%)	153 (29.0%)	
Age (years)	71.4 (0.7)	70.6 (0.5)	70.3 (0.7)	0.5 [b]	69.2 (0.7)	69.3 (0.5)	67.6 (0.7)	0.09 [b]
BMI (kg/m²)	26.8 (0.3)	26.8 (0.2)	26.7 (0.3)	0.9 [b]	25.5 (0.3)	25.5 (0.2)	26.0 (0.2)	0.3 [b]
Age at menopause (years)	48.5 (0.4)	48.7 (0.3)	49.4 (0.4)	0.1 [c]				
Years since menopause (years)	22.9 (0.8)	21.9 (0.6)	20.9 (0.8)	0.07 [c]				
Smoking (%)				0.9 [d]				0.3 [d]
Current	19.4%	19.8%	21.3%		31.0%	30.8%	26.5%	
Past	27.8%	27.4%	23.8%		56.0%	61.3%	66.2%	
Time of blood draw (hr.min)	11.31 (0.11)	11.21 (0.08)	11.50 (0.10)	0.1 [b]	11.39 (0.13)	11.30 (0.09)	11.38 (0.12)	0.8 [b]
Testosterone (nmol/L)								
Crude	1.32 (0.06)	1.38 (0.04)	1.36 (0.06)	0.7 [b]	11.59 (0.35)	11.17 (0.23)	11.56 (0.30)	0.5 [b]
Adjusted [e]	1.35 (0.06)	1.40 (0.04)	1.38 (0.06)	0.8 [b]	11.71 (0.35)	11.30 (0.27)	11.61 (0.33)	0.5 [b]
Estradiol (pmol/L)								
Crude	17.45 (1.24)	15.17 (0.83)	14.05 (1.15)	0.05 [c]	48.11 (2.34)	48.21 (1.57)	50.65 (2.04)	0.4 [c]
Adjusted [e]	17.80 (1.23)	15.25 (0.85)	13.87 (1.16)	0.02 [c]	49.52 (2.45)	49.41 (1.87)	51.76 (2.29)	0.6 [c]
Adjusted [f]	17.89 (1.14)	14.97 (0.79)	13.73 (1.08)	0.008 [c]	48.90 (2.36)	49.57 (1.80)	51.33 (2.21)	0.5 [c]

Values are expressed as mean (standard error or percentage)

[a] P-value for Hardy-Weinberg Equilibrium is 0.8 for women and 0.7 for men

[b] ANOVA

[c] Linear regression

[d] Chi-square

[e] Adjusted for age, BMI, smoking (and years since menopause in women)

[f] Adjusted for age, BMI, smoking, testosterone (and years since menopause in women)

Table 3. Characteristics of 446 postmenopausal women and 362 men from the Rotterdam Study by genotype for ERα haplotype 1 (T-A)

Number of allele-copies ER haplotype 1	Women				Men			
	0	1	2	P-value[b]	0	1	2	P-value[b]
Numbers (%)[a]	105 (23.5 %)	225 (50.4%)	116 (26.0%)		81 (22.4%)	181 (50.0%)	100 (27.6%)	
DHEA sulfate (μmol/L)[c]	3.17 (0.20)	2.78 (0.14)	2.94 (0.19)	0.5	4.76 (0.34)	4.76 (0.26)	5.05 (0.32)	0.5
Androstenedione (nmol/L)[c]	3.64 (0.17)	3.57 (0.12)	3.41 (0.16)	0.3	3.85 (0.20)	4.05 (0.15)	4.21 (0.18)	0.1
Estrone (pmol/L)								
Crude	39.01 (3.19)	42.62 (2.18)	42.34 (3.04)	0.5	89.35 (4.95)	85.83 (3.31)	89.71 (4.46)	0.9
Adjusted[d]	41.02 (3.17)	43.30 (2.19)	44.15 (3.03)	0.5	87.43 (5.18)	83.37 (3.94)	87.54 (4.80)	0.9
Testosterone (nmol/L)[d]	1.25 (0.06)	1.34 (0.04)	1.26 (0.06)	0.9	11.62 (0.44)	11.00 (0.34)	11.49 (0.41)	0.9
Free testosterone (nmol/L)	0.025 (0.001)	0.026 (0.001)	0.025 (0.001)	0.8	0.264 (0.010)	0.251 (0.008)	0.258 (0.009)	0.7
Estradiol (pmol/L)								
Crude	17.67 (1.39)	15.20 (0.95)	14.87 (1.32)	0.1	45.08 (2.77)	47.76 (1.85)	50.38 (2.49)	0.2
Adjusted[e]	17.94 (1.38)	15.12 (0.95)	14.51 (1.32)	0.07	47.22 (2.94)	49.63 (2.24)	52.41 (2.73)	0.2
Adjusted[e]	18.00 (1.16)	14.70 (0.80)	14.02 (1.11)	0.01	47.73 (2.61)	50.96 (1.99)	52.47 (2.42)	0.2
Free estradiol (pmol/L)[e]	0.46 (0.03)	0.36 (0.02)	0.36 (0.03)	0.02	1.28 (0.07)	1.40 (0.05)	1.43 (0.07)	0.1

Values are expressed as mean (standard error or percentage)

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)

subset *Pvu*II-*Xba*I haplotypes were not significantly associated with age, BMI, age at menopause, years since menopause or smoking. Furthermore the larger sample of 1159 participants was not statistically different from this subset of 808 participants in any of the above mentioned baseline characteristics. A significant association between *Pvu*II-*Xba*I haplotype 1 and serum E_2 levels, as well as bioavailable E_2 levels, was also observed in postmenopausal women in this subset, but not in men (Table 3). No association between any of the *Pvu*II-*Xba*I haplotypes and DHEA sulfate, androstenedione, E_1, testosterone or free testosterone was observed in either men or women.

Discussion

The present population-based association study demonstrates that in postmenopausal women ERα *Pvu*II-*Xba*I haplotype 1 is associated with decreased serum E_2 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 E_2 levels as compared to non-carriers. This is a 22% reduction in E_2 level.

Our a priori hypothesis for studying the association between ERα gene polymorphisms and E_2 levels was driven by recent evidence that ERα can modulate *CYP19* expression in breast cancer cells.[5] The authors of that experimental study found that in human breast cancer cells E_2 up-regulates aromatase gene expression via ERα. We hypothesized that ERα gene polymorphisms may modulate the effect of E_2 on *CYP19* expression. Indeed, the ERα gene polymorphisms we studied were associated with E_2 levels. However, aromatase not only catalyzes the conversion of testosterone to E_2, but also the conversion of androstenedione to E_1. If *CYP19* expression is modulated by ERα polymorphisms then we would expect serum E_1 levels to be influenced in parallel with levels of E_2. Apart from chance, there is another explanation for this finding. When studying our results we noticed that, carriers of haplotype 1 did not have lower E_1 levels. In fact, we observed a non-significant trend in the opposite direction; carriers of haplotype 1 had higher E_1 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 E_1 into E_2.[20] Although regulation of 17β-HSD by E_2 or ERα has not been shown so far, our results do suggest a role for ERα in 17β-HSD expression or activity. Future research will be necessary to show if this hypothesis is true.

How do these specific polymorphisms influence ERα gene expression and consequently serum E_2 levels? The *Pvu*II and *Xba*I polymorphisms have been an important area of research in diseases such as osteoporosis, cardiovascular disease, and cancer.[7-11] A number of hypotheses for the functional significance of these polymorphisms have been reported in the literature. Given their location, 397 and

351 basepairs upstream from the start of exon 2, possible functional mechanisms include changed ERα expression via altered binding of transcription factors and influence on alternative splicing of the ERα 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 ERα gene. The first mechanism was recently supported by findings of Herrington et al.[10] and were confirmed in our own laboratory (manuscript in preparation). Herrington et al. showed that the T-allele of the *Pvu*II RFLP eliminates a functional binding site for the transcription factor B-myb. This implies that the presence of this allele may result in lower ERα transcription. Our study reports that the *Pvu*II T-allele, represented in haplotype 1, is associated with decreased serum E_2 levels in an allele dose-dependent manner in postmenopausal women. This suggests that the potentially lower ERα expression caused by the *Pvu*II T-allele leads to a lower expression of an enzyme in the estrogen synthesis pathway, such as 17β-HSD, and, subsequently, reduced E_2 synthesis. These findings are further supported by the observation in our study population, as well as in others, that this T-allele of the *Pvu*II polymorphism is associated with increased risk of osteoporosis,[9] decreased risk of osteoarthritis[19] and hysterectomy,[14] lower BMI,[21] shorter stature,[18] and later age at menopause.[14] These phenotypes are known to be related to decreased E_2 effects. The fact that the *Xba*I polymorphism A-allele is also associated with E_2 levels may be due to linkage disequilibrium with the *Pvu*II SNP or another functional polymorphism, or to functional significance of the *Xba*I polymorphism itself.

We observed the association between ERα polymorphisms and E_2 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 (HPG) axis and E_2 still plays an important role in the regulation of gonadotropin release. Therefore, in men the influence of ERα polymorphisms on enzymes involved in the peripheral biosynthesis of E_2 may be less important. Secondly, in men E_2 levels are three times higher than in postmenopausal women; therefore, levels of ligand for the ERα may be sufficiently high so that differences in ERα expression may not influence feed-back to other genes. Thirdly, the serum levels of the direct precursors to E_2 in the biosynthesis pathway are a great deal higher in men than in postmenopausal women of the same age. Perhaps, in the presence of larger amounts of E_2 precursors, genotype dependent differences in 17β-HSD expression may not lead to changes in E_2 levels. Finally, in men testosterone is the main precursor to E_2 and although the conversion of androstenedione to testosterone is also catalyzed by 17β-HSD, this is another subtype and not the subtypes 1 and 7 that catalyze the conversion of estrone to E_2.

In interpreting the clinical implications of these results we must consider two important aspects. First, plasma levels of E_2 do not necessarily reflect local tissue levels. The circulating E_2 level in postmenopausal women originates in extrago-

nadal sites where it also acts locally. If this peripherally produced E_2 escapes local metabolism, it enters the circulation. However, plasma levels of E_2 are important in the pathology of a number of diseases. Individual variation in circulation levels of E_2 has been shown to influence the risk of diseases such as osteoporosis[2,3] and breast cancer.[4] Secondly, the variance in E_2 levels explained by these ERα polymorphisms is 1%. Clearly the impact of this polymorphism on absolute E_2 levels is not great. However, it is expected that a large number, perhaps hundreds, of genes and "low penetrance" polymorphisms will contribute to individual variation in E_2 levels. Each of these polymorphisms will thus explain only a small fraction. For example, polymorphisms in genes encoding other enzymes in the estradiol synthesis pathway, such as the *CYP19* gene encoding aromatase, will likely contribute to individual estradiol levels. Furthermore, interactions between these polymorphisms and environmental factors will also prove to be important. Although the contribution of these ERα polymorphisms to the variance in E_2 levels may be low, its impact may be much greater. Average E_2 levels for the extreme genotypes are above and below 15.5 pmol/L. Our research group has recently shown that individuals with E_2 below this "cut-off" value have an increased risk of osteoporosis.[3] These findings suggest that the genotype dependent differences in E_2 levels created by these polymorphisms may be clinically significant.

The only other study showing an association between ERα gene polymorphisms and sex-hormone levels found an association with increased androstenedione and not E_2.[22] This is in contrast to our findings. Zofkova et al., in a study of 114 postmenopausal women of Czech origin, found that the *Pvu*II and *Xba*I polymorphism were not significantly associated with E_2 levels. Although the association was not significant in their study, the *Pvu*II T-allele and *Xba*I A-allele did also show a trend for lower E_2 levels. Perhaps, the power of that study was insufficient to show a significant association. Zofkova et al. did find a significant association between these alleles and higher androstenedione levels. We were not able to replicate these results 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 *Pvu*II-*Xba*I genotypes were in Hardy-Weinberg equilibrium and haplotype frequencies were similar to those found in studies of other Caucasian subjects.[23] 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 that the entire Rotterdam Study cohort. However, 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_2 assay with an very low detection limit (4.8 pmol/L), and we excluded all participants with medication, such as hormone replacement therapy 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_2 levels in carriers of ERα *Pvu*II-*Xba*I 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_2 levels are, while E_1 levels are not, associated with these ERα polymorphisms, we hypothesize that it is likely that these common sequences variants alter 17β-HSD expression or activity.

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Chapter 4.2

Aromatase (CYP19) Gene Exon 10
Polymorphism and Sex-hormone Levels

Abstract

Individual variation in postmenopausal estradiol (E_2) level is an important determinant of diseases such as breast cancer and osteoporosis. A large portion of this variation may be determined by genetic variations (i.e. polymorphisms) in genes that control hormone biosynthesis. The aim of this study was to determine if a common, possibly functional polymorphism, located in exon 10 (a T to C substitution in the 3'UTR) of the aromatase (*CYP19*) gene is associated with variation in serum estradiol levels in the general population.

In 719 postmenopausal women and 589 men from the Rotterdam Study, a population-based cohort study of individuals 55 years and older, *CYP19* genotypes and serum E_2 levels were determined. The C-allele, which was previously related to decreased *CYP19* mRNA expression by others, was significantly associated with decreased serum E_2 in postmenopausal women in an allele-dose dependent manner. Serum E_2 levels decreased by 2.23 pmol/L per C-allele copy (p-trend 0.005); extreme genotypes varied 4.53 pmol/L. In a subset of 836 participants DHEA-sulfate, androstenedione, testosterone, bioavailable testosterone, estrone (E_1) and bioavailable E_2 were determined. In women, the C-allele was associated with decreased serum E_1 and bioavailable E_2 levels (p-trend 0.0001 and 0.001, respectively). In men, no association between the *CYP19* polymorphism and sex-hormone levels was observed.

We previously demonstrated the estrogen receptor (ER) alpha gene *Pvu*II-*Xba*I haplotype 1 to be associated with a 2 pmol/L decrease in E_2 levels in postmenopausal women. An additive effect of the *CYP19* exon polymorphism and ER alpha haplotype 1 on E_2 levels was observed. There was a 40% reduction in E_2 levels in homozygous carriers of both risk alleles as compared to non-carriers.

In conclusion, we have demonstrated a role for a polymorphism located in exon 10 of the *CYP19* gene in determining postmenopausal E_2 and E_1 levels.

INTRODUCTION

Fluctuation of serum estradiol (E_2) levels is a characteristic feature of the female reproductive cycle. During the reproductive years, the ovaries are the principal source of blood E_2 levels and circulating levels are controlled by a sensitive feedback mechanism, the hypothalamic-pituitary-gonadal (HPG) axis, to maintain cycle regularity and reproductive function. However, after menopause the ovaries stop producing E_2, this feedback mechanism ceases to function, and women are dependent on peripheral aromatization of adrenal androgens to produce E_2. The peripheral conversion to estrogens is insufficient to produce circulating E_2 levels resembling those before menopause and, consequently, circulating E_2 levels in postmenopausal women are drastically reduced. After menopause E_2 levels in women are 3 times lower than in men. Postmenopausal withdrawal of the effects of E_2 from non-reproductive tissues such as the skeleton, the cardiovascular system, and the brain, is a major risk factor for the development of osteoporosis, coronary artery disease and stroke.[1-5]

Large individual variations in postmenopausal E_2 levels can be observed and this individual variation in endogenous E_2 exposure has been shown to result in differences in the risk of diseases such as osteoporosis and breast cancer.[6-8] It can be assumed that, aside from known environmental factors such as smoking and body weight, individual variation in E_2 levels can also be determined by genetic variations (i.e. polymorphisms) in genes that affect hormone biosynthesis. These polymorphisms could, therefore, influence the risk of hormone related disease. Thus, unraveling the genetic origins of variations in E_2 levels will not only give important information about the physiology of hormone biosynthesis, but may also provide new insights into the etiology of these diseases. A logical candidate gene to study for its effects on E_2 levels is the aromatase (*CYP19*) gene. Aromatase catalyzes the rate limiting step in the biosynthesis of E_2, the conversion of androgens to estrogens.

In 1994, Sourdaine et al. identified a potentially interesting polymorphism in the *CYP19* gene.[9] Recently, Kristensen et al. showed that this C-T substitution, located in the untranslated region (UTR) of exon 10 (rs10046), is frequently found in Caucasians (~50%) and is associated with increased aromatase mRNA levels and a switch from the adipose promoter to ovary promoter.[10] The aim of the present study was to determine if this common *CYP19* polymorphism is associated with serum E_2 and E_1 levels in a population of both men and postmenopausal women aged 55 years and older.

Polymorphisms in *CYP19* alone may cause variation in E_2 levels, but presumably they also do so through interactions with other polymorphisms. Recently, our group has found that two polymorphisms in the estrogen receptor alpha gene (ERα) are associated with serum E_2 levels.[11] Therefore, the second aim of our study was to determine if there is interaction between these two genes in influencing E_2 levels.

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.[12] All residents age 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. Base-line 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 serum E_2 levels in a gender-stratified random sample of 1308 subjects (719 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, levels of additional sex-hormones were available for 836 subjects (473 women).

Clinical examination

At baseline, interview information, including smoking habits and age at menopause, was obtained by a trained research assistant during a home interview. Smoking was categorized as current, past or never. Age at menopause responses were validated as described previously.[13] 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 venapuncture at the baseline examination in the research center between 0830 and 1600 h. 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 x g at 4 C and then for 30 min at 7000 x 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 prior to hormone assessment. For the present study, plasma levels of E_2, testosterone, androstenedione, estrone (E_1), dehydroepiandrosterone sulfate (DHEAS), and sex hormone binding globulin (SHBG) were estimated in 12 separate batches of samples using coated tube or double antibody radioimmunoassays purchased from

Diagnostic Systems Laboratories, Inc. (Webster, TX). For the estimation of E_2 we used an ultra-sensitive assay with a mean minimum detection limit of 4.8 pmol/l, which enabled us to study the association with ERα polymorphism at very low levels.[7] Non-detectable estradiol 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) serum pools with 3 different levels of each analyte, were below 15% for all assays, with the exception of E_2 (18%) and E_1 (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. As described previously, this reduced inter-assay variations to acceptable levels (all under 18%) and was considered justified because the pattern of the results of these pools and the mean results for male and female sera in one assay batch showed very similar patterns.[14] 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, non-SHBG-bound testosterone and E_2 were calculated on the basis of hormone, SHBG and albumin levels, and respective affinity constants according to the method described by Södergård et al.[15] and van den Beld et al.[16]

Genotyping

Genomic DNA was isolated from peripheral leucocytes by standard procedures. Genotypes were determined using the Taqman allelic discrimination assay. Primer and probe sequences were optimized for the *CYP19* and ERα gene polymorphisms using the SNP assay-by-design service of Applied Biosystems. For details see store.appliedbiosystems.com. Reactions were preformed on the Taqman Prism 7900HT 384 wells format.

All participants were genotyped for the *CYP19* exon 10 and ERα *Pvu*II and *Xba*I polymorphisms The *CYP19* exon 10 polymorphism is located 1531 basepairs downstream of the first nucleotide of the transcription initiation site (ATG). Participants were categorized by the presence of the T- or C-allele. The ERα *Pvu*II and *Xba*I polymorphisms are located 397 and 351 basepairs, respectively, upstream of the start of exon 2. Haplotypes were constructed for the ERα polymorphisms as described previously.[17] The ERα *Pvu*II-*Xba*I haplotype 1 has previously been associated with lower serum E_2 levels and was analyzed for interaction with the *CYP19* polymorphism.[11] Haplotype 1 represents a T-allele at the *Pvu*II restriction site and an A-allele at the *Xba*I restriction site.

Statistical analysis

One-way analysis of covariance (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 *CYP19* genotypes, sex-hormone levels and possible confounders was evaluated using ANCOVA, linear regression or Pearson's chi-square tests. 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.

We allowed for three possible models to explain differences between genotype groups, i.e., an allele dose effect, a dominant effect, or a recessive effect. Allele dose was defined as the number of copies of the allele of interest.

For the analyses of gene-gene interaction, we categorized participants for both the *CYP19* polymorphism and ERα haplotype. For each of the 9 possible genotype categories mean E_2 levels were calculated. Stepwise multiple linear regression was used to test for independent predictors for E_2 entering, age, years since menopause, BMI, smoking, ERα haplotype, and *CYP19* genotype into the model.

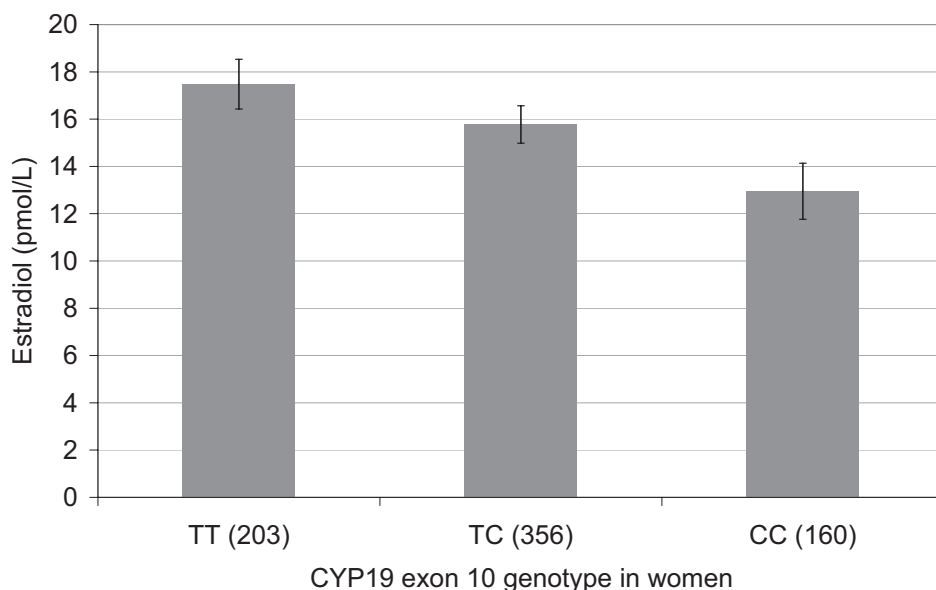All statistical analyses were performed using SPSS version 11.0.1 (SPSS Inc., Chicago, USA).

Figure 1.
Association of CYP19 exon 10 polymorphism with E_2 levels in 719 postmenopausal women. Analysis was adjusted for age (mean ± SE).

Table 1. Baseline characteristics by CYP19 exon 10 polymorphism in 719 postmenopausal women and 589 men from the Rotterdam Study

	Women				Men			
	TT	TC	CC	P-value	TT	TC	CC	P-value
Numbers[a]	203	356	160		159	288	142	
(%)	(28.2 %)	(49.5%)	(22.3%)		(27.0%)	(48.9%)	(24.1%)	
Age (years)	72.2 (0.7)	71.9 (0.5)	69.8 (0.7)	0.03 [b]	67.8 (0.7)	69.3 (0.5)	68.7 (0.7)	0.2 [b]
BMI (kg/m²)	27.0 (0.3)	26.7 (0.2)	26.4 (0.3)	0.3 [b]	25.6 (0.2)	25.7 (0.2)	25.8 (0.3)	0.9 [b]
Age at menopause (yrs)	49,1 (0.4)	48.6 (0.3)	48.7 (0.4)	0.4 [c]				
Years since menopause (yrs)	22.3 (0.7)	22.7 (0.5)	20.4 (0.8)	0.09 [c]				
Smoking (%)				0.2 [d]				0.1 [d]
Current	16.7%	20.8%	20.6%		31.4%	31.6%	22.5%	
Former	31.5%	23.9%	22.5%		58.5%	59.7%	64.1%	

Values are expressed as mean (standard error or percentage)

a   P-value for Hardy-Weinberg Equilibrium is 0.9 for women and 0.6 for men

b   ANOVA

c   Linear regression

d   Chi-square

Table 2. Sex-hormone levels by CYP19 exon 10 polymorphism in in 473 postmenopausal women and 363 men from the Rotterdam Study

	Women				Men			
	TT	TC	CC	P-value[a]	TT	TC	CC	P-value[a]
Numbers (%)	132 (27.9 %)	234 (49.5%)	107 (22.6%)		102 (28.1%)	189 (52.1%)	72 (19.8%)	
DHEA sulfate (μmol/L)								
Adjusted for age	2.95 (0.17)	2.92 (0.13)	2.43 (0.19)	0.06	4.44 (0.29)	4.88 (0.21)	4.92 (0.34)	0.2
Full model[b]	3.05 (0.22)	3.01 (0.18)	2.49 (0.23)	0.07	4.42 (0.40)	4.92 (0.37)	4.92 (0.44)	0.2
Androstenedione (nmol/L)								
Adjusted for age	3.67 (0.15)	3.42 (0.11)	3.31 (0.16)	0.09	4.17 (0.17)	4.13 (0.12)	4.14 (0.20)	0.9
Full model[b]	3.73 (0.19)	3.48 (0.15)	3.36 (0.19)	0.1	4.13 (0.23)	4.09 (0.22)	4.11 (0.26)	0.9
Estrone (pmol/L)								
Adjusted for age	51.71 (2.82)	40.56 (2.12)	35.59 (3.14)	0.0001	88.00 (4.45)	91.95 (3.25)	78.04 (5.26)	0.2
Full model[c]	55.35 (3.50)	45.49 (2.85)	41.12 (3.52)	0.001	80.83 (6.03)	84.03 (5.64)	71.39 (6.66)	0.2
Testosterone (nmol/L)								
Adjusted for age	1.36 (0.06)	1.22 (0.04)	1.26 (0.07)	0.2	11.43 (0.37)	10.93 (0.27)	11.48 (0.44)	0.9
Full model[c]	1.35 (0.07)	1.28 (0.05)	1.34 (0.07)	0.9	11.95 (0.47)	11.59 (0.44)	12.04 (0.52)	1.0

Table 2. cont.

Free testosterone (nmol/L)								
Adjusted for age	0.027 (0.001)	0.024 (0.001)	0.025 (0.001)	0.1	0.251 (0.009)	0.249 (0.007)	0.270 (0.011)	0.3
Full model[c]	0.028 (0.001)	0.026 (0.001)	0.027 (0.001)	0.7	0.267 (0.012)	0.267 (0.011)	0.286 (0.013)	0.2
Estradiol (pmol/L)								
Adjusted for age	18.70 (1.26)	15.80 (0.95)	12.75 (1.40)	0.002	46.60 (2.47)	50.19 (1.81)	44.70 (2.93)	0.8
Full model[d]	19.17 (1.48)	17.41 (1.20)	14.08 (1.48)	0.004	49.95 (3.30)	54.52 (3.07)	48.10 (3.64)	0.8
Free estradiol (pmol/L)								
Adjusted for age	0.48 (0.03)	0.39 (0.02)	0.32 (0.04)	0.001	1.25 (0.07)	1.37 (0.05)	1.25 (0.08)	0.9
Full model[d]	0.49 (0.04)	0.44 (0.03)	0.36 (0.04)	0.003	1.36 (0.09)	1.50 (0.08)	1.36 (0.10)	0.9

Values are expressed as mean (standard error or percentage)

a   Linear regression

b   Adjusted for age, BMI, smoking (and years since menopause in women)

c   Adjusted for age, BMI, smoking, androstenedione levels (and years since menopause in women)

d   Adjusted for age, BMI, smoking, testosterone levels (and years since menopause in women)

RESULTS

Approximately 16% of the Rotterdam Study cohort was included in our study. We compared our study participants to the entire Rotterdam Study cohort and found no differences in baseline characteristics (data not shown).

In the 1308 participants of our study the frequency of the *CYP19* exon 10 C-allele was 47.7%. Genotype distributions were in Hardy-Weinberg equilibrium (HWE) and similar between genders and across age categories.

In the 719 postmenopausal women, age was significantly associated with *CYP19* genotype (Table 1), therefore we adjusted all analyses for age. None of the other baseline characteristics was associated with *CYP19* genotype (Table 1). The C-allele was associated with decreased serum E_2 levels in an allele-dose dependent manner (Figure 1). Mean serum E_2 levels decreased by 2.23 pmol/L per C-allele copy (p-trend 0.005, adjusted for age); extreme genotypes varied 4.53 pmol/L. Further adjustments for the number of years since menopause, BMI, and smoking did not change the results (p-trend 0.006). In the 589 men in our study, the *CYP19* polymorphism was not associated with serum E_2 levels (results shown for subset in Table 2).

Within a subset of 473 postmenopausal women and 363 men, serum levels of other sex-hormones were also determined. In line with the observations in the larger set, the *CYP19* C-allele was significantly associated with lower serum E_2 levels in postmenopausal women, but not in men (Table 2). Furthermore, the C-allele was also significantly associated with serum E_1 and free E_2 levels in women. Testosterone, free testosterone, androstenedione and dehydroepiandrosterone (DHEA) sulfate levels were not significantly associated with *CYP19* genotype. The associations with E_1 and E_2 levels were adjusted for the direct precursors, androstenedione and testosterone, respectively, along with age, years since menopause, BMI, and smoking. These adjustments did not change the results. In men, the *CYP19* polymorphism was not associated with any of the sex-hormone levels we analyzed (Table 2).

When we further stratified the 719 female participants by ERα *Pvu*II-*Xba*I haplotype 1 as well as *CYP19* genotype, we observed an additive effect of both genotypes on E_2 levels. The mean E_2 levels of homozygous carriers of both the *CYP19* C-allele and ERα haplotype 1 was 8.86 pmol/L lower than non-carriers (p=0.004, Figure 2). When both genotypes were taken together in a multivariate regression model there was no significant interaction between the *CYP19* genotype and ERα *Pvu*II-*Xba*I haplotype 1. In a multiple linear regression model, we investigated the relation between E_2 and the ERα and *CYP19* polymorphisms, age, years since menopause, BMI, and smoking. Years since menopause, BMI, and the ERα and *CYP19* polymorphisms were significant predictors of E_2. The model shown in Table 3 explained 7.5% of the variance in E_2 in postmenopausal women. BMI accounted for the majority of the total variance (2.7%). The *CYP19* exon 10

Table 3. Predictors of estradiol in 719 women

Predictor	Coefficient	R^2	p-value
Age	-0.136	0.024	0.2
Years since menopause	-0.212	0.030	0.03
BMI	0.624	0.057	0.00002
Smoking	0.792	0.059	0.08
ERα PvuII-XbaI haplotype 1	-1.657	0.066	0.04
CYP19 exon 10 polymorphism	-2.064	0.075	0.008
Constant	15.069	-	0.03
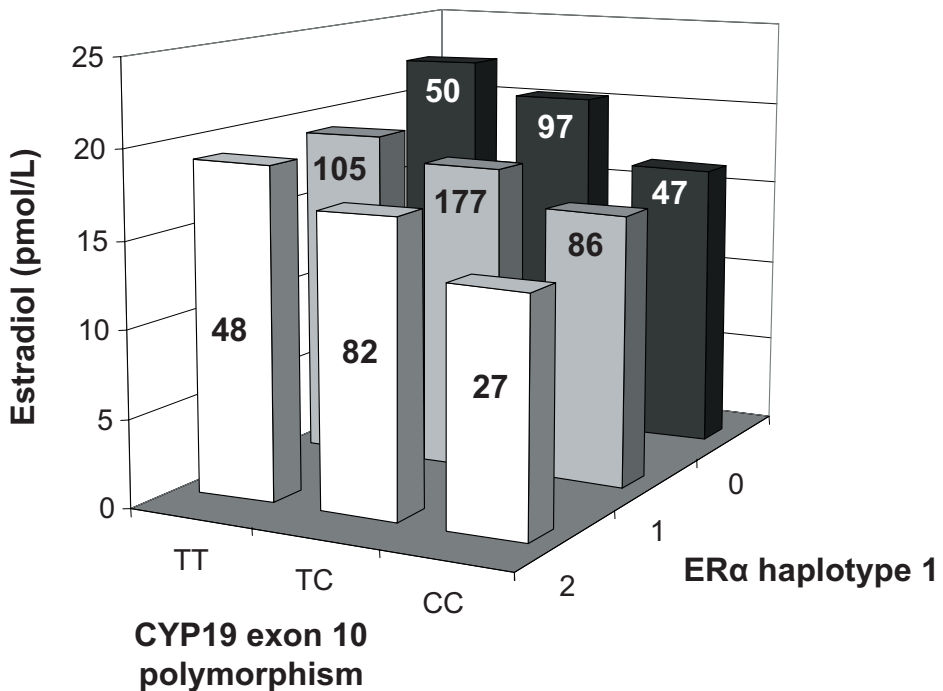
Figure 2.
Effect of CYP19 polymorphism and ERα haplotype 1 on E_2 levels in 719 post-menopausal women. Numbers in the bars represent the number of women in each genotype category.

polymorphism accounted for approximately 1% of the variance in E_2 and the ERα and *CYP19* polymorphisms together accounted for 1.6%.

Discussion

The present population-based study demonstrates that in postmenopausal women a polymorphism located in exon 10 of the *CYP19* gene is associated with decreased serum E_1, E_2 and bioavailable E_2 levels in an allele-dose dependent manner. There was a 26% reduction in these hormone levels in homozygous carriers of the C-allele as compared to non-carriers. This polymorphism explained approximately 1% of the variance of E_2 in postmenopausal women. Furthermore, we observed an additive effect of the *CYP19* exon polymorphism and the ERα haplotype 1 on E_2 levels. There was a 40% reduction in E_2 levels in homozygous carriers of both risk alleles as compared to non-carriers. Together polymorphisms in these two genes explained 1.6% of the variance of E_2.

Aromatase catalyzes the rate limiting step in the peripheral biosynthesis of E_2, the conversion of androgens to estrogens, and is therefore essential for maintaining E_2 levels after menopause. Polymorphisms in the gene encoding this enzyme, *CYP19*, have been associated with risk of a number of diseases which are known to be related to endogenous E_2 levels such as breast cancer, endometriosis, endometrial cancer, and osteoporosis.[18-23] However, previous studies on this subject are rare and inconsistent. The mechanism of action for these associations with polymorphisms in the *CYP19* gene is most likely via effects on circulating and/or local E_2 levels.

The T-allele of the *CYP19* exon 10 polymorphism was associated with higher E_2 levels in an allele-dose dependent manner, but how does this particular polymorphism cause such variation? Kristensen et al. found that the T-allele was associated with higher *CYP19* mRNA levels and a switch from the normally used adipose promoter to the more potent ovarian promoter.[10] The T-allele was also associated with breast cancer in this study. These findings suggest that the T-allele causes higher expression of *CYP19*, accelerated production of estrogen and, therefore, higher risk of developing estrogen dependent tumors. However, we cannot rule out the possibility that this polymorphism acts merely as a marker through LD with a truly functional variation elsewhere in the gene. A recently published comprehensive haplotype analysis found that the *CYP19* locus contains five blocks of LD. One of these blocks spans the entire coding region of the *CYP19* gene and includes the exon 10 polymorphism we studied, the $(TTTA)_n$ VNTR and many other polymorphisms. Therefore, linkage between the exon 10 polymorphism and another functional polymorphism in this haplotype block as the source of our results can not be excluded.

Several single nucleotide polymorphisms (SNP) and variable number tandem repeats (VNTR) polymorphisms have been identified in *CYP19*, the gene encoding aromatase (http://www.ncbi.nlm.nih.gov). Of the polymorphisms identified,

the tetranucleotide repeat polymorphism, $(TTTA)_n$, in intron 5 has been the most well-studied so far. This polymorphism has been associated with breast cancer, endometriosis, endometrial cancer, and osteoporosis.[18-22] Although, it has been speculated that the $(TTTA)_n$ polymorphism may have a direct functional significance in mRNA splicing and therefore in cellular aromatase activity,[19] there is not evidence to support this theory and based on the location of this repeat polymorphism, this proposed functional significance is not likely. If this polymorphism is a marker of risk it is probably because it is in linkage disequilibrium (LD) with a truly functional polymorphism elsewhere in the aromatase gene. Kristensen et al. have shown strong linkage between this polymorphism and the $(TTTA)_n$ VNTR.[10] The exon 10 polymorphism we have studied may be the functional polymorphism that is driving the associations observed with the $(TTTA)_n$ VNTR.

There are two previous studies of *CYP19* gene polymorphisms and sex-hormone levels in postmenopausal women and the results are contradictory, possibly because both studies investigated the $(TTTA)_n$ polymorphism.[18,24] As discussed above, it is likely that the $(TTTA)_n$ VNTR polymorphism is not functionally significant in itself, but acts as a marker through LD with a truly functional polymorphism elsewhere in the gene. This may explain why Haiman et al.[18] did observe an association with E_2 and E_1 levels while Probst-Hensch et al.[24] did not. The first study was performed among Caucasians, while the latter study investigated the association with E_1 in African-American subjects. LD is known to vary widely between ethnic groups and especially in the African-American race there is greater genetic diversity.

We observed the association with estrogen levels only in postmenopausal women and not in men. In contrast to women, men do not experience a sudden cessation of gonadal function similar to menopause. Elderly men still have a largely intact hypothalamic-pituitary-gonadal (HPG) axis and E_2 may still play important role in the regulation of gonadotropin release. Therefore, in men, the influence of polymorphisms in enzymes involved in the peripheral biosynthesis of E_2 may be less important.

Are these genotype dependent differences clinically significant? To answer this question we must consider two important aspects of this study. First, plasma levels of E_2 do not necessarily reflect local tissue levels because circulating E_2 levels in postmenopausal women originate in extragonadal sites where E_2 also acts locally. If this peripherally produced E_2 escapes local metabolism, it can enter the circulation. However, individual variation in postmenopausal circulating E_2 levels have been shown to influence the risk of diseases such as osteoporosis[6,7] and breast cancer risk,[8] indicating that circulating E_2 is an important determinant of disease. Secondly, the variance in E_2 levels explained by the *CYP19* polymorphism is approximately 1%. Clearly the impact of this polymorphism is not large. However, it is expected that a large number, perhaps hundreds, of genes and "low penetrance" polymorphisms will contribute to individual variation in E_2 levels. Each of these polymorphisms will thus explain only a small fraction. Although this polymorphism's contribution

to the variance in E_2 levels may be low, its impact may be much greater since average E_2 levels for the extreme genotypes may be just above or below cut-off values that lead to difference in disease susceptibility.

Interactions between polymorphisms are also likely to be important determinants of individual variation in E_2 levels. Our research group has previously reported an association between polymorphisms in the ERα gene and postmenopausal E_2 levels.[11] The observed association may be due to the influence of these polymorphisms on expression or activity of the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) subtype 1 or 7. We observed that haplotype 1, which represents a T-allele and an A-allele, respectively, at the *Pvu*II and *Xba*I restriction sites, was associated with a 22% reduction in E_2 levels. These findings are similar to the results we report here for the C-allele of the *CYP19* exon polymorphism. Since interaction or synergy between polymorphisms in different genes is expected to play a role in phenotype variation in the general population, we investigated what portion of the variance in E_2 levels is explained by these polymorphisms. We observed an additive effect of these polymorphisms, but no significant interaction. This is not surprising since these polymorphism influence different enzymes in the E_2 biosynthesis pathway, namely the conversion of E_1 to E_2 and the conversion of androgens to estrogens.

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 *CYP19* genotypes were in Hardy-Weinberg equilibrium and genotype frequencies were similar to those found in studies of other Caucasian populations.[10,25] Therefore, our study population may be considered ethnically homogeneous and representative of the Dutch population. Another limitation of association studies is the definition of the phenotype of interest. We used assays with extremely low detection limits, 4.8 pmol/l, and excluded all participants with medication, such as hormone replacement therapy 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_1, E_2, and bioavailable E_2 levels in carriers of the C-allele of a polymorphism located in exon 10 of the *CYP19* gene 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 precursor levels. We also observed an additive effect of the *CYP19* exon polymorphism and the *Pvu*II and *Xba*I polymorphisms in the ERα gene.

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5 | Polymorphism Functionality

Chapter 5.1

Functionality of the Estrogen Receptor Alpha
Gene *Pvu*II polymorphism

Abstract

There are a large number of known polymorphisms in the estrogen receptor alpha (ERα) gene. The most well-studied polymorphism so far is the *Pvu*II C/T polymorphism located 397 basepairs upstream of the start exon 2, in exon 1. The T-allele of this polymorphism, which has an allele frequency of 54% in the Caucasian population, has previously been associated with diseases ranging from osteoporosis to cardiovascular disease. Recently, it was reported that the *Pvu*II T-allele leads to the loss of a binding site for the transcription factor B-myb in monkey kidney (CV1) cells. Regulation of transcription may differ between cells of various origins, and as we have studied the association of this ERα polymorphism with skeletal phenotypes, the aim of this study was to determine whether this process could also be demonstrated in human osteoblasts.

For this study we used the human osteoblast cell line MG-63. MG-63 cells were cotransfected with luceriferase reporter constructs carrying either the *Pvu*II T- or C-allele and a B-myb expression plasmid or a negative control. In the presence of the B-myb expression plasmid, the *Pvu*II C-allele gave a more than 40-fold induction of luciferase activity compared to the almost 10-fold induction of MG-63 cells cotransfected with the T-allele (p-value 0.004).

In conclusion, this study demonstrates that the previously reported finding that in the presence of B-myb the ERα gene *Pvu*II RFLP C-allele leads to augmented ERα expression can also be demonstrated in human osteoblasts. It is hypothesized that carriers of the T-allele will be less sensitive to the effects of estradiol due to presence of lower numbers of ERα.

INTRODUCTION

Current generation of estrogen deficiency models in mice with targeted disruption of the estrogen receptor α (ERα, also known as estrogen receptor 1, *ESR1*) gene provides insight into the role of estrogen in the pathophysiology of a number of diseases outside the reproductive tract.[1] The discovery of a male with a disruptive mutation in the ERα gene leading to complete resistance to estrogen and the consequential occurrence of bone and cardiovascular disease has established the importance of estrogen in humans as well.[2] The pleiotrophy in effects of estradiol beyond the reproductive tissues led the hypothesis that more frequent mutations in the ERα gene, i.e., polymorphisms, may be related to differences in the risk of diseases such as osteoporosis in the general population. Indeed, many studies have reported associations between polymorphisms in the ERα gene with diseases ranging from osteoporosis[3] to cardiovascular disease.[4]

The first polymorphism to be discovered in the ERα gene and the most well-studied so far is the *Pvu*II restriction fragment length polymorphism (RFLP) located 397 basepairs upstream of the start exon 2, in intron 1. Recent history has seen the discovery of a number of other polymorphisms, but none have been as consistently associated with different phenotypes as the *Pvu*II RFLP. The T-allele of the *Pvu*II RFLP, alone or in combination with the *Xba*I polymorphism (located 351 basepairs upstream of exon 2), has been associated with a number of phenotypes known to be related to decreased estradiol effects. These associations include an increased risk of osteoporosis,[3] myocardial infarction,[4] and stroke,[5] decreased risk of osteoarthritis[6] and hysterectomy,[7] lower BMI,[8] shorter stature,[9] and later age at menopause.[7]

Given the socioeconomic impact of the diseases the *Pvu*II RFLP has been associated with and the great frequency in which this polymorphism is present in the Caucasian population (T-allele frequency is 54%) it is important to find if there is a functional significance of this C to T substitution. Until recently, the *Pvu*II RFLP was not in itself expected to be functionally significant to the expression of the ERα protein. The observed associations with disease phenotypes were predicted to be due to linkage disequilibrium with a functional polymorphism elsewhere in the ERα gene or perhaps even in another gene in the surrounding genome. However, recently Herrington et al. suggested that the C-allele in the *Pvu*II RFLP creates a possible binding site for the transcription factor B-myb (Figure 1).[10] If this allele is present this could lead to increased transcription of the ERα gene. However, when the T-allele is present this B-myb binding site is absent, leading to a lower transcription rate of the ERα gene. Therefore, carriers of the T-allele would be at a higher risk of diseases, such as osteoporosis, that are known to result from lower estradiol effects.

Herrington et al. tested the functionality of this RFLP in monkey kidney cells (CV1 cells) and human embryonic kidney cells (293T cells).[10] However, regulation of transcription may differ between cells of various origins, and as we have studied
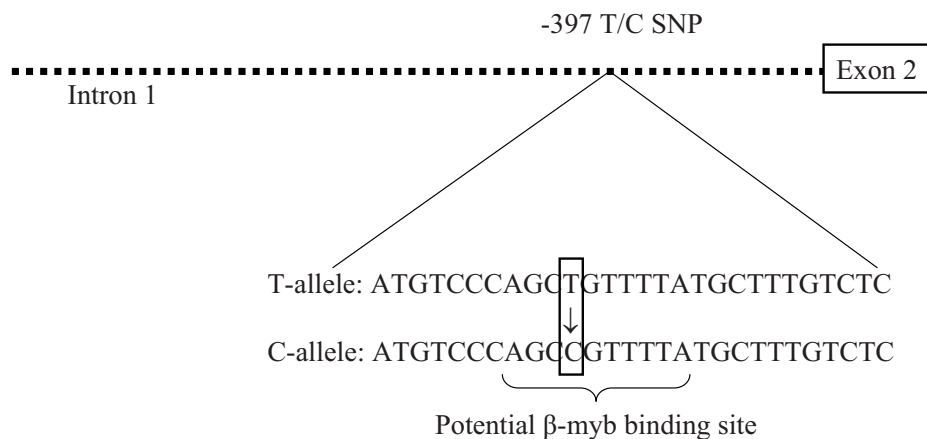
-397 T/C SNP

Figure 1.
The -397 T/C RFLP in intron 1 creates a potential B-myb binding site in the presence of the C-allele, while in the presence of the T-allele the transcription factor binding site is absent.

the association of this ERα polymorphism with skeletal phenotypes,[3,9] the aim of this study was to determine whether this process could also be demonstrated in human osteoblasts. For this purpose we tested the functionality of this RFLP in a human osteosarcoma cell line (MG-63 cells).

MATERIALS & METHODS

Vector plasmids

In total five different plasmids were used. A B-myb expression plasmid and a negative control were cloned into a pcDNA3 plasmid from Invitrogen using HindIII-EcoRI restriction enzyme recognition sites (provided by Dr. Roger Watson, Imperial College School of Medicine, London). The clones containing the C- or the T-allelewere a generous gift from Dr. D. McDonald (Duke University). They were subcloned into a PGL3-Luc vector. The three constructs created were: TATA-Luc (negative control) and a TATA box plus the 28 base pair sequence that contain the ERα *Pvu*II T-allele (C allele-TATA-Luc) or the C-allele (T allele-TATA-Luc).

Cell transfections

MG-63 cells are established as an osteoblastic cell line from a human osteosarcoma. The cells were grown in α-MEM (5% penicillin/streptomycin, 2.39 g HEPES ($C_8H_{18}N_2O_4S$)/500 ml, 0.898 μM $CaCl_2$, 10% FCS, pH 7.5) at 37°C. Cultures of MG-63 cells in the 6-11th passage were cotransfected with 200 ng B-myb expression

plasmid or the B-myb negative control and 100 ng of either one of the 3 different luciferase reporter constructs with 1 µl FuGENE (Roche), creating six different conditions.

Luciferase activity was measured using a beetle luciferin substrate. To release the luciferase from the cells, they were pre-lysed with 75µl pre-lysis buffer (25 mM Tris; 15% glycerol; 1% Triton X-100; 1mM DTT) with 8mM $MgCl_2$ freshly added. After 10 minutes of shaking, 50ul was put in a Costar 96-wells white assay plate, together with 50ul (16mg/ml) Steady-Glo luciferase assay substrate (Promega) in Steady-Glo luciferase assay buffer. The released photons were measured at the Packard Topcount 3.01 Microplate Scintillation Counter.

All transfection experiments were carried out in triplicate and luciferase activity results were averaged and standard deviations were calculated. Fold activation was calculated for each of the three luciferase reporter constructs by dividing the normalized luciferase activity of the cells cotransfected with the B-myb expression plasmid by the normalized luciferase activity of the cells cotransfected with the negative control for the B-myb expression plasmid. Statistical significance was calculated with the student-T test by comparing the reporter construct carrying the C-allele to the T-allele construct.

RESULTS

Cotransfection of the MG-63 cells with the B-myb expression vector and the luciferase reporter C-allele construct gave a more than 40-fold increase in luciferase activity compared to the approximately 10-fold increase in MG-63 cells cotransfected with the T-allele construct or the negative control luciferase construct (Figure 2). This difference was statistically significant when we compared the T-allele construct to the C-allele construct (p-value 0.003 for normalized luciferase activity and 0.004 for fold activation). The T-allele construct was similar to the negative control luciferase construct in luciferase activity (p-value 0.7 for normalized luciferase activity and 0.8 for fold activation).

DISCUSSION

With these transfection experiments in MG-63 cells we have shown that the *Pvu*II RFLP C-allele constitutes a binding site for the transcription factor B-myb, while the T-allele does not. B-myb induces a 4-fold higher expression in the presence of the C-allele when compared to the T-allele of the negative control luciferase construct. These findings suggest that in the presence of B-myb the *Pvu*II C-allele may lead to higher ERα expression compared to the T-allele. Therefore, it is tempting to hypothesize that carriers of the T-allele will be less sensitive to the effects of estradiol due to presence of smaller numbers of estrogen receptors α. These find-
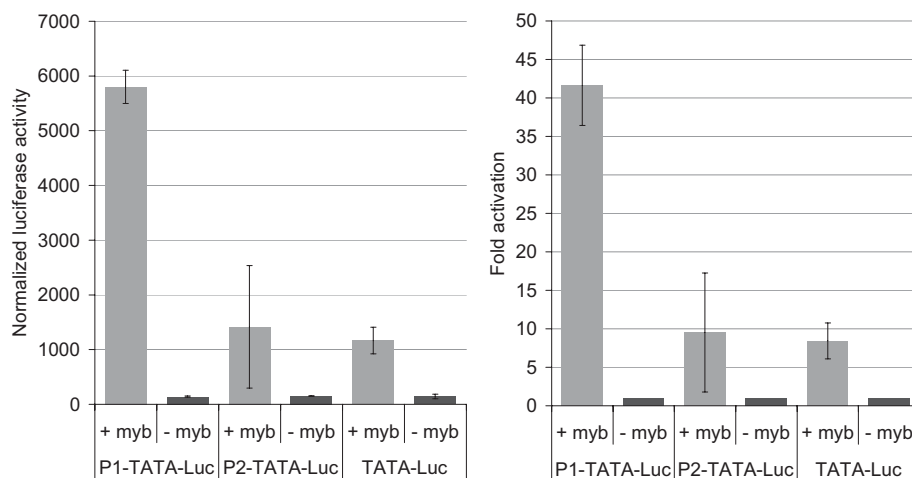
Figure 2.
Normalized luciferase activity and fold activation for the three reporter construct and the B-myb expression plasmid and the negative control B-myb plasmid.

ings are very similar to the results Herrington et al. obtained in their experiment in human and monkey kidney cells (CV1 and 293T cells).[10] In this study we have shown that these same findings can also be obtained in a human bone cell-line, the human osteosarcoma MG-63 cells.

The ERα *Pvu*II RFLP T-allele has been associated with a number of phenotypes known to be related to decreased estradiol effects. These associations include an increased risk of osteoporosis,[3] myocardial infarction,[4] and stroke,[5] decreased risk of osteoarthritis,[6] and hysterectomy,[7] lower BMI,[8] shorter stature,[9] and later age at menopause.[7] Even before these data and the data by Herrington et al. reported that the *Pvu*II RFLP may lead to augmented ERα expression, the associations found in disease phenotypes led to the suggestion that carries of the *Pvu*II T-allele were less sensitive to estradiol. The results from this study support this previously postulated hypothesis. We now hypothesize that carriers of the T-allele are more susceptible to osteoporosis, for example, due to lower ERα expression in bone and consequently decreased sensitivity to estradiol.

The transcription factor B-Myb is expressed in all cell lines analyzed so far regardless of their embryonic origin and expression is stringently coupled to the proliferative state of the cell.[11-13] Although the exact function of the B-myb transcription factor is unknown, a number of studies have provided evidence to support the notion that B-Myb is required for cell cycling and may be essential to the proliferation of many different tissues.[13] More interestingly, B-myb expression is in itself responsive to estrogen activation,[14] therefore, a signal-amplifying

system producing augmented responses to estrogen has been suggested previously by Herrington et al.[10]

Our findings confirm the findings reported Herrington et al. that the ERα *Pvu*II RFLP C-allele creates a bindings site for the transcription factor B-myb, while the T-allele does not. Furthermore, our study demonstrates that these results can also be obtained in human osteoblasts. This is of great interest since the *Pvu*II RFLP has previously been associated with a number of bone related phenotypes including osteoporosis. However, B-myb is only one of a great many transcription factors likely to play a role in osteoblast function. Therefore, we can not be certain that this is a functional process in normal bone cells. To prove that this process takes place in normal bone cells under normal conditions further studies will be needed.

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6 | General Discussion

The objective of the studies presented in this thesis was to determine the role of candidate genes within the estrogen signaling pathway in common diseases of the elderly. Two candidate genes that play a pivotal role in this pathway were chosen: the estrogen receptor alpha (ERα, also known as *ESR1*) and the aromatase (*CYP19*) gene. Potentially functional polymorphisms in these genes were identified by consulting publicly available polymorphism databases and literature. Association studies were performed with bone and cardiovascular phenotypes, and sex-hormone levels. The shortcomings and merits of the individual studies presented have been discussed in the previous chapters. In this discussion, the main findings of this thesis are brought together and placed in a broader perspective. Differences in penetrance of the risk alleles between genders are discussed and an attempt is made to indicate the relevance of these findings for clinical practice. Finally, suggestions are made for future research.

6.1 BONE

Osteoporotic fractures, the clinical endpoint of osteoporosis, are associated with increased morbidity, mortality and high socio-economic costs.[1,2] Due to increased life expectancy fracture incidence is increasing over time, hereby increasing the population burden of fractures.[1] Many different risk factors for osteoporosis, including genetic risk factors, have been identified. Since up to 80% of individual variation in bone mineral density (BMD) is estimated to be heritable,[3,4] a large effort is being made worldwide to determine the genetic origins of osteoporosis. Figure 1 gives a general representation of the role of gene polymorphisms in a complex disease such as osteoporosis. Gene polymorphisms may influence fracture risk through a number of different pathways. These pathways may be as diverse as BMD or an individual's risk of falling. A number of environmental factors may modify fracture risk alone or in combination with gene polymorphisms. Such a representation can also be made for other complex diseases.

6.1.1 Epidemiology

The first chapter in the section on bone discusses the incidence of non-vertebral fractures in the Rotterdam Study and the relation with BMD. Hip, wrist and upper humerus fractures are the most frequent fractures in elderly men and women and they are the fractures that are most clearly related to osteoporosis. Since collecting data on incident fractures is time-consuming, expensive and requires a long-term follow-up study-design, many genetic association studies use BMD as a surrogate phenotype for osteoporosis. A prerequisite for this method is that BMD accurately predicts who will fracture. However, our study has shown that this provision is not absolutely true. While each standard deviation decrease in BMD does increase the risk of fracture, only 21 to 34% of all fractures occur in men and women with a BMD that is considered to be in the osteoporotic range (T-score < -2.5). Therefore, we conclude that although it is time-consuming and expensive it is essential to test
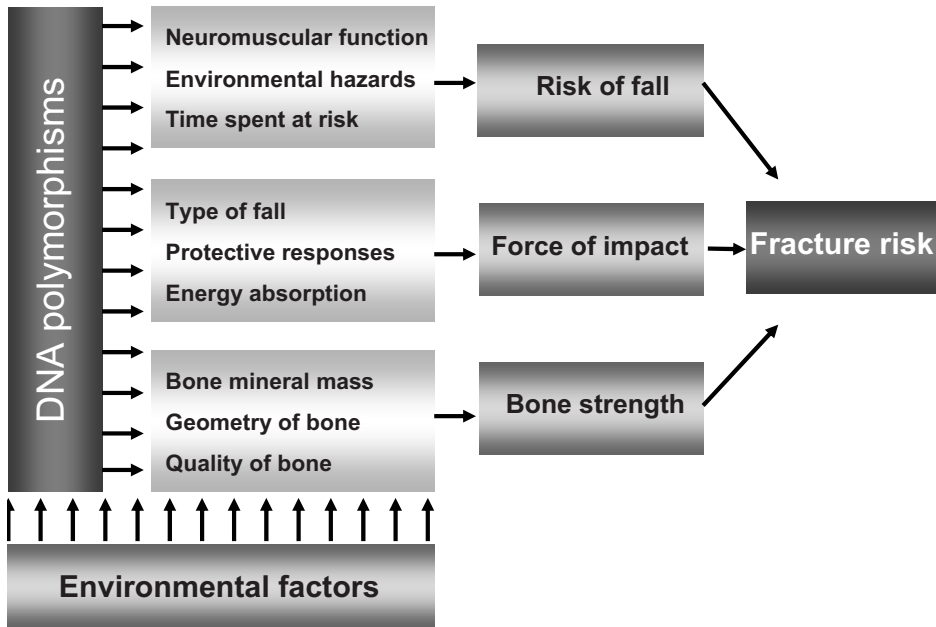
Figure 1.
Complex disease risk: the example of osteoporosis

candidate genes in osteoporosis by investigating the clinical outcome of osteoporosis, i.e., fractures, and not just surrogate markers. However, these markers for osteoporosis may help us in determining how (genetic) risk factors for osteoporosis increase fracture risk. In later stages, when a candidate gene has been shown to be related to fracture risk, these markers, such as BMD or fall risk, can help in determining the pathway through which the increased risk in incurred.

In this study the incidence of non-vertebral fractures in the Rotterdam study is well documented. However, the incidence of clinically diagnosed vertebral fractures was not included. The reason for this is that only about one third of all vertebral fractures spontaneously come to clinical attention.[5] Therefore, the incidence rate calculated from studying clinically diagnosed vertebral fracture would be highly underestimated and the relation to BMD inaccurate. The incidence of radiologically detected vertebral fractures in the Rotterdam Study and its relation to BMD has previously been well documented by van der Klift et al.[6]

6.1.2 Estrogen receptor α gene and bone

Some of the most striking consequences of estradiol (E_2) resistance are seen in bone. In 1994, a man with a loss-of-function mutation in the ERα gene was

reported.[7] Lack of functional α estrogen receptors in this young adult male led to absence of the pubertal growth spurt, delayed bone maturation, unfused epiphyses, continued bone growth into adulthood, and low bone mineral density (BMD). The pivotal role of ERα in bone development is also seen in mouse ERα knockout models where males and females show low BMD and pathological bone growth.[8,9] The ERα gene is therefore a logical candidate gene to study in bone development and osteoporosis.

The *Pvu*II and *Xba*I SNPs are the first known polymorphisms in the ERα gene and by far the most studied. These polymorphisms are located 397 and 352 base pairs, respectively, upstream of the start of exon 2 in intron 1 and are named after the presence of a binding site for the restriction enzymes *Pvu*II and *Xba*I, respectively, when the variant allele is present. Since they were first presented in the literature these SNPs have been known under a number of different names and codes. The *Pvu*II polymorphism is also commonly known as c.454-397T>C, IVS1-397 T/C and rs2234693, and the *Xba*I polymorphism is also known as c.454-351A>G SNPs, IVS1-351 A/G and rs9340799.

Given their intronic location, the *Pvu*II and *Xba*I polymorphisms were not in themselves expected to be functionally significant to the expression of the ERα protein. The observed associations with disease phenotypes were expected to be due to linkage disequilibrium (LD) with a truly functional polymorphism elsewhere in the ERα gene or perhaps even in another gene in the surrounding area. Therefore, we and other research groups determined direct haplotypes for these two polymorphisms. In so doing we could increase the "genetic resolution" at that locus and have a better chance of pinpointing the real culprit, the disease-causing, functionally significant polymorphism. The objective is to explore the genomic region by combining information from multiple markers. While the degree of LD between each of these polymorphisms and the true disease-causing mutation may be large, the LD between the combined haplotype of these SNPs is by definition larger. Therefore, by using haplotypes we can more correctly predict the real disease-causing mutation. Due to strong LD between these SNPs, out of the four possible haplotypes only three were seen frequently in the Caucasian population.

6.1.3 Stature and osteoporosis

The ERα *Pvu*II-*Xba*I haplotype 1 is strongly associated with shorter stature, lower lumbar spine BMD and increased risk of vertebral fractures in postmenopausal women, but not in men. A so-called allele-dose effect was observed in these associations, i.e., the associations increased in magnitude with increasing allele copy number. When such an allele-dose dependent effect, rather than a dominant or recessive effect, is observed in a complex disease researchers are most confident that a true genetic association is present. A linear relation can be expected to exist between gene expression rate and the clinical effect of that gene. Therefore, a polymorphism with an important influence on, for example, the expression of the

ERα protein can be expected to demonstrate an allele dose-response.

Since we had observed that the ERα polymorphisms were associated with increased vertebral fracture risk in elderly, this was a likely explanation for the finding that carriers of haplotype 1 were shorter. Therefore, we excluded all individuals who had a vertebral fracture at the time of height measurement. This did not influence the association we had found.

6.1.4 Menopausal status and ERα polymorphisms associations

After we had excluded all participants with vertebral fractures from the height analysis, however, the possibility still remained that the association was not caused by differences in linear bone growth earlier in life, but by age-related changes in the spine such as kyphosis or vertebral fractures that did not meet the McCloskey-Kanis criteria. Therefore, we verified our finding in a group of 1500 premenopausal women. In this group we observed the same association as in postmenopausal women. Not only did we validate our findings in postmenopausal women, but these findings also indicate that the difference in stature caused by the ERα polymorphisms occurred earlier in life, most likely at puberty, rather than after menopause. During puberty small differences in circulating estradiol levels have great consequences. Estradiol levels will stimulate bone growth at one level, but will cause closure of the epiphyses and cessation of growth at a slightly higher level. Therefore, it is imaginable that genotype dependent differences in ERα expression and, therefore, estradiol sensitivity may lead differences in linear bone growth.

In this same group of premenopausal women we also looked for ERα genotype related differences in lumbar spine BMD, such as we had seen in the postmenopausal women. This finding could not be reproduced in premenopausal women (data not presented in this thesis), indicating that the genotype-dependent changes in BMD are most likely related to menopausal status. We hypothesize that genotype-dependent differences in BMD occur after menopause when estradiol levels are greatly decreased. We were unable to examine the association with vertebral fractures in premenopausal women since the number of vertebral fractures prior to menopause is so low that the statistical power for such an analysis was insufficient. Taking these results together, we hypothesize that in times of rapidly changing estradiol levels, such as at puberty and after menopause, tissues are most sensitive to genotype-dependent differences in ERα expression. A graphic representation of this effect is shown in figure 2.

6.1.5 ERα polymorphisms: an independent risk factor for osteoporosis

One intriguing and clinically important aspect of the association we observed between ERα polymorphisms and vertebral fracture risk is that this increased risk is independent of BMD. This implies that the increased risk of vertebral fractures is not caused by the genotype-dependent differences in lumbar spine BMD. Through what other parameter of bone strength does the ERα gene influence fracture risk?
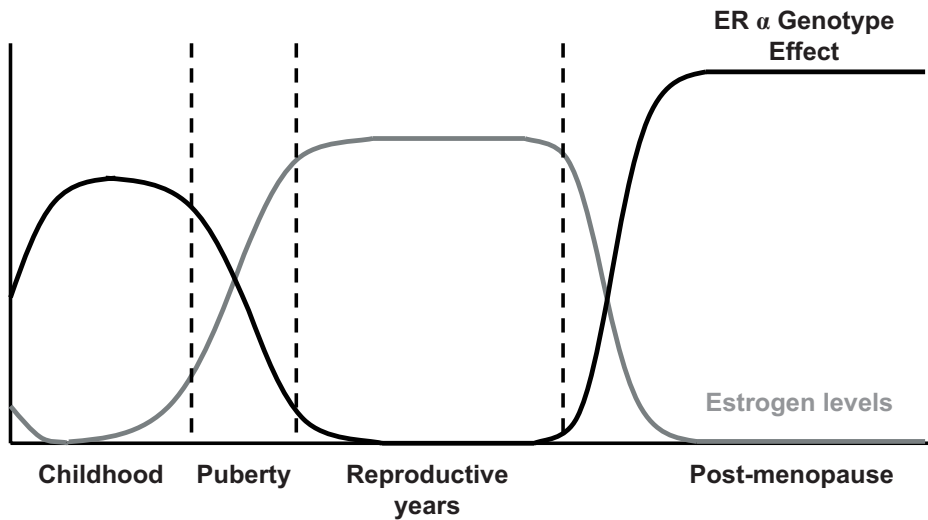
Figure 2.
Effect of ERα polymorphisms during a female life-time

Perhaps bone geometry or micro-architecture play in important role? Our finding that *Pvu*II-*Xba*I haplotype 1 is not only associated with height, but also with vertebral body size, show that ERα polymorphisms influence bone geometry. However, the genotype-dependent differences in vertebral body size did not explain the increased vertebral fracture risk, just as BMD in itself had not explained it either. On the other hand, we used DEXA scans to calculate vertebral body size. This method is crude and based on the assumption that the vertebral body is a perfect cube. It is the imperfection of this calculation that may have decreased the statistical power of this analysis. Other parameters of vertebral body geometry that we have not been able to measure may be paramount in conveying the increased fracture risk by ERα polymorphisms. More specialized DEXA scans and sophisticated software for bone structural analysis such as the method recently employed at the hip may help determining the role of the ERα in bone geometry in the future.[10]

6.1.6 ERα polymorphisms and fracture risk

A priori, we had hypothesized that the estrogen pathway candidate genes would determine osteoporotic fracture risk, including vertebral as well as non-vertebral fractures. However, ERα polymorphisms were only associated with vertebral fracture risk and not with other osteoporotic fracture types, such as hip, wrist or upper humerus. Furthermore the associations found with BMD were strongest at the lumbar spine, as compared to the femoral neck. 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.[11-14] The vertebral body is rich in trabecular bone which has a higher rate of bone turnover than cortical bone because trabecular bone presents relatively more surface per unit of bone volume. The higher bone turnover rate of trabecular bone may be the reason why the effect of the ERα gene is more pronounced in the vertebral body.

Several studies in women have reported inconsistent associations between polymorphisms in the ERα gene and BMD.[14-23] The existence of ethnic differences between the populations, the case-control designs and a health-based selection bias in several studies could explain such discordant findings. The present study confirms data of a number of previous studies.[15,16,22,24] In contrast, two other studies found the *Pvu*II-*Xba*I haplotype 3 (Px) to be associated with decreased BMD,[17,18] whereas others showed no association.[19,21,25] Statistical reasons (such as lack of power and study design) could contribute to the discordant results published so far. In addition, our findings together with those of others suggest that there could be allelic heterogeneity at the ERα locus among different populations. This should be accompanied by differences in genotype distributions, which we indeed found when we studied the allelic frequencies among different ethnic populations. The two Asian populations studied[18,21] showed different frequencies of the 2nd (PX) and 3rd (Px) haplotype compared to 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 ERα variations and bone characteristics.

6.1.7 Gender differences

An intriguing aspect of the studies on bone presented in this thesis is that all associations were significantly observed in women, while in men no associations seen. There are two major reasons this gender difference may occur. The first is based on circulating estradiol levels. An important difference between men and women is the dynamics of circulating estradiol levels. During the reproductive years estradiol levels in women are many times higher than in men. However, after the cessation of gonadal function at menopause estradiol levels in women are approximately 3 times lower than in men. If ERα *Pvu*II-*Xba*I haplotype 1 decreases expression of ERα, sensitivity to estradiol may be decreased because there are fewer α receptors for estradiol present in each cell. In postmenopausal women, circulating estradiol is so low that a difference in estradiol sensitivity may be clinically relevant. Such lower effective estradiol signaling could eventually translate to increased risk of osteoporosis and cardiovascular disease. In men, circulating estradiol levels are still sufficiently high for genotype dependent differences in ERα expression not to lead to differences in estradiol effectiveness. The second reason for the observed gender differences might be that men may be able to compensate for differences in estra-

diol sensitivity by means of an intact HPG-axis. This compensation mechanism may also occur in premenopausal women since their HPG-axis is also intact.

6.1.7 Distinguishing the effects of the different polymorphic sites

Three polymorphisms within the ERα gene have been studied in this thesis: the *Pvu*II and *Xba*I polymorphisms in intron 1 and the TA-repeat in the promoter region. At the start of the research described in this thesis, we suspected that even though the *Pvu*II and *Xba*I polymorphisms showed strong associations with different phenotypes, they were not functionally involved in regulating the expression of ERα. Our hypothesis was that the associations observed with the ERα *Pvu*II polymorphism are driven by LD with a truly functional polymorphism elsewhere in the ERα gene. In searching for this functional polymorphism, we tested a variable number of tandem repeat (VNTR) polymorphism located 1 kb upstream of the first exon at –1118 bp from the transcription start site and –1351 bp from the translation start site. The TA-repeat is located in front of the B-promoter (figure 3); a promoter speculated to be important in ERα expression in bone.[26,27] It is conceivable that the number of TA-repeats in that region may change the transcription rate of ERα mRNA. Furthermore, as is seen in other populations, in The Rotterdam Study strong LD exists between the intron 1 *Pvu*II and *Xba*I polymorphic sites and the TA-repeat VNTR, located approximately 35 kb upstream of these polymorphisms.[15,17,28,29] Therefore, linkage with the TA-repeat may explain the associations found with the *Pvu*II and *Xba*I polymorphism.

In the studies on bone, we determined both the *Pvu*II-*Xba*I haplotype and the TA-repeat genotype in our cohort. The TA-repeat polymorphism showed a bimodal distribution. Between 9 and 33 TA-repeats were present in individuals in the Rotterdam Study, while 13 through 15 repeats and 21 through 24 repeats were seen
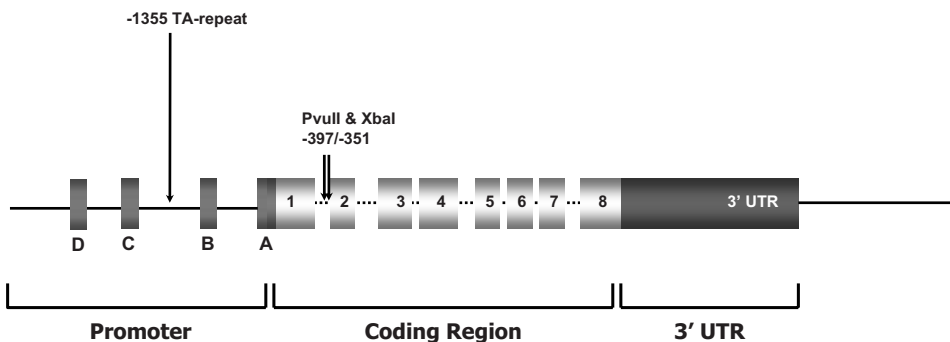

Figure 3.
Estrogen receptor α gene polymorphisms discussed in this thesis

most often. When genotyping the TA-repeat based on the individual TA-repeat alleles numbers the genotype groups were very small and the statistical power to discern a risk allele was limited. However, when we examined the associations for each TA-repeat allele separately we noticed that a bimodal distribution could also be discerned in the associations. Female carriers of alleles with a low number of TA-repeats ($<(TA)_{18}$) were consistently shorter, had a lower BMD and had more vertebral fractures than carriers of the high number of TA-repeats ($\geq(TA)_{18}$) (Table 1). To increase the statistical power of our study, we genotyped the Rotterdam Study cohort by grouping in categories of high and low TA-repeat number. We hypothesize that the number of TA-repeats in that region may change the transcription rate of ERα B-promoter. Perhaps the presence of a low or high number of TA-repeat changes the 3-dimensional positioning of the B-promoter and may augment the transcription rate.

The alleles with a low number of TA-repeats are in strong LD with the *Pvu*II-*Xba*I haplotype 1. It is, therefore, possible that the associations observed for the *Pvu*II-*Xba*I haplotype are driven by LD with the TA-repeat polymorphism. However, it may also be the other way round. To distinguish the individual role of each of these polymorphisms we used long-range haplotyping. In this method the haplotypes in which the polymorphic sites are not in linkage are the most informative. Specifically, alleles that carry a low number of TA-repeats, but do not carry the *Pvu*II-*Xba*I haplotype 1 and the other way round (high number of TA-repeats and haplotype 1) can tell us which polymorphic site is causing shorter stature and osteoporosis. Unfortunately, the LD between these polymorphic sites is so strong that we were unable to distinguish the different effects of the two polymorphic sites. Even though the cohort of more than one thousand individuals used for these analyses is quite large, the statistical power was insufficient. However, the association studies with the long range haplotypes tend to suggest that both sites are contributing, since the long range haplotype defined by carrier status for both the *Pvu*II-*Xba*I as well as the TA-repeat VNTR risk alleles shows the strongest effects on stature and osteoporosis. Larger (meta-analysis) studies are necessary to prove this hypothesis.

When we continued our research we realized that it would be most efficient to choose one these polymorphisms to test in the entire Rotterdam Study cohort. The TA-repeat polymorphism can only be determined by an expensive and time-consuming (3000 samples in three weeks) fragment sizing method, while the *Pvu*II and *Xba*I polymorphisms can be genotyped with the inexpensive and fast (3000 samples in one day) TaqMan method. Since LD between these polymorphisms is very strong, we chose to continue our research with only the *Pvu*II and *Xba*I polymorphisms.

Table 1. Association bone parameters with individual TA-repeat alleles.

TA-repeat alleles	Number of carriers	Δ % Vertebral fractures	Δ Lumbar spine BMD	Δ Height	Δ Vertebral area
11	17	+3.6	-0.02	-1.74	-0.23
12	9	.	0	-2.89	-0.61
13	158	+7.9	-0.03	-0.99	-0.24
14	597	+3.3	-0.01	-0.21	+0.09
15	203	+2.7	-0.01	-0.64	-0.31
16	58	+5	-0.01	-0.17	-0.21
17	75	+3.7	+0.02	-0.26	0.09
18	28	-5.3	+0.01	-0.49	-0.31
19	89	-3.8	+0.01	-0.4	-0.2
20	70	-5.4	-0.02	+0.75	+0.28
21	206	-3.6	+0.02	+1.54	+0.33
22	203	-1.5	+0.02	+0.19	+0.01
23	249	-3.4	+0.01	+0.56	+0.09
24	175	-5.7	+0.03	+0.82	+0.19
25	41	-0.8	-0.03	-0.52	-0.29
26	25	-5.3	+0.02	-0.22	0
27	4	.	+0.01	+0.46	-1.02

Δ = (carriers of the test TA-repeat allele) – (non-carriers)

6.2 CARDIOVASCULAR DISEASE

6.2.1 Ischemic heart disease

Aside from osteoporosis, another striking manifestation of estradiol resistance in the ERα deficient male is the presence of premature atherosclerotic coronary artery disease and endothelial dysfunction.[30] Not only the human estradiol resistance model, but also mouse ERα-knockout models have shown the importance of ERα in vascular physiology.[31-35] It was speculated that ERα polymorphisms may influence cardiovascular disease risk in the general population. In the Rotterdam study, we reported that the ERα *Pvu*II-*Xba*I haplotype 1 is significantly associated with an increased risk of myocardial infarction in postmenopausal women.

There was a two-fold increased risk of myocardial infarction in female carriers of ERα haplotype 1. We assumed that if the ERα polymorphism increased the risk of myocardial infarction then they would also increase the risk of other ischemic

heart disease events such as revascularization procedures (CABG and PTCA) and death due to chronic ischemic heart disease. When we combined these forms of ischemic heart disease (IHD) we found that ERα haplotype 1 also increased the risk approximately two-fold in postmenopausal women. The combined ischemic heart diseases variable included approximately 50% more cases then myocardial infarction alone thereby increasing the statistical power of the study significantly. Although the statistical power was limited when studying the revascularization procedures and death due to chronic ischemic heart disease categories separately, similar non-significant trends were observed. These findings support our theory that ERα gene variants influence ischemic heart disease, in every form, and confirm our findings for myocardial infarction.

Further analyses revealed that ERα polymorphisms most strongly influenced the risk of fatal ischemic heart disease. Homozygous carriers of ERα haplotype 1 had a 6 times increased risk of fatal ischemic heart disease as compared to non-carriers. Furthermore, the first year cumulative mortality after IHD was two times higher in women homozygous for haplotype 1 as compared to non-carriers. This is mainly due to genotype-dependent differences in mortality within the first month after an IHD event.

A number of possible indirect as well as direct ERα-dependent mechanisms via which estradiol may exert their cardioprotective effects have been presented in the literature.[36,37] Some of the protective effects of estradiol could potentially be mediated through systemic effects.[36] However, ERα *Pvu*II-*Xba*I genotypes were not associated with differences in a number of cardiovascular risk factors such as hypertension, hypercholesterolemia and diabetes mellitus. This implies that it is not through these pathophysiological pathways that ERα gene polymorphisms influence ischemic heart disease.

One plausible functional mechanism for the influence of ERα ischemic heart disease is through endothelial derived nitric oxide (NO) synthesis. Normal endothelium secretes nitric oxide, which both relaxes vascular smooth muscle and inhibits platelet activation.[38] One of the effects relevant to the development of atherosclerosis is that NO prevents the activation of platelets that are in close contact with the endothelium of the vessels. As platelets can recruit inflammatory cells, the inhibition of platelet activation helps prevent atherosclerosis. In addition, by dilating blood vessels, NO prevents coronary spasms that take place at the level of plaques and result in ischemia.[39] As discussed previously in chapter 3, estradiol and the ERα gene are both essential to vascular NO production by increasing synthesis and release.[40-43] The short-term coronary vasodilatory effects of estradiol in humans are largely mediated by increased NO production.[44] In cultured endothelial cells, physiological concentrations of estradiol cause a rapid release of NO;[45,46] a reaction which is blocked by specific estrogen receptor antagonists.[40] Furthermore, ERα can directly induce mRNA expression of the enzyme responsible for the production of vascular NO, endothelial NO synthase.[40] This proposed mechanism could explain

why the association is independent of known cardiovascular risk factors. Taken together, we hypothesize that female carriers of haplotype 1 have lower expression of ERα, therefore, estradiol is less effective in preventing ischemia in these women. Female carriers of haplotype 1 are more likely to suffer an ischemic event and such an event is more likely to be fatal.

6.2.2 Stroke

If these ERα SNPs influence ischemic heart disease risk then one could hypothesize that other vascular tissues may also be predisposed to occlusion and ischemia. We had previously hypothesized that ERα SNPs influence ischemia through vascular NO production. Knockout mice deficient in endothelial NO synthase are highly sensitive to focal cerebral ischemia.[47] Therefore, we hypothesized that ERα polymorphisms may influence stroke risk.

Within a subset of almost 1000 individuals from the Rotterdam Study (the Rotterdam Scan Study), MRI scans of the brain were available. At baseline of the Rotterdam Scan Study (between 1995 and 1996), MRI were evaluated for the presence of cerebral infarcts and white matter lesions; the latter being strongly related to cerebral small vessel disease. Although, the large majority (84%) of strokes diagnosed in this manner is silent (that is there are no clinical manifestations) they are still clinically quite relevant. The presence of a silent stroke is a major risk factor for the occurrence of a new stroke and of cognitive decline.[48,49] However, Similar to what we had found in ischemic heart disease, strokes identified by MRI (in particular the lacunar subtype) and white matter lesions were strongly increased in female carriers of ERα *Pvu*II-*Xba*I haplotype 1.

Within the Rotterdam Study, the incidence of many common diseases in the elderly has been well documented. Aside from ischemic heart disease, information on incident strokes has also been collected. As we had seen for ischemic myocardial infarction there was an increased risk of stroke in female carriers of haplotype 1 (hazard ratio homozygous carriers compared to non-carriers was 1.4, 95% confidence interval 0.9-2.1). However, statistical significance was not reached and these results were not described in detail in this thesis. There are a number of reasons why this may have occurred. First, data collection for stroke was not as extensive as for ischemic heart disease, therefore, the statistical power of the study was lower. Second, the later onset of stroke as compared to ischemic heart disease makes genetic comparisons between individuals difficult. Third, in contrast to MRI defined stroke, clinically diagnosed stroke includes a large variety of subtypes. These include the major subdivision between cerebral hemorrhages and cerebral infarctions. Within the ischemic strokes there are also a number of subtypes such as stroke due to cardioembolism, stroke secondary to intracerebral and extracerebral large artery disease, and small vessel disease arising from occlusion of the deep perforating arterioles. Different classification methods can be employed to identify different stroke subtypes, however, a significant number (approximately 40% in

the Rotterdam Study) are of unknown cause. The variety of stroke subtypes/phenotypes is likely to reflect different etiologies, including different genetic etiologies. Therefore, the results of our study of clinical strokes may be diluted by phenotype heterogeneity. However, the majority of MRI defined stroke are lacunar in origin (75%), which is the most common form of cerebral small vessel disease.[49,50] Therefore, association studies MRI defined stroke suffer from inaccuracy due to phenotype heterogeneity.

6.2.3 Opposing effects in men?

In our study population, ERα *Pvu*II-*Xba*I haplotype 1 was associated with lower lumbar spine BMD, increased risk of vertebral fractures, decreased height, and increased risk of ischemic heart disease, lacunar cerebral infarcts and white matter lesions in women, but not in men. A recent study by Shearman et al. from the Framingham Heart Study reported an decreased risk of cardiovascular disease in men carrying the *Pvu*II T-allele.[51] The association was only reported for men in the cohort, as women contributed very few cases (5 myocardial infarctions in women and 14 ischemic heart disease events). Therefore, no conclusions can be drawn from this study about the association in women. This is seemingly in contrast to our findings that the T-allele prevents IHD in women in our study. However, there is not a clear conflict with the seemingly opposite results reported by Shearman et al. since the men in our study showed a non-statistical trend for IHD and stroke opposite to the women. Furthermore, the men in the Rotterdam Study were much older than the men from Framingham Heart Study and we know that effects of (genetic) risk factors often change in older cohorts. Moreover, the effect of ERα on cerebral white matter lesions was statistically significant and opposite in men and women.

A great many explanations can be given for these seemingly contradictory findings between men and women. It is intriguing to hypothesize that such opposite effects observed in men and women may be due to different pathophysiological mechanisms between genders. The most obvious difference between men and postmenopausal women is the cessation of gonadal function in women. Perhaps the presence of an intact hypothalamic-pituitary-gonadal (HPG) axis in men leads to protection of the ERα *Pvu*II T-allele on cardiovascular disease, while in postmenopausal women, who are completely dependent on peripheral conversion to estradiol, an opposing effect occurs. We reported an association between haplotype 1 and lower estradiol levels in postmenopausal women, a finding which may be in line with lower lumbar spine BMD, increased risk of vertebral fractures, decreased height, and increased risk of ischemic heart disease, lacunar cerebral infarcts and white matter lesions reported in women. This study also reported a non-significant trend in the opposite direction in men, male carriers of haplotype 1 tended to have higher estradiol levels then their non-carrier counterparts. It is hypothesized that the reason for such an opposite effect is that elderly men, in contrast to postmeno-

pausal women, still have a largely intact hypothalamic-pituitary-gonadal (HPG) axis and estradiol still plays an important role in the regulation of gonadotropin release. These findings fit with our observation that these polymorphisms seem to play a protective role against cerebrovascular disease in men, but are a risk factor in women. Future research and in particular meta-analyses of large data sets are needed to establish whether there are truly opposing effects of the ERα *Pvu*II polymorphism in men and women.

6.3 Sex-hormone levels

6.3.1 Estrogen receptor α gene

As we and other studies have previously reported, female carriers of *Pvu*II-*Xba*I haplotype 1 are at an increased risk of a number of diseases associated with reduced estradiol effects. We hypothesize that this is due to reduced expression of ERα and thus decreased end-organ estradiol sensitivity. Alternatively, ERα genotype dependent differences in estradiol production may also partially explain the observed associations with these diseases.

Human and mouse ERα knockout models both have elevated serum levels of estradiol levels.[52,53] This is not an unexpected finding since sex-hormone production is regulated by the HPG axis, a system upon which estradiol negatively feeds back. Therefore, resistance to estradiol, due to lack of functional ERα, may lead to a higher "set-point" in the HPG-axis and, consequently, higher circulating estradiol levels. However, a prerequisite for this effect is the presence of an intact HPG-axis. However, the main characteristic of menopause is the cessation of gonadal function and the ineffectiveness of the HPG-axis. Postmenopausal women are, therefore, completely dependent on peripheral synthesis of estradiol.

One of the most important enzymes in the peripheral conversion of estradiol
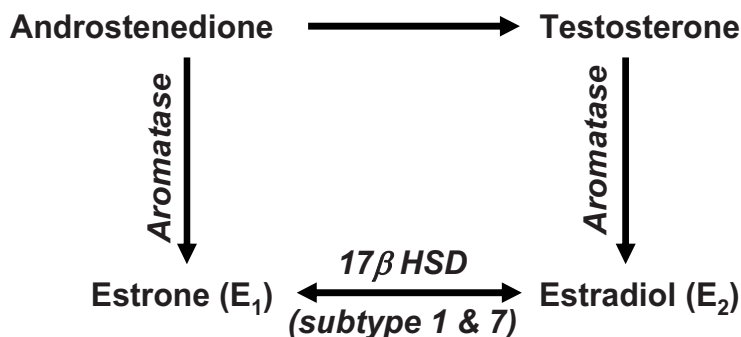

Figure 4.
Last steps in the synthesis of estradiol

is aromatase. This enzyme, produced by the gene *CYP19*, is responsible for the conversion of androgens to estrogens. It has recently been reported that estradiol, through ERα, can modulate *CYP19* gene expression in human breast cancer cells through a positive feed-back system.[54] This finding is not only seen in humans, but has also been shown for other vertebrates.[55] It is tempting to hypothesize that if ERα expression is reduced, the expression of *CYP19* may also be reduced. Therefore, postmenopausal carriers of haplotype 1, who are less sensitive to estradiol, may have lower expression of aromatase and, consequently, lower circulation estradiol levels. This is what we observed in our study of postmenopausal women from the Rotterdam Study. ERα *Pvu*II-*Xba*I haplotype 1 was associated with significantly lower estradiol levels in postmenopausal females, while in males no association was observed. We can, therefore, conclude that postmenopausal female carriers of haplotype 1 appear to be more susceptible to certain diseases not only due to decreased end-organ sensitivity to estradiol, but also due to decreased circulating estradiol levels.

We had expected estradiol levels in women to be influenced by ERα polymorphisms due to the effect of ERα on *CYP19* expression. In that case we would also expect circulating estrone (E_1) levels to be affected in the same direction since aromatase not only catalyzes the conversion of testosterone to estradiol, but also the conversion of androstenedione to estrone (Figure 4). Unexpectedly, we observed an opposite effect; carriers of haplotype 1 had higher estrone levels in an allele-dose dependent manner. Although this association was not significant, it is strange that the direction of the association was the other-way-round. This led us to consider that it is not *CYP19* expression that is influenced by ERα, 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 conversion of estrone into estradiol.[56] Although regulation of 17β-HSD by estradiol or ERα has not been shown so far, our results do suggest a role for ERα in 17β-HSD expression or activity.

The variance in estradiol level explained by the ERα *Pvu*II-*Xba*I haplotype in postmenopausal women is only 1%. However, the difference in circulating estradiol between extreme genotypes (17.5 pmol/L in non-carriers versus 14.1 pmol/L in homozygous carriers) may be clinically significant. A recent study in the Rotterdam cohort reported that individuals with estradiol levels below a "cut-off" value of 15.5 pmol/L have an increased risk of osteoporosis [57]. These findings suggest that the genotype dependent differences in estradiol levels created by these polymorphisms may be clinically relevant.

6.3.2 Aromatase (*CYP19*) gene

Genes that determine the variance in circulating estradiol levels in postmenopausal women may also be involved in determining the risk of diseases such as osteoporosis[57,58] and breast cancer.[59] As discussed previously, the enzyme aromatase

catalyzes the rate-limiting step in the synthesis of estradiol. Within the gene encoding aromatase, *CYP19*, a number of polymorphisms have been documented. One very interesting polymorphism is located in exon 10 in the 3'UTR. Although this single nucleotide polymorphism (SNP) does not lead to an amino acid variation in the gene, its location in the 3'UTR could imply it is important for *CYP19* mRNA stability. In fact it has been reported that the T-allele of this C-T substitution is associated with increased aromatase mRNA levels and a switch from the adipose promoter to ovarian promoter.[60] The exact mechanism for the switch in promoter is uncertain. Kirstensen et al. speculated that two different folding patterns for the 3' end of the *CYP19* transcript occur depending on the nucleotide in the polymorphic position.

The aim of our study was to determine if this SNP in the *CYP19* gene could influence estradiol levels in postmenopausal women. Female carriers of the C-allele, the allele associated with significantly lower *CYP19* mRNA expression, had lower estradiol levels in an allele-dose dependent manner. In men, no association with estradiol was observed. An explanation for this finding may be that in elderly men, the HPG-axis is still largely intact and, thus, men still rely on neuroendocrine driven estradiol production. Through negative feedback the quantity of sex-hormones synthesizes is thus regulated by gonadotropins. In contrast, in postmenopausal women a negative feedback system no longer exists. Therefore, estradiol production is more dependent on aromatase expression.

6.4 Functionality of ERα PvuII polymorphism

In view of the fact that the PvuII and XbaI polymorphisms have been associated with many different phenotypes by different research groups their possible functional significance to ERα expression have been amply speculated about. Given their location, 397 and 351 basepairs upstream from the start of exon 2, possible functional mechanisms include altering ERα expression via altered binding of transcription factors and influencing alternative splicing of the ERα gene. Both these mechanisms can be a direct result of either of these polymorphisms or through LD with a truly functional, but so far unknown, sequence variation elsewhere in the ERα gene. The first mechanism was recently supported by findings of Herrington et al.,61 who showed that the T-allele of the PvuII RFLP eliminates a functional binding site for the transcription factor B-myb. This suggests that presence of this allele may result in lower ERα transcription. This mechanism has previously been reported in the collagen type Iα1 (COLIA1) gene, where a change in a transcription factor binding site caused by a polymorphism in intron was shown to alter gene expression.62

Herrington et al. tested the functionality of this SNP in monkey kidney cells (CV1 cells) and human embryonic kidney cells (293T cells).[61] However, regulation of transcription may differ between cells of various origins, therefore, we have studied functional aspects of the ERα polymorphism in human osteoblasts. We found

that in a human osteosarcoma cell line (MG-63 cells) the *Pvu*II C-allele creates a binding site for the transcription factor B-myb. However, these in vitro studies can not simply be translated to in vivo mechanisms. In these experiments MG-63 cells were used as a reaction unit; the cell's own B-myb reserves were not used since the transcription factor was cotransfected into the cells. If we had observed a significant increase in luciferase expression in C-allele cells that were not cotransfected with B-myb (B-myb negative control) then we could have concluded that the *Pvu*II C-allele leads to higher ERα expression in human bone cells. However, we did not observe this. There may be many reasons why we could not observe such an increased expression. These include the fact that B-myb expression is most likely cell-cycle related and that increased expression caused by the *Pvu*II C-allele may be very small (but nonetheless significant to the function of the cell) and, therefore, undetectable in these experiments. Furthermore, B-myb is only one of a great many transcription factors likely to play a role in osteoblast function. Therefore, we can not be certain that this is a functional process in normal bone cells.

The findings by Herrington et al.[61] and this study that the *Pvu*II polymorphism T-allele may lead to lower expression of ERα are in line with observations in our study population, as well as in others, that the T-allele is associated with a number of phenotypes that are known to be related to low estradiol levels and therefore low estradiol activity. These phenotypes include an increased risk of osteoporosis, decreased risk of osteoarthritis and hysterectomy, shorter stature, and later age at menopause.[28,29,63,64]

If the *Pvu*II polymorphism is truly the functional SNP, are the associations reported for the *Xba*I SNP not driven solely by LD? We studied the *Pvu*II-*Xba*I haplotype and not the *Pvu*II polymorphism alone. Although the mechanism sketched above is very plausible it is by no means a certainty. Many other possible functional mechanisms are still possible, including LD with a functional polymorphism. Therefore, haplotyping a gene locus is the best way to increase genetic resolution at that point in the genome. However, since the presence of haplotype 4 is negligible, haplotype 1 fully represents the *Pvu*II polymorphism. Therefore, the associations with ERα haplotype 1 reported in this thesis are exactly the same as for the *Pvu*II polymorphism alone.

A next step in proving support for the functional significance of the *Pvu*II polymorphism would be to show that it is this polymorphism that is driving the associations with the *Pvu*II-*Xba*I haplotype. However, since LD between the *Pvu*II and *Xba*I polymorphisms is so strong is not possible to distinguish the effects of *Pvu*II and *Xba*I even in a cohort 6600 individuals such as the Rotterdam Study. The statistical power needed for such an effort can only be realized by doing large meta-analyses. A meta-analysis of more than five thousand women from 30 study cohorts has recently shown that it may be the *Xba*I polymorphism which is driving the haplotype associations with BMD.[65] In this meta-analysis, only the *Xba*I A-allele was significantly associated with lower BMD, while the association with the *Pvu*II

polymorphism T-allele was not significant associated with decreased BMD. More studies of this magnitude are needed to verify these findings. At this moment, a large European collaboration (GENOMOS) is underway in which the ERα *Pvu*II and *Xba*I polymorphisms are genotyped in more than 20.000 women and 5000 fracture cases are available. This study will hopefully answer these questions.

There are also other plausible mechanisms for a functional significance of the *Pvu*II or the *Xba*I polymorphism. One of these is that one or both of these polymorphisms may influence alternative splicing of the ERα gene. Several studies have demonstrated the expression of an exon 1-truncated human ERα isoform (46-kDa ERα) in both endothelial cells and osteoblasts.[66-70] Two recent studies have shown that this ERα isoform, primarily located at the plasma membrane, specifically binds estradiol and mediates acute estradiol actions, in particular eNOS activation, more efficiently that the full-length (66-kDa) classical ERα.[66,69] Furthermore, this truncated ERα isoform is unable to mediate transcriptional responses to estradiol. This unique ability of the truncated ERα isoform to mediate acute but not transcriptional responses to estradiol suggests that it may play an important role in rapid estradiol signaling. The location of the *Pvu*II and *Xba*I polymorphisms 397 and 351 basepairs from the start of exon 2, the transcription start site for the exon 1-truncated ERα isoform, suggests that these polymorphisms may influence alternative splicing and/or transcription and, therefore, influence the 46-kDa to 66-kDa ERα ratio. Further studies are needed to support this hypothesis.

We can not exclude the possibility that the observed associations with the ERα TA-repeat, *Pvu*II and *Xba*I polymorphisms are driven by LD with another, so far unknown, functional polymorphism. LD seems to have a block-like structure in genes. For example, in the aromatase gene a haplotype block exists that spans a portion of the promoter region and entire coding region.[71] Since such strong linkage exists between the polymorphisms in the promoter region and the first intron, we have likely found such a block in the ERα gene. Another functional polymorphism may be located somewhere within this block which drives the associations we observed. Sequencing of the promoter and coding sequence of the ERα gene will be necessary to find this potential polymorphism.

6.5 Identifying Polymorphisms

We identified polymorphisms in the candidate genes in our studies by consulting polymorphism and literature databases. This is an inexpensive and fast method to find polymorphisms when compared to sequencing. But how thorough is it? So far, the public SNP consortium has identified 1.4 million polymorphisms.[72] Although each collection contains a modest false-positive rate (10–15%), the false-negative rate is of greater concern.[73] Because the SNP collection was not based on the sequence of a large number of subjects, numerous lower frequency (<10%) SNPs, especially those that are specific to a single population, were not detected. A more comprehensive sequencing effort (84 ethnically diverse individuals) has been

carried out for 313 genes and 720 kb of genomic sequence.[74] Only 2% of the SNPs identified are in the public SNP database, suggesting that there are many more SNPs than the roughly 1.2 million SNPs in this database. Are we employing the right method by consulting these databases? As stated previously, LD seems to have a block-like structure in genes. It has been suggested that a judiciously chosen subset of SNPs can identify most of the genetic variation in any genomic region through haplotypes.[75] A previous study has demonstrated that, within LD blocks, more than 90% of the diversity of common haplotypes (>5%) may be captured by six to eight common SNPs and that these common haplotypes explain the vast majority of genetic variation contributed by unmeasured or undiscovered SNPs.[76] Therefore, in the initial stages of studying a candidate gene, choosing polymorphisms through the SNP databases may be the most efficient method to determine if a gene of interest is indeed involved in determining the phenotype. By choosing a number of polymorphisms spaced similarly a good representation of the polymorphisms in the gene can be tested. If associations with one or more of the polymorphisms are observed, such as for the promoter and intron 1 polymorphisms in the ERα gene in our studies, in the next step fine mapping of the region should take place to find the disease causing locus. During the fine mapping stage, the region containing the associated polymorphisms should be sequenced to identify all present polymorphism. For the ERα gene, sequencing of the region surrounding the TA-repeat, *Pvu*II and *Xba*I polymorphisms will be the next step in identifying the disease causing locus.

6.6 CLINICAL RELEVANCE

The question being raised in all areas of genetic association research today is: "What is the clinical relevance?" The importance and relevance to human biology of identifying and characterizing genes for complex disorders has been clearly demonstrated. However, research still has a long way to go before we are able to use the knowledge obtained so far in clinical practice. We can envision that in the future polymorphisms such as the ERα polymorphisms discussed in this thesis will be used to predict risk for the general population. For example, a general practitioner may opt to treat mild hypercholesterolemia or hypertension more quickly in a female patient who also carriers *Pvu*II-*Xba*I haplotype 1 than in a woman who does not carry the risk allele. However, to use such polymorphisms in general practice more must be known about that particular sequence variant and its functional significance to gene expression. Another example of how polymorphisms may be clinically relevant in the future is in identifying women who will benefit most treatments such as hormone replacement therapy.

Even though the polymorphisms discussed in this thesis are by no means exploitable in clinical practice at this moment, the associations described do have a number of characteristics that make them potentially very interesting to clinicians. First, the frequency of haplotype 1 is quite high; approximately one quarter of the Caucasian population is homozygous carrier of haplotype 1, one half is heterozy-

gous carrier and one quarter is homozygous carrier. Secondly, the disease pheno-types this haplotype is associated with, osteoporosis and cardiovascular disease, are common disorders in the elderly. Taken together with the high frequency of the haplotype, the population-attributable risk due to these polymorphisms can be quite high. Thirdly, the associations with osteoporosis and cardiovascular disease are independent of known risk factors. The increased risk of cardiovascular disease, for example, conveyed by the ERα haplotype is not mediated by any of the known risk factors such as cholesterol and blood pressure. Therefore, there are no easily evaluated parameters that can predict this increased disease risk; the only way to identify individuals at an increased risk is through genotyping.

6.7 FUTURE RESEARCH

There are several factors future researchers should consider in my opinion. With respect to genetic association research in general a number of comments should be made. Genotyping methods are quickly becoming faster and easier to do. In the five years I have been involved in genetic association research giant leaps in technology have been made. Just five years ago, months of planning and laboratory work proceeded association studies. Today a polymorphism can be genotyped in a large cohort within a few hours. We run the risk of publishing false positive results purely due to multiple testing, but at the same time we risk missing important gene effects, such as interactions with environmental risk factors, due to the large amount of data being produced. Therefore, a structured method for studying candidate gene pathways should be developed within each research group to prevent these pit-falls.

Within ERα and *CYP19* gene research, studies of polymorphism functionality will be a main focus in the future. In the last two decades of genetic association studies many polymorphisms have been associated with even more phenotypes, but the functional background has been largely ignored. Although, dozens of reports of associations of ERα gene polymorphisms have been published, the first two studies of a possible functional mechanism behind the observed associations have only recently been reported. The general opinion today is that an explanation for the functional mechanism behind reported associations is essential. However, simply testing the functional significance of polymorphisms is not efficient. The first step must always be to first test a polymorphism for association with the phenotype of interest. Without a clinical association functional testing of a polymorphism is of no value.

An area of clinically relevant research for the ERα gene in particular is in identifying women who will benefit most treatments such as hormone replacement therapy. The combined estrogen plus progestin part of the Women's Health Initiative randomized clinical trial was recently stopped early because of an excess cardiovascular and breast cancer risk in the active arm compared with the placebo group.[77] This surprising and unexpected finding, coupled with the results of

other recently completed randomized clinical trials, has dramatically transformed opinions concerning use of hormone replacement therapy (HRT). Specifically with respect to cardiovascular disease, HRT is migrating from a position of presumed benefit to one of confirmed harm. One important question concerning the cardiovascular effects of HRT is whether important genetic factors can substantially modify individual women's responses to estrogen treatment. If subgroups of women exist who are genetically predisposed to a greater or lesser response to HRT, for good or ill, HRT could be directed to women most able to benefit from it, and withheld from women likely to be harmed. Knowledge of such genetic factors may also contribute to the understanding of the biological variability in many other physiological processes influenced by both endogenous and exogenous estrogen. Since we have already shown that ERα gene polymorphisms probably influence sensitivity to estradiol, common sequence variants in the ERα gene may also alter the effects of estrogen treatment at the cellular, biochemical, or clinical level in postmenopausal women. The fact that only half of all estrogen-receptor-positive breast carcinomas are responsive to hormone therapy is additional clinical evidence for the existence of estrogen receptor variants that respond in different ways to estrogen treatment.[78] Recently, several studies have examined the effects of one or more ERα polymorphisms on the response to estrogen treatment in randomized clinical trials.[61] Within the Estrogen Replacement and Atherosclerosis (ERA) trial, 309 women with coronary artery disease were genotyped for ERα polymorphisms. [61] Postmenopausal women who carried the ERα *Pvu*II C-allele had a greater effect of HRT on HLD cholesterol than non-carriers. In two small clinical trials of the effects of estrogen on bone mineral density, the *Pvu*II C allele was associated with greater effects of HRT on bone mineral density.[14,79] However, a study of 248 Korean women found no such association.[80] It is of great interest to repeat these studies in larger randomized control trials and particularly compare the risk of cardiovascular disease in response to HRT treatment between genotype groups.

Verification of reported associations is another area of interest for the future. Many hundred to thousands genes are involved in complex disease risk. Since each individual gene will only confer a small relative risk in complex disorders, replication of results is often difficult. The inability to replicate many results has led to increasing skepticism about the value of simple association study designs for detection of genetic variants contributing to common complex traits. It has been suggested that a genetic association should always be replicated before an effect is considered valid.[81] Large scale collaborations between studies to increase power by pooling cohorts will play a key role in the future. The recently set up European consortium GENOMOS is an excellent example of such an effort in osteoporosis research.

In this thesis, we have shown that ERα gene polymorphisms lead to difference in the risk of diseases such as osteoporosis and cardiovascular disease. These diseases are evidently related to circulating estradiol levels since their incidence

increases after menopause. The incidence of a number of other disorders is also increased after menopause including dementia, anxiety disorders, and breast, colon and endometrial cancer. Furthermore, the occurrence of menopausal complaints such as "hot flashes" may also be influenced by estrogen pathway genetics. If carriers of ERα haplotype 1 are less sensitive to estradiol then perhaps these women also have more menopausal complaints. It is plausible that polymorphisms in genes involved in estrogen production and signaling may also influence the risk of these disorders and future research into these associations is of great interest.

Within the estrogen pathway, other candidate genes may prove of great importance. As stated in the introduction, there are five broad classes of human genes of relevance for estrogen production and action. These include genes involved in the neuroendocrine regulation of sex-hormone production; enzymes involved in the synthesis of estradiol; the estrogen receptors α and β; genes involved in the downstream pathways of estrogen action; and genes involved in drug absorption, transport, and metabolism. In this thesis we have studied one of the estrogen receptors and a protein that catalyzes a rate-limiting step in estradiol production, aromatase. However, many other interesting candidate genes remain to be studied. The newly identified ERβ gene is one of the most important candidate genes for future research. ERβ seems to be an important factor in the mechanism of action of estrogen, and it is expressed in many tissues, including the central nervous system, the cardiovascular system, the immune system, the urogenital tract, the gastrointestinal tract, the kidneys and the lungs.[82-88] Research in knockout mice has suggest an antagonistic effect of the ERα and β genes.[53,89] For example, while ERα knockout mice have decreased BMD and tend to show more aggression, ERβ knockout mice tend to have higher BMD and show less aggression. This ying-yang effect of these genes implies that polymorphisms in the ERβ gene may have an important and clinically relevant effect. Many other candidate genes in this pathway can also be identified. One method to choose these genes is to determine the rate-limiting protein in each step of estradiol production and action. For neuroendocrine regulation of sex-hormone production the gene encoding gonadotropin releasing hormone (GnRH) may then be the first candidate gene to examine.

In conclusion, with the rapid advancement of new technologies for high throughput genotyping and a greater understanding of the molecular mechanisms involved in conveying disease risk there are a great number of directions future research should focus. However, we must keep in mind that now, more than ever, knowledge about biological explanations for observed associations should not be ignored, but sought after actively. An approach, such as the one used for the studies in this thesis, where observed associations and functional experiments are brought together to create a unifying explanation for the findings reported may be a good example of how to approach genetic research in complex diseases in the future.

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7 | Summary

Chapter 7.1

Summary

Estradiol, one of the sex hormones responsible for gender dimorphism and reproduction, is a pleitropic hormone with widespread biological actions far beyond human reproduction alone. Several lines of evidence suggest that estradiol may be instrumental in the etiology of diseases such as osteoporosis and cardio-vascular disease. In searching for candidate genes for these diseases the estrogen signaling pathway is an obvious area of research. Two candidate genes that play a pivotal role in this pathway were chosen: the estrogen receptor alpha (ERα, also known as *ESR1*) and the aromatase (*CYP19*) gene. This thesis aims to elucidate the role of common sequence variations (polymorphisms) in these candidate genes in determining the incidence of estrogen related diseases and phenotypes in the general population. The diseases and phenotypes discussed in this thesis are related to bone, cardiovascular disease and circulating hormone levels. Most studies presented in this thesis were based on data from the Rotterdam Study, a large prospective population-based cohort study in the Netherlands. Participants of this study were men and women aged 55 years or over living in Ommoord, a suburb of Rotterdam.

In **chapter 2**, studies of the association between estrogen receptor α gene polymorphisms and bone phenotypes such as vertebral fracture risk, bone mineral density (BMD) and stature are presented. The overall fracture incidence in the Rotterdam Study and the correlation with BMD are discussed in **chapter 2.1**. In summary, hip, wrist and upper humerus fractures are the most frequent fractures in both men and women. In many genetic association studies due to logistic or financial reasons BMD is used as a surrogate marker for osteoporosis instead of fractures. While each standard deviation decrease in BMD does increase the risk of fracture, only 21 to 34% of all fractures occur in men and women with a BMD that is considered to be in the osteoporotic range (T-score < -2.5). Therefore, we conclude that although it is time-consuming and expensive, it is essential to investigate the clinical outcome of osteoporosis, i.e., fractures, when testing candidate genes for osteoporosis.

In **chapter 2.2**, the association between the ERα *Pvu*II, *Xba*I and TA-repeat polymorphisms and osteoporosis is presented. One of the haplotype alleles spanning the *Pvu*II and *Xba*I polymorphic sites (ERα haplotype 1 (*Pvu*II T-allele and *Xba*I A-allele)) is associated with decreased lumbar spine BMD and an increased risk of vertebral fractures in postmenopausal women. The impact of this haplotype on the incidence of osteoporosis in the general population may be considerable since the risk allele is quite common. Approximately one quarter of the population is homozygous carrier of the risk allele, 50% is heterozygous carrier and one quarter is non-carrier. An interesting aspect of the association with vertebral fracture risk is that it is independent of BMD. Therefore, it appears that it is not only through BMD that ERα influences fracture risk, but also through another aspect of bone quality that is not reflected in BMD measurements. One of these parameters may be

bone geometry or microarchitecture. In contrast to the findings in postmenopausal women, no association with osteoporosis was observed in men.

The same ERα haplotype allele associated with osteoporosis is also associated with shorter stature and smaller lumbar vertebral body area in women, as described in **chapter 2.3**. The association with stature was not only seen in the postmenopausal participants of the Rotterdam Study, but also in the pre- and perimenopausal participants of the Eindhoven Study. These findings indicate that the effects of the ERα gene polymorphisms on height occur prior to menopause, perhaps at puberty.

Chapter 3 focuses on the association between ERα polymorphisms and cardiovascular disease. In postmenopausal women, the same ERα haplotype allele associated with osteoporosis was found to increase the risk of ischemic heart disease, including myocardial infarction, approximately two-fold (**chapter 3.1**). This ERα risk allele is an independent risk factor for ischemic heart disease since the association was unaffected by known cardiovascular risk factor such as weight, cholesterol and hypertension. As observed previously for the bone phenotypes, no associations were observed in men. Further analysis revealed that the ERα risk allele is most strongly associated with fatal ischemic heart disease events. Female carriers of ERα haplotype 1 are not only twice as likely to suffer a myocardial infarction, they are also twice as likely to die from it.

In **chapter 3.2** MRI (Magnetic Resonance Imaging) defined stroke and cerebral white matter lesions are studied. Similar to what was found for ischemic heart disease, in postmenopausal women ERα *Pvu*II-*Xba*I haplotype 1 is also an independent risk factor for the presence of brain infarcts on MRI. In particular, lacunar infarcts, a stroke subtype thought to be largely due to ischemic cerebral small vessel disease, were significantly associated with ERα haplotype 1. Furthermore, in women ERα haplotype 1 was also associated with increased severity of white matter lesions which are also considered to be caused by ischemic small vessel disease. An intriguing aspect of these associations with cerebral vascular disease is that while haplotype 1 is a risk factor in women, it appears to be a protective factor in men. This is possibly also seen in the association with ischemic heart disease, where haplotype 1 - although it is not statistically significant – seems to have a protective effect in men. The protective effects of ERα haplotype 1 on cardiovascular disease in men in have also recently reported in the literature in the Framingham Heart Study, lending further support to our findings.[1] However, further research is needed to clarify the gender specific mechanisms behind this association.

Individual variation in postmenopausal estradiol levels is an important determinant of diseases such as osteoporosis. A large part of the individual variation may be determined by genetic variations (i.e. polymorphisms) in genes that control hormone biosynthesis. Studies of the influence of genetic variations in the ERα

and aromatase (*CYP19*) genes on circulating sex-hormone levels are presented in **chapter 4**. The ERα haplotype 1, previously associated with osteoporosis, stature and cardiovascular disease is also associated with decreased circulating estradiol levels in postmenopausal women (**chapter 4.1**). In men no association was observed, perhaps due to the presence of an intact hypothalamic-pituitary-gonadal (HPG) axis in men, as opposed to postmenopausal women. From our finding that estradiol (E_2) levels are, while estrone (E_1) levels are not, associated with these ERα polymorphisms, we hypothesize that it is likely that these common sequences variants alter expression or activity of the enzyme 17β-HSD subtype 1 or 7. This enzyme is responsible for the conversion of estrone to estradiol. The variance in postmenopausal estradiol level explained by the ERα *Pvu*II-*Xba*I haplotype is only 1%. However, the difference in circulating estradiol between extreme genotypes (17.5 pmol/L in non-carriers versus 14.1 pmol/L in homozygous carriers) may be clinically significant. A recent study in the Rotterdam cohort reported that individuals with estradiol levels below a "cut-off" value of 15.5 pmol/L have an increased risk of osteoporosis. These findings suggest that the genotype dependent differences in estradiol levels created by these polymorphisms may be clinically relevant.

The association of a possibly functional polymorphism in the aromatase (*CYP19*) gene with circulating estrogen levels in postmenopausal women is discussed in **chapter 4.2**. The *CYP19* polymorphism was associated with estradiol as well as estrone levels in postmenopausal women. This findings is in line with the a priori hypothesis that variations in *CYP19* expression will influence all conversions catalyzed by the aromatase enzyme. In men, who still have an intact HPG axis, no associations with sex-hormone levels were observed.

Three polymorphisms within the ERα gene have been studied in this thesis: the *Pvu*II and *Xba*I polymorphisms in intron 1 and the TA-repeat in the promoter region. Until recently it was suspected that although these polymorphisms show strong associations with different phenotypes they do not have functional consequences on ERα expression. The observed associations with among others osteoporosis were though to be most likely due to linkage disequilibrium with a functional polymorphism elsewhere in the ERα gene or perhaps even in another gene in the surrounding area. However, the findings reported in **chapter 5.1** provide the alternative hypothesis that the *Pvu*II polymorphism may be functionally significant to ERα expression. The presence of the *Pvu*II T-allele (the allele also present in ERα *Pvu*II-*Xba*I haplotype 1) eliminates a functional binding site for the transcription factor B-myb. This suggests that presence of this allele may result in lower ERα transcription. It is hypothesized that carriers of the T-allele (and also ERα haplotype 1) will be less sensitive to the effects of estradiol due to the presence of a lower number of estrogen receptors.

In the general discussion in **chapter 6**, the results described in this thesis are brought together and placed in a broader perspective. The different clinical phenotypes associated with the candidate genes are compared to the known functional studies. The discussion also focuses on the gender differences observed in the genetic association studies and the possible biological basis. In addition, the significance of this research in clinical practice is discussed. Finally, recommendations for future research are given.

Reference

1. **Shearman AM, Cupples LA, Demissie S, et al.** Association between estrogen receptor alpha gene variation and cardiovascular disease. *JAMA.* 2003;290(17):2263-2270.

Chapter 7.2

Samenvatting

Oestradiol, één van de geslachtshormonen verantwoordelijk voor geslachts-differentiatie en voortplanting, is een pleiotroop hormoon met een verscheidenheid aan biologische functies buiten alleen de voortplanting. Verschillende bewijsvoeringen suggereren dat oestradiol cruciaal zou kunnen zijn in de etiologie van ziekten zoals osteoporose (botontkalking) en hart- en vaatziekten. Bij het zoeken naar kandidaatgenen voor deze ziekten is de oestrogeen signaal transductie een voor de hand liggend onderzoeksgebied. Uit de verschillende genen betrokken bij de oestrogeen signaal transductie zijn twee kandidaatgenen onderzocht die een centrale rol spelen in deze signaal transductie. Het betreft het oestrogeen receptor alfa (ERα, ook wel bekend als *ESR1*) en het aromatase (*CYP19*) gen. Dit proefschrift richt zich op het verhelderen van de rol van veel voorkomende DNA sequentievarianten (polymorfismen) in deze kandidaatgenen bij het bepalen van de incidentie van oestrogeen gerelateerde ziekten en individuele variatie in fenotypen in de bevolking. De ziekten en fenotypen die behandeld worden zijn osteoporose, hart- en vaatziekten, lichaamslengte en circulerend geslachtshormoon spiegels. De meeste studies die hier gepresenteerd worden zijn gebaseerd op het Rotterdamse ERGO-onderzoek (Erasmus Rotterdam Gezondheid en Ouderen), internationaal bekend als "the Rotterdam Study". Dit is een groot prospectief bevolkingsonderzoek onder mannen en vrouwen van 55 jaar en ouder uit de Rotterdamse deelgemeente Ommoord.

In **hoofdstuk 2** worden de associaties tussen polymorfismen in het ERα gen en botfenotypen zoals risico op wervelinzakkingen (vertebrale fracturen), bot mineraal dichtheid (BMD) en lichaamslengte gepresenteerd. De algehele incidentie van fracturen binnen het ERGO-onderzoek en de correlatie met BMD wordt bestudeerd in **hoofdstuk 2.1**. Samenvattend komen heup, pols en proximale humerus facturen het meeste voor bij zowel mannen als vrouwen. Om logistieke en financiële redenen wordt bij veel genetische associatie studies BMD in plaats van fractuurrisico gebruikt als surrogaat marker voor osteoporose. Terwijl iedere standaard deviatie vermindering in BMD inderdaad lijdt tot een toename in fractuurrisico, ontstaan slechts respectievelijk 21% en 34% van alle fracturen in mannen en vrouwen met een BMD welke geacht wordt binnen het osteoporotische gebied te liggen (T-score < -2.5). Wij concluderen dat, alhoewel het tijdrovend en duur is, het essentieel is om de klinische gevolgen van osteoporose te bestuderen wanneer we de kandidaatgenen onderzoeken.

In **hoofdstuk 2.2** wordt de associatie tussen de ERα *Pvu*II-, *Xba*I- en TA-repeat polymorfismen en osteoporose bestudeerd. Van combinaties van deze drie polymorfismen komen drie varianten ("haplotype allelen" genoemd) voor in de bevolking: haplotype 1, -2, en -3. Één van de haplotype allelen, ERα haplotype 1 (*Pvu*II T-allel en *Xba*I A-allel) is bij postmenopauzale vrouwen geassocieerd met verlaagde lumbale wervelkolom BMD en een toegenomen risico op wervelinzakkingen. De invloed van dit haplotype op de incidentie van osteoporose in de algemene bevolk-

ing kan aanzienlijk zijn, aangezien het risico allel frequent voorkomt. Ongeveer een kwart van de bevolking is homozygoot drager van het risico allel, 50% is heterozygoot drager en een kwart draagt het risico allel niet. Een interessant aspect van de gevonden associatie met wervelinzakkingen is dat het onafhankelijk is van BMD. Het is dus niet alleen via BMD dat het ERα het risico van wervelinzakkingen beïnvloed maar ook en met name via andere aspecten van bot kwaliteit. Één van de aspecten zou bot geometrie of microarchitectuur kunnen zijn. In tegenstelling tot de bevinding bij postmenopauzale vrouwen, werd er bij mannen geen associatie met osteoporose gevonden van dit risicoallel.

Hetzelfde ERα haplotype dat een risicofactor is voor osteoporose, is ook geassocieerd met een kortere lichaamslengte en een kleiner werveloppervlak in vrouwen zoals beschreven in **hoofdstuk 2.3**. Deze associatie werd niet alleen gezien in de postmenopauzale deelnemers aan het ERGO-onderzoek maar werd ook gezien in pre- en perimenopauzale deelnemers van de Eindhoven Perimenopauzale Osteoporose Studie (EPOS). Deze bevindingen tonen aan dat de effecten van het ERα gen op lichaamslengte al voor de menopauze ontstaan, waarschijnlijk tijdens de puberteit.

Hoofdstuk 3 richt zich op de associatie tussen ERα polymorfismen en hart- en vaatziekten. Bij postmenopauzale vrouwen veroorzaakt hetzelfde ERα haplotype welke geassocieerd is met osteoporose een twee maal verhoogd risico op ischemische hartziekten waaronder hartinfarcten (**hoofdstuk 3.1**). Aangezien de relatie niet wordt beïnvloed door bekende risicofactoren voor hart- en vaatziekten zoals gewicht, cholesterol en hypertensie is dit ERα risico allel een onafhankelijke risicofactor voor ischemische hartziekten. Nader onderzoek onthulde dat het ERα risico allel de sterkste relatie had met fatale hartinfarcten. Vrouwelijke dragers van ERα haplotype lopen niet alleen een twee maal verhoogde kans om een hartinfarct te krijgen, zij hebben ook een twee maal verhoogde kans om te overlijden aan het hartinfarct.

In **hoofdstuk 3.2** worden beroerten en cerebrale witte stof afwijkingen bestudeerd die met behulp van MRI (Magnetic Resonance Imaging) zijn vastgesteld. Net als wat gevonden werd bij ischemische hartziekten, werd bij postmenopauzale vrouwen gevonden dat het ERα haplotype 1 ook een onafhankelijke risicofactor is voor het bestaan van herseninfarcten op de MRI. Lacunaire infarcten in het bijzonder waren geassocieerd met het ERα risico allel. Het lacunaire herseninfarct is een subtype herseninfarct grotendeels veroorzaakt wordt door ischemie van de kleine cerebrale vaten. Bovendien was ERα haplotype 1 bij vrouwen ook geassocieerd met de ernst van cerebrale witte stof afwijkingen welke ook geacht wordt veroorzaakt te worden door ischemie van de kleine cerebrale vaten. Een intrigerend aspect aan deze bevindingen voor cerebrale vaatziekten is dat terwijl haplotype 1 een risicofactor is voor postmenopauzale vrouwen het een beschermende factor is voor mannen. Dit wordt ook tot op zekere hoogte gezien in de associatie met

ischemische hartziekten waar haplotype 1 een beschermende werking had bij mannen, alhoewel deze bevinding niet statistisch significant is. Dit beschermende effect van ERα haplotype 1 op hart- en vaatziekten bij mannen werd recent ook gerapporteerd in de Framingham Heart studie, die onze bevindingen dus ondersteunt.[1] Verder onderzoek zal nodig zijn om de geslachtspecifieke mechanismen van deze bevindingen te identificeren.

Individuele variatie in postmenopauzale oestradiol spiegels zijn deels bepalend voor ziekten zoals osteoporose. Een groot deel van de individuele variatie wordt waarschijnlijk veroorzaakt door genetische variaties (polymorfismen) in genen welke de geslachtshormoon synthese reguleren. Studies naar de invloed van het ERα en aromatase (*CYP19*) gen op circulerende geslachtshormoon spiegels worden gepresenteerd in **hoofdstuk 4**. Het ERα haplotype 1, eerder geassocieerd met osteoporose, lichaamslengte en hart- en vaatziekten, werd gerelateerd aan verlaagde circulerende oestradiol spiegels in postmenopauzale vrouwen (**hoofdstuk 4.1**). Bij mannen werd geen associatie gevonden, mogelijk ten gevolge van het bestaan van een intacte hypothalamus-hypofyse-gonade (HHG) as in mannen, in tegenstelling tot de situatie bij postmenopauzale vrouwen. Uit onze bevindingen dat oestradiol (E_2) spiegels, in tegenstelling tot oestron (E_1) spiegels, gerelateerd zijn met het ERα haplotype 1 allel stellen wij de hypothese op dat dit haplotype de expressie of activiteit van het 17β-HSD (subtype 1 of 7) enzym beïnvloedt. Dit enzym is verantwoordelijk voor de conversie van oestron naar oestradiol. De variatie in postmenopauzale oestradiol spiegels welke veroorzaakt worden door het ERα *Pvu*II-*Xba*I haplotype 1 allel is slechts 1%. Echter het verschil in oestradiol spiegels (17,5 pmol/L in niet dragers versus 14,1 pmol/L in homozygoot dragers) zou klinisch significant kunnen zijn. Een recente studie binnen het ERGO-onderzoek rapporteerde dat individuen met een oestradiol spiegel beneden de grenswaarde van 15,5 pmol/L een verhoogde kans op osteoporose hadden. De bevindingen suggereren dat de genotype-afhankelijke verschillen in oestradiol spiegels veroorzaakt door deze ERα gen polymorfismen dus klinisch relevant zouden kunnen zijn.

De associatie tussen een mogelijk functioneel polymorfisme in het aromatase (*CYP19*) gen en de circulerende oestradiol spiegels wordt behandeld in **hoofdstuk 4.2**. Het *CYP19* polymorfisme was niet alleen geassocieerd met oestradiol spiegels maar ook met oestron spiegels. Deze bevinding is in overeenstemming met de a-priori hypothese dat variaties in *CYP19* expressie alle conversies gekatalyseerd door het aromatase enzym zouden beïnvloeden. In mannen, waarin de HHG-as nog intact is, werden geen associaties met geslachtshormoon spiegels gevonden.

Drie polymorfismen binnen het ERα gen werden bestudeerd in dit proefschrift: de *Pvu*II en *Xba*I polymorfismen in intron 1 en de TA-repeat in het promotor gebied. Tot recent werd aangenomen dat alhoewel deze polymorfismen sterk geassocieerd zijn met verschillende fenotypen ze geen functionele gevolgen hebben

voor ERα expressie. De waargenomen associaties met onder andere osteoporose werden geacht het gevolg te zijn van "koppeling" tussen deze polymorfismen en een werkelijk functioneel polymorfisme elders in het ERα gen of mogelijk zelfs in een omliggend gen. De bevindingen die in **hoofdstuk 5.1** worden gerapporteerd suggereren echter de alternatieve hypothese dat het *Pvu*II polymorfisme functionele gevolgen voor ERα expressie zou kunnen hebben. De aanwezigheid van het *Pvu*II T-allel (het allel van het *Pvu*II polymorfisme dat ook aanwezig is in het *Pvu*II-*Xba*I haplotype 1 allel) elimineert een functionele bindingsplaats voor de transcriptiefactor B-myb. Hierdoor zou de aanwezigheid van het T-allel resulteren in een lagere ERα transcriptie. De hypothese is dat dragers van het T-allel hierdoor minder gevoelig zullen zijn voor de effecten van oestradiol door de aanwezigheid van minder receptoren (ERα) voor dit hormoon.

In de algemene discussie in **hoofdstuk 6** worden de resultaten beschreven in dit proefschrift samengebracht en geplaatst in een breder perspectief. De verschillende klinische fenotypen welke geassocieerd werden met de kandidaatgenen worden in verband gebracht met de functionele studies. Tevens worden de geslachtsverschillen waargenomen in de genetische associatie studies en de mogelijke biologische verklaringen hiervoor besproken. De relevantie van dit onderzoek voor de kliniek wordt besproken en tenslotte worden aanbevelingen gedaan voor toekomstig onderzoek.

Referentie

1. **Shearman AM, Cupples LA, Demissie S, et al.** Association between estrogen receptor alpha gene variation and cardiovascular disease. *JAMA*. 2003;290(17):2263-2270.

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Stephanie Schuit was born on June 6th, 1974 in Rotterdam, The Netherlands. After spending a large part of her youth living in the United States, she graduated from the Awty International School in Houston, Texas in 1991, where she obtained the International Baccalaureate Diploma. From 1991 to 1993 she studied at the University of St. Thomas in Houston, Texas, where her major was "International Studies". From 1993 to 1994, she studied economics at the Erasmus University in Rotterdam.

In 1994, Stephanie started her medical training at the Erasmus Medical Center in Rotterdam. During medical school she worked at the Genetics Laboratory of the Department of Internal Medicine (dr. A.G. Uitterlinden, Prof.dr. H.A.P. Pols) as a research student and started part of the work described in this thesis. In November 2000 she obtained her medical degree cum laude. In December 2000 and April 2001 she obtained Step I and II of The United States Medical Licensing Examination (USMLE).

In 2001 Stephanie worked as a resident in internal medicine at the Medical Center Rijnmond Zuid (dr. A. Berghout) for three months before starting the work described in this thesis in May 2001 at the Departments of Internal Medicine (Prof. dr. H.A.P. Pols) and Epidemiology & Biostatistics (Prof.dr. A. Hofman) of the Erasmus Medical Center, Rotterdam. During this period she obtained a Master of Science degree in Genetic Epidemiology from the Netherlands Institute for Health Sciences in Rotterdam. In May 2003 and June 2004 she received "Young Investigator Awards" from the European Calcified Tissue Society and October 2003 she received the same award from the Dutch Society for Calcium and Bone Metabolism. In May 2004 she started her residency in internal medicine at the Medical Center Rijnmond Zuid (dr. A. Berghout).

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