Timo Salmén

Genetic Influences of the Sex Steroid Pathway on Fracture Risk and the Effect of Hormone Replacement Therapy in Early Postmenopausal Women

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium L23, Snellmania building, University of Kuopio, on Friday 29th November 2002, at 12 noon

Department of Biochemistry
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Osteoporosis is a disease with a strong genetic background. Genetic factors play an important role in determining peak bone mass and regulating bone loss. With hormone replacement therapy (HRT) it is possible to prevent or retard the rate of rapid bone loss typical for menopause and the response to HRT may also be under genetic control.

The purpose of this long-term prospective population-based study was to evaluate genetic influences of the sex steroid pathway on fracture risk and the effect of HRT in early postmenopausal women taking part in the Kuopio Osteoporosis Risk Factor and Prevention (OSTPRE) Study. The final study groups in the 5-year randomized HRT trial on 331 early postmenopausal women were the HRT group (n=151): Sequential combination of 2 mg E2Val (days 1-21) and 1 mg CPA (days 12-21), with a treatment-free interval (days 22-28) alone or in combination with vitamin D3 (100-300 IU) + calcium lactate, 500 mg/day (equivalent to 93 mg Ca2+). The Non-HRT group (n=180): Calcium lactate, 500 mg/day, equivalent to 93 mg Ca2+ alone or in combination with vitamin D3, 100-300 IU/day. BMDs of the lumbar spine (L2-4) and proximal femur were measured by using dual X-ray absorptiometry (DXA). Grip strength was measured with a dynamometer. The estrogen receptor-α (ERα) gene polymorphism in intron 1 was analyzed using PCR and PvuII restriction enzyme digestion. The length of the polymorphic TTTA repeat region in intron 4 of the aromatase (CYP19) gene and of the polymorphic CAG repeat region in exon 1 of the androgen receptor (AR) gene were also evaluated. All new symptomatic, radiographically defined fractures were recorded. A 5-year postal inquiry in May 1994 included questions on falls during the previous 12 months.

The results of this randomized trial show that in the non-HRT-group the lumbar spine BMD decreased more in women with the ERα genotypes PP (6.4%) and Pp (5.2%) than in subjects with the pp genotype (2.9%) (p<0.01). In the HRT group, the relative changes of the lumbar spine BMD were similar in all three ERα genotype groups. Additionally, the incidence of new fractures in the HRT group was significantly reduced in women with the P allele (p<0.05) with the relative risk (HR) of 0.25 (95% CI, 0.07-0.98), in comparison with the non-P allele group (pp genotype). In the non-HRT group, the ERα genotype was not significantly associated with fracture risk. The ERα genotype was also associated with fall risk in the HRT group (p<0.01), women with the PP genotype had an increased risk of falls. The grip strength values were not influenced by the ERα genotype. In addition, the CYP19 and AR gene polymorphisms did not influence the circulating E2 levels, grip strength values, BMD values, or fracture risk in these women.

A new and clinically potentially important finding was that the ERα genotype appears to modulate lumbar spine bone loss rates, fracture risk, and the responsiveness to HRT. The results suggest that the P allele is a relatively hormone sensitive allele and it appears that women with the P allele may benefit more from the bone protective effect of HRT than women without the P allele.

National Library of Medicine Classifications: QZ 50, WE 175, WP 522

Medical subjects headings: accidental falls; aromatase genetics; bone density; fractures; hormone replacement therapy; human; osteoporosis, postmenopausal; polymorphism (genetics); randomized controlled trials; receptors, estrogen genetics; receptors, androgen genetics
To Pappa
ACKNOWLEDGMENTS

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In a study as large as the OSTPRE Study, a skilled secretary is needed. Ms. Seija Oinonen has provided me with fast and reliable help when needed, thank you. Additionally, my thanks belong to Mrs. Pirjo Halonen, M.Sc., for clear and professional advice on statistical problems.

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Kuopio, October 2002

Timo Salmén
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>1,25(OH)₂D</td>
<td>1,25-dihydroxyvitamin D = calcitriol</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25-hydroxyvitamin D = calcidiol</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BUA</td>
<td>broadband ultrasound attenuation</td>
</tr>
<tr>
<td>Ca</td>
<td>calcium</td>
</tr>
<tr>
<td>CPA</td>
<td>cyproterone acetate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DXA</td>
<td>dual energy X-ray absorptiometry</td>
</tr>
<tr>
<td>E₂</td>
<td>estradiol</td>
</tr>
<tr>
<td>E₂Val</td>
<td>estradiol valerate</td>
</tr>
<tr>
<td>ERα</td>
<td>estrogen receptor-α</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>HRT</td>
<td>hormone replacement therapy</td>
</tr>
<tr>
<td>LD</td>
<td>linkage disequilibrium</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on five original articles, which are referred in the text by their
Roman numerals I-V, and on unpublished results:

I  Salmén T, Heikkinen AM, Mahonen A, Kröger H, Komulainen M, Saarikoski
    S, Honkanen R, Mäenpää PH. Early postmenopausal bone loss is associated
    with PvUII estrogen receptor gene polymorphism in Finnish women: effect of

II Salmén T, Heikkinen AM, Mahonen A, Kröger H, Komulainen M, Saarikoski
    S, Honkanen R, Mäenpää PH. The protective effect of hormone-replacement
    therapy on fracture risk is modulated by estrogen receptor alpha genotype in

III Salmén T, Heikkinen AM, Mahonen A, Kröger H, Komulainen M, Saarikoski
    S, Honkanen R, Mäenpää PH. Relation of estrogen receptor-α gene
    polymorphism and hormone replacement therapy to fall risk and muscle

IV Salmén T, Heikkinen AM, Mahonen A, Kröger H, Komulainen M, Saarikoski
    S, Honkanen R, Mäenpää PH. Relation of aromatase gene polymorphism and
    hormone replacement therapy to serum estradiol levels, bone mineral density,
    and fracture risk in early postmenopausal women. Submitted.

V Salmén T, Heikkinen AM, Mahonen A, Kröger H, Komulainen M, Saarikoski
    S, Honkanen R, Mäenpää PH. Relation of androgen receptor gene
    polymorphism to bone mineral density and fracture risk in early
    postmenopausal women during a 5-year randomized hormone replacement
    therapy trial. J Bone Miner Res, in press.
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APPENDIX: 5-YEAR POSTAL INQUIRY
1. INTRODUCTION

Osteoporosis is a multifactorial disease characterized by reduced bone mass and increased fracture risk. Up to 80% of bone mineral density (BMD) is estimated to be heritable (Pocock et al. 1987, Howard et al. 1998) and BMD is under multigene control (Guéguen et al. 1995). There are two periods in bone development, first is the gaining of peak bone mass which is attained sometime between the ages of 20 and 30 and then the phase of bone loss which is accelerated after menopause. The genes which determine peak bone mass probably are not the same as those which determine bone loss. Also the strength of genetic influence varies at different parts of the skeleton i.e. lumbar spine contains more metabolically active trabecular bone than femoral neck making the lumbar spine more prone to genetic influences.

The genes which affect BMD are not known yet. There are several candidate genes. Most studies concerning gene polymorphism and BMD have been focused on the influence of vitamin D receptor (VDR) gene polymorphism (Morrison et al. 1994). The results of meta-analyses suggest that polymorphisms of the VDR gene explain only a small proportion of the genetic effect on BMD (Cooper & Umbach 1996, Gong et al. 1999). There are several studies concerning the influence of estrogen receptor-α (ERα) genotype on BMD (Figure 1), but further studies are required. The third candidate gene having effects on bone is collagen Iα1 (COLIA1) (Uitterlinden et al. 1998). However, it is common to all candidate gene studies that the results have not been consistent. There are no good explanations for these inconsistent results. Environmental factors (e.g. calcium intake) and population differences (genetic background and experimental design, e.g. hormonal status of women) are some of the confounding factors. Other potential candidate genes (Figure 1) are androgen receptor (AR), apolipoprotein E (apoE), aromatase (CYP19), biglycan (BGN), calcitonin receptor, estrogen receptor-β (ERβ), insulin-like growth factor–I (IGF-1), interleukin-1 (IL-1) interleukin-6 (IL-6), matrix Gla protein (Mgp), parathyroid hormone (PTH), and transforming growth factor β1 (TGF-β1).
Figure 1. The sex steroid metabolic pathways.

Decreased BMD is an important predictor of fracture risk (Seeley et al. 1991, Kröger et al. 1995a). Also other risk factors for fractures have been recognized (Cummings et al. 1995, Hans et al. 1996, Garnero et al. 1996b). Hip BMD, heel broadband ultrasound attenuation (BUA), and bone resorption markers appear to be independent predictors of hip fractures in elderly women (Hans et al. 1996, Garnero et al. 1996b). In elderly women, the functional ability and the tendency to fall predict hip fracture (Cummings et al. 1995).

Postmenopausal estrogen deficiency is closely related to the risk of osteoporosis whereas hormone replacement therapy (HRT) prevents or retards postmenopausal bone loss. Postmenopausal women can be classified as fast and slow bone losers (Christiansen et al. 1987), but the reasons for the different loss rates are not well
understood. AR, CYP19, and ERα are candidate genes which may affect postmenopausal bone loss. Although postmenopausal HRT is effective in preventing bone loss, individual variation exists concerning the bone response to HRT (Hassager et al. 1994). This variation could be explained by a genetically determined response to HRT, especially by the ERα gene.

The aim of the present population-based, randomized trial was to investigate the genetic influences of the sex steroid action (Figure 1) on bone health, fracture risk, and responsiveness to HRT in nonosteoporotic early postmenopausal Finnish women.
2. REVIEW OF THE LITERATURE

2.1. Genetic determinants of bone health

2.1.1. Acquisition of peak bone mass and bone loss

The amount of skeletal mass acquired during adolescence is an essential determinant for the risk of postmenopausal osteoporosis. During puberty, bone mineral mass of skeletal sites such as the lumbar spine more than doubles (Bonjour et al. 1991, Theintz et al. 1992). The phase of bone loss commences after acquisition of the peak bone mass. The rate of bone loss is highly accelerated during the postmenopausal years, when predominantly cancellous bone is lost (Riggs et al. 2002). During the subsequent slow phase of bone loss cortical losses are greater than cancellous losses. In aging men, the continuous phase of bone loss is similar to that of the late, slow phase in aging women (Riggs et al. 2002). However, in men the continued periosteal apposition (3-fold greater in men than in women) partially compensates the endosteal resorption consequently increasing the width of the long bones (Riggs et al. 2002).

Twin and family studies show that genetic factors have an important role in determining peak bone mass and up to 80% of BMD may be heritable (Pocock et al. 1987, Seeman et al. 1989, Ferrari et al. 1998, Howard et al. 1998). Twin studies show greater similarity of BMD in monozygotic than in dizygotic twins (Pocock et al. 1987, Howard et al. 1998). Data from family studies support these results (Seeman et al. 1989, Ferrari et al. 1998).

Also in postmenopausal women genetic factors have a substantial role in explaining variation in BMD (Flicker et al. 1995, Hunter et al. 2001b). Recently, Hunter et al. reported that the genetic proportion of total variance for, e.g. lumbar spine BMD, was 88% premenopausally and 77% postmenopausally. These results demonstrate the strong role of genetic factors in regulation of BMD from late childhood until old age.
2.1.2. Bone mineral density and quantitative ultrasound

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture risk (Anon. 1993). With dual X-ray absorptiometry (DXA) and quantitative ultrasound (QUS) it is possible, to some extent, to evaluate these alterations in bone tissue.

DXA is a fast (2-5 min scanning time) and reliable (stable calibration and low precision error) method to measure BMD with a low effective radiation dose (<1 μSv). DXA is able to measure both axial and peripheral bone (e.g. spine, proximal femur, and total body). However, the presence of aortic calcification, radio-opaque contrast media, metallic objects, distribution of fat tissue, previous vertebral fractures, scoliosis, osteomalacia, or ostearthrosis at the lumbar spine may affect the results.

QUS is an inexpensive, portable, and radiation-free technique to measure peripheral bones, usually the calcaneus. The most commonly used variables are speed of sound and broadband ultrasound attenuation. However, results from different QUS devices are not convergent and the measurement of QUS present several inaccuracies.

Currently, measuring BMD is the predominant method to assess bone strength and DXA is the standard in the diagnosis of osteoporosis. However, DXA measurement provides incomplete information on bone structure and properties (Glüer 1997). When compared with DXA, QUS appears to be associated with different bone properties, which are also associated with fracture susceptibility (Hans et al. 1996, Bauer et al. 1997). A recent review concluded: the amount of ultrasound attenuation is dependent on structural parameters and these variables are also dependent on density (Njeh et al. 2001).

In a twin study, the QUS measurement was an equally heritable trait as BMD (as much as 80%) (Howard et al. 1998). Similarly, in other twin studies the genetic component in the QUS measurements was substantial, although smaller (Arden et al. 1996, Hunter et al. 2001b).
2.1.3. Metabolic state of bone

It has been reported that in elderly women some bone resorption markers predict hip fracture risk independently of BMD (Garnero et al. 1996b, Garnero et al. 2000). Thus a combination of measuring bone resorption markers and BMD could improve fracture risk prediction (Garnero et al. 1998b).

As with BMD and QUS measurements, also bone metabolism markers have a strong genetic component (Kelly et al. 1991, Hunter et al. 2001a). The heritabilities for bone-specific alkaline phosphatase (bone formation) and deoxypyridinoline (bone resorption) were 74% and 58%, respectively (Hunter et al. 2001a).

2.2. Methods to assess genes affecting bone health

2.2.1. Candidate gene studies

2.2.1.1. Vitamin D receptor gene

In candidate gene studies single nucleotide, microsatellite, and minisatellite polymorphisms have been investigated. Mainly restriction fragment length (RFLP) and repeat polymorphisms have been determined. DNA-chip technology is a novel method for detecting single nucleotide polymorphisms (SNPs). Most of these polymorphisms are located in introns and do not alter the protein structure.

Candidate gene approach takes advantage of the accumulated knowledge of the pathophysiology of bone and bone disorders to develop specific hypotheses (Table 1).
Table 1. The most commonly studied candidate genes for association with bone health

<table>
<thead>
<tr>
<th>A large number of studies</th>
<th>Some studies</th>
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<tr>
<td>Collagen type IαI</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>Estrogen receptor-α</td>
<td>Apolipoprotein E</td>
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<tr>
<td>Vitamin D receptor</td>
<td>Aromatase</td>
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<td>Calcitonin</td>
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<td></td>
<td>Calcitonin receptor</td>
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<td>Glucocorticoid receptor</td>
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<td></td>
<td>Insulin-like growth factor-1</td>
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<tr>
<td></td>
<td>Interleukin-1 receptor antagonist</td>
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<tr>
<td></td>
<td>Interleukin-6</td>
</tr>
<tr>
<td></td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td></td>
<td>Transforming growth factor β1</td>
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Vitamin D receptor (VDR) gene polymorphism was the first candidate gene to be studied by molecular genetic methods (Morrison et al. 1994). Initially, Morrison et al. reported that VDR gene polymorphisms predict up to 75% of the genetic effect on bone density. However, due to genotyping errors the figure was an overestimation (Eisman 1999). Since then a large number of papers have been published investigating the possible association between different VDR polymorphisms and bone health describing variables (Kröger et al. 1995b, Garnero et al. 1996a, Uitterlinden et al. 1996, Salamone et al. 1996, Gennari et al. 1999, Sowers et al. 1999, Ensrud et al. 1999). The results of meta-analyses suggest that polymorphisms of the VDR gene explain only a small proportion of the genetic effect on BMD (Cooper & Umbach 1996, Gong et al. 1999).

intestinal calcium absorption (Gennari et al. 1997, Dawson-Hughes et al. 1995). Also gene-gene interactions between VDR gene polymorphisms and other candidate genes (estrogen receptor-α and collagen Iα1 genes) in BMD levels, QUS values, and fracture risk have been observed (Gennari et al. 1998, Willing et al. 1998, Giguere et al. 2000, Uitterlinden et al. 2001).

Thus, the evidently small effect of the VDR gene polymorphisms on bone health is easily confounded by environmental and genetic factors.

2.2.1.2. Estrogen receptor-α gene

Lack of functional estrogen receptor in a 28-year-old man has been reported to result in severe osteoporosis and incomplete epiphyseal closure (Smith et al. 1994). Similarly, two adult men with aromatase deficiency were tall and presented continued linear growth with a decrease in BMD (Grumbach & Auchus 1999). Also studies on transgenic estrogen receptor-α (ERα) knock-out mice have shown that both female and male knock-out mice have 20-25% lower BMD than wild-type (Korach 1994). These studies show that the absence of a functional estrogen receptor influence bone health. Additionally, these findings show the importance of estrogen for bone health also in men.

In the first study on 144 postmenopausal Japanese women, a TA repeat polymorphism in the ERα gene was associated with bone mass and bone metabolism (Sano et al. 1995). In addition to the TA repeat polymorphism (located in the promoter), polymorphic PvuII and XbaI restriction enzyme sites, located in intron 1, have been widely used (Figure 2). In 238 postmenopausal Japanese women the combination of genotypes PvuII and XbaI was associated with Z score (the number of SDs from the mean of age-matched women) values of BMD, but not with bone turnover markers (Kobayashi et al. 1996). Since then several studies have investigated the effect of ERα gene polymorphisms on bone health in Asian populations. However, the results have not been consistent (Han et al. 1997, Mizunuma et al. 1997, Kurabayashi et al. 1999, Han et al. 1999b, Lau et al. 2001, Kim et al. 2001).
Studies on Caucasian women have shown strong and highly significant linkage disequilibrium (LD) between ERα gene polymorphisms (TA repeat, *PvuII*, and *XbaI*) (Deng et al. 1998, Becherini et al. 2000, Langdahl et al. 2000, Patel et al. 2000, Albagha et al. 2001). Haplotype analysis, using *PvuII* and *XbaI* polymorphisms (resulting in PX, Px, pX, and px haplotypes), in relation to the bone health describing parameters have been widely used (Kobayashi et al. 1996, Albagha et al. 2001). Several studies have reported that ERα gene polymorphisms are associated with BMD, QUS values, bone loss rates, and fracture risk (Willing et al. 1998, Deng et al. 1998, Sowers et al. 1999, Patel et al. 2000, Becherini et al. 2000, Langdahl et al. 2000b, Albagha et al. 2001). A connective factor for these studies is the age of women, since most of the women were pre-, peri-, or early postmenopausal, i.e., they still were hormonally active. Additionally, ERα gene polymorphisms (*XbaI* and/or *PvuII* polymorphisms used) have been associated with age of menarche and onset of natural menopause (Weel et al. 1999, Stavrou et al. 2002). Most of the studies which have not observed an association between ERα gene polymorphisms and bone health describing variables have consisted of late postmenopausal women (Gennari et al. 1998, Vandevyver et al. 1999, Bagger et al. 2000, Giguere et al. 2000, Brown et al. 2001, Efstratiadou et al. 2001a, Aerssens et al. 2001), i.e., the women had been hormonally inactive for a fairly long time.

### 2.2.1.3. Collagen type Iα1 gene

Type I collagen is the major protein of bone. Sp1 polymorphism (DNA recognition site for the transcription factor Sp1) of the collagen Iα1 (COL1A1) gene is one of the most
widely studied candidate gene markers in the bone field (Grant et al. 1996, Uitterlinden et al. 1998, Garnero et al. 1998a, Langdahl et al. 1998, Keen et al. 1999, Harris et al. 2000, McGuigan et al. 2001, Uitterlinden et al. 2001, Brown et al. 2001, MacDonald et al. 2001). In these studies the “ss” genotype or “s” allele have been associated with reduced BMD, increased bone loss, bone turnover, and increased fracture risk. However, these results have not been confirmed in all studies (Liden et al. 1998, Hustmyer et al. 1999, Heegaard et al. 2000). Interestingly, the effect on fracture risk appears to be, at least in part, independent on BMD (Uitterlinden et al. 1998, Langdahl et al. 1998, McGuigan et al. 2001, Uitterlinden et al. 2001).

Recently, two studies have assessed the possible gene-gene and gene-environment interactions in relation to COLIA1 polymorphism (Uitterlinden et al. 2001, Brown et al. 2001). In a large study on 1004 postmenopausal women, a significant interaction between VDR and COLIA1 genotype effects on fracture risk was observed (Uitterlinden et al. 2001). VDR gene is a transcription factor regulating the expression of the COLIA1 gene (Slack et al. 1993, Pavlin et al. 1994) and this connection may be an important element in the observed interaction. In the study by Brown et al., an association between COLIA1 polymorphism and the rate of lumbar spine bone loss was observed with a significant gene-environment interaction related to dietary calcium intake (Brown et al. 2001). Interestingly, they also observed that the association between the VDR (Taq1) polymorphism and the bone loss rates was strongest in those individuals in the lowest tertile of calcium intake.

Two recent meta-analyses have investigated the effect of the COLIA1 gene polymorphism on the prevalence of fractures (Mann et al. 2001, Efstathiadou et al. 2001b). The meta-analyses suggests an important role for the Sp1 polymorphism in the regulation of fracture risk. However, the importance of COLIA1 gene polymorphism as a possible genetic marker for fracture risk depends on its prevalence in different populations, e.g., this polymorphism has not been found in Asian populations (Beavan et al. 1998, Han et al. 1999a, Nakajima et al. 1999).
2.2.1.4. Other genes

In addition to the VDR, ERα, and COLIA1 genes a large number of other candidate genes have been studied. Among the sex steroids only ERα gene polymorphism and its possible effects on bone health have been widely studied. Only a few studies have addressed the possible genetic variation of the androgen effect on bone health, directly via androgen receptor (AR) or indirectly via aromatization. One candidate gene study has investigated the role of AR polymorphism (CAG repeat region in exon 1) on BMD in pre- and perimenopausal women, aged 28-48 years (mean age 37 years, n=261) (Sowers et al. 1999). Sowers and co-workers reported that the AR genotype distribution was different at the lumbar and femoral neck BMD groups (low, average, and high BMD groups). Women with a relatively high number of repeats were overrepresented in the low BMD group. Similarly, only one study has investigated the role of the CYP19 (aromatase) polymorphism on bone health (Masi et al. 2001). Recently, Masi and co-workers reported that the TTTA repeat polymorphism in intron 4 of the CYP19 gene was associated with lumbar BMD and spine fracture incidence in postmenopausal Italian women (mean age 58 years, n=350).

Several other genes coding for hormones and/or the receptor have been investigated in relation to bone health, besides the VDR, ERα, and AR genes. Insulin-like growth factor-I (IGF-I) gene is a candidate gene due to its anabolic effects on bone mass. So far, a CA repeat polymorphism upstream from the IGF-I gene transcription initiation site has not been associated with BMD (Miyao et al. 1998, Takacs et al. 1999). Parathyroid hormone (PTH) gene polymorphism and its association with BMD has been studied in 383 postmenopausal Japanese women (Hosoi et al. 1999). The results suggested that the PTH gene polymorphism in intron 2 (BsrBI restriction enzyme) may be associated with BMD. Calcitonin or calcitonin receptor gene polymorphisms have also been associated with BMD (Masi et al. 1998, Taboulet et al. 1998, Miyao et al. 2000, Braga et al. 2000), although the results have been contradictory (Masi et al. 1998, Taboulet et al. 1998, Braga et al. 2000). An interesting observation was done by Huizenga et al. when they investigated glucocorticoid receptor gene polymorphism and its association with different variables, including dexamethasone suppression tests,
BMD, circulating cortisol, and insulin concentrations (Huizenga et al. 1998). Individuals carrying the N363S polymorphism (asparagine-to-serine substitution) were more sensitive to exogenously administrated glucocorticoids and a trend towards lower lumbar BMD values was observed.

Cytokines are obvious candidate genes for regulation of bone health. Interleukin-1 receptor antagonist (IL-1ra) inhibits the actions of interleukin-1, which is a potent bone resorption stimulant. A 86 bp variable number tandem repeat (VNTR) polymorphism in intron 2 of the IL-1ra gene has been associated with lumbar BMD, lumbar bone loss rates, and fracture risk (Keen et al. 1998, Langdahl et al. 2000a). However, Bajnok et al. did not observe any association between BMD and the VNTR polymorphism (Bajnok et al. 2000). IL-6 stimulates cells of the osteoclast lineage and several studies have investigated the role of IL-6 polymorphisms on bone health (Murray et al. 1997, Tsukamoto et al. 1999, Ferrari et al. 2001, Ota et al. 2001). In these studies, different IL-6 polymorphisms have been associated with bone resorption (C-telopeptide of type I collagen) and BMD. Transforming growth factor β1 (TGF-β1) is an important regulator of bone metabolism (Hughes et al. 1996, Yanagisawa et al. 1999). In several studies, polymorphisms of the TGF-β1 gene have been associated with bone turnover, BMD, bone loss rates, and fracture risk (Langdahl et al. 1997, Yamada et al. 1998, Yamada et al. 2000, Bertoldo et al. 2000, Yamada et al. 2001, Hinke et al. 2001). Interestingly, in the Japanese population the TT genotype or T allele (T29>C polymorphism in exon 1 resulting in a leucine-to-proline substitution) has been associated with reduced BMD, increased bone loss, and increased fracture risk (Yamada et al. 1998, Yamada et al. 2000), whereas in a small Caucasian population the TT genotype was associated with higher BMD values (Hinke et al. 2001).

Apolipoprotein E (APOE) gene polymorphism (resulting in amino acid substitution) and its APOE*4 allele have been associated with several chronic diseases, including Alzheimer’s dementia (Corder et al. 1993), making it an intriguing candidate gene. An association between APOE polymorphism, bone turnover, BMD, bone loss rates, and fracture risk has been observed in some studies (Shiraki et al. 1997, Cauley et al. 1999, Salamone et al. 2000, Gerdes et al. 2001). However, there has been discrepancy among these results (Salamone et al. 2000, Gerdes et al. 2001).
Additionally, all studies have not observed an association between APOE genotype, BMD, and the bone loss rates (Heikkinen et al. 2000, von Muhlen et al. 2001). In a large study by Cauley et al. on 1750 postmenopausal women, the APOE*4 allele was associated with increased fracture risk independent of BMD, impaired cognitive function, or falling (Cauley et al., 1999).

The number of studies investigating these candidate genes is very limited and the results have not been consistent. More studies, on pre- and postmenopausal women, are needed before the influence of these candidate genes on bone health in different populations can be evaluated.

2.2.1.5. Functional relevance of polymorphisms

Very few of the polymorphisms described previously cause any coding region differences. However, some polymorphisms appear to influence the structure and function of the protein (Chamberlain et al. 1994, Tut et al. 1997, Irvine et al. 2000). When the structure of the protein is intact, transcriptional activity, mRNA levels, and cell growth have mostly been studied to reveal possible functional differences caused by the different polymorphisms.

Most of the studies concerning gene polymorphism and BMD have focused on VDR polymorphisms. Similarly, studies on possible functional differences have almost solely focused on VDR polymorphisms. In a recent study on 261 Japanese women, the polymorphism in a Cdx-2 binding site in the promoter region of the VDR gene was associated with the expression of VDR in the small intestine (Arai et al. 2001). Similarly, several other VDR gene polymorphisms have been reported to be associated with functional differences (Arai et al. 1997, Carling et al. 1998, Yamagata et al. 1999, Colin et al. 2000, Whitfield et al. 2001). However, functional differences between VDR polymorphisms have not been found in all studies (Mocharla et al. 1997, Gross et al. 1998, Correa et al. 1999).

There is only two studies investigating possible functional differences caused by ERα gene polymorphisms (Maruyama et al. 2000, Herrington et al. 2002b). The P allele of the PvulII polymorphism produces a potential binding site for the myb family of transcription factors (Herrington et al. 2002b). Maruyama and co-workers transfected
HeLa S3 cells with experimental and control vectors and luciferase activity was measured. The enhancer activity differed among the haplotypes (PvuII and XbaI RFLPs used). Similarly, in a recent study ERα gene polymorphism (PvuII RFLP used) affected luciferase activity (Herrington et al. 2002b). Cotransfection of CV1 cells with a luciferase reporter construct containing the P allele and a myb expression vector produced a >10-fold increase in luciferase activity compared with an only 2.5-fold increase observed in cells transfected with the p allele reporter (Herrington et al. 2002b). Thus, the polymorphic variants of ERα gene, particularly PvuII polymorphism, may regulate transcription (Maruyama et al. 2000, Herrington et al. 2002b). Further research is needed to determine what is the significance of the observed variation in transcription activity (alteration of quantity/quality of ERα transcripts or protein). Additionally, it remains to be solved whether one or a combination (haplotypes) of the commonly studied polymorphisms (TA repeat, PvuII, and XbaI) or sequence variation in LD with these markers causes the observed associations.

The mechanism of the possible effect of the COLIA1 genotypes on bone has been partly elucidated. Recently Mann et al. reported that the COLIA1 “s” allele had increased affinity for Sp1 binding and that the primary RNA transcripts derived from the “s” allele were approximately three times more abundant than the “S” allele-derived transcripts in “Ss” heterozygotes (Mann et al. 2001). Collagen produced from osteoblasts cultured from “Ss” heterozygotes had an increased ratio of α1(I) protein relative to α2(I) and this was accompanied by an increased ratio of COLIA1 mRNA relative to COLIA2. Lastly, the yield strength of bone derived from “Ss” individuals was reduced when compared with bone derived from the “SS” subjects, possibly via [α1(I)S] formation.

In summary, there is now increasing evidence that at least some candidate gene polymorphisms are functionally relevant. However, almost all polymorphisms studied produce a normal protein, suggesting that the bone phenotype caused by these polymorphisms results from a functional or regulatory change in the candidate gene.
2.2.2. Linkage analysis

In the bone field linkage analysis is generally used to study quantitative traits (e.g. BMD) in families. Polymorphic genetic markers can be used on a genome-wide basis or within specific genomic regions (e.g. candidate gene approach).

A genome screen in 595 sister pairs found significant evidence of linkage to lumbar BMD at chromosome 1q21-23 (Koller et al. 2000). In another genome screen in 309 sister pairs, three chromosomal regions (chromosomes 5q, 4q, and 17q) were identified with significant evidence of linkage to femoral structure phenotype (Koller et al. 2001).

Koller et al. have also performed a candidate locus linkage study in 835 premenopausal sisters, where they found suggestive evidence of linkage with femoral neck BMD near the marker D11S987 (Koller et al. 1998). This marker is in the same chromosomal region (11q12-13) as three Mendelian BMD-related phenotypes: an autosomal dominant high bone mass, autosomal recessive osteoporosis pseudoglioma, and autosomal recessive osteopetrosis (Gong et al. 1996, Johnson et al. 1997, Heaney et al. 1997). However, Deng et al. found no evidence of linkage with BMD of the marker D11S987 in 595 sibling pairs (Deng et al. 2001).

Duncan et al. investigated the role of 23 candidate genes in the control of BMD by linkage studies in families of probands with osteoporosis and low BMD (Duncan et al. 1999). Similar to the candidate gene approach discussed before, this approach takes advantage of the large body of knowledge that has already been accumulated on osteoporosis to develop specific hypotheses. In 614 individuals they found suggestive evidence of linkage between BMD and the parathyroid hormone receptor type 1 (Duncan et al. 1999).

2.2.3. Animal models

When linkage studies are performed on laboratory animals, mouse is the main animal model used. Identification of chromosomal regions (quantitative trait loci, QTL) which regulate BMD (Klein et al. 1998, Beamer et al. 2001) or some other bone trait (Turner
et al. 2000), has been mostly done by crossing two inbred mouse strains (one with low BMD and the other with high BMD).

Klein et al. found evidence of linkage at 10 chromosomal sites to peak bone mass development in the female (Klein et al. 1998). In a recent study, analyses of markers on BMD revealed ten chromosomal locations carrying QTLs for femurs and seven chromosomal locations carrying QTLs for vertebrae (Beamer et al. 2001). Interestingly, five QTLs were unique to femur, whereas two QTLs were unique to vertebrae.

A large number of genetically manipulated mouse models have been created to evaluate the role of specific candidate genes in bone biology (Korach 1994, Lazner et al. 1999). An extensively studied model has been the ERα (and ERβ) knock-out mouse. Studies on ERα knock-out mice have shown that both female and male knock-out mice have 20-25% lower BMD than wild-type mice (Korach 1994).

The utility of the animal models is based on the assumption that some of the genes, which determine BMD in animals, will also be involved in humans. This limitation must be remembered when considering results from the animal studies. Additionally, mouse models are limited to analysis of some inbred strains, which is equivalent to studying a limited number of human individuals. However, experimental animal models provide a means to circumvent complicating environmental factors.

2.3. Hereditary determinants of fracture risk

2.3.1. Bone health

Osteoporosis is a common disease characterized by reduced BMD, deterioration of the skeletal microarchitecture, and increased fracture risk. Bone mass (BMD) results from the amount of bone acquired and the age-related bone loss, which is accelerated after menopause. To some extent, it is possible to measure structural and microstructural integrity of bone with QUS. Additionally, biochemical markers of bone turnover can be used to assess fracture risk. Currently, these are the methods available for the evaluation of bone health and all these methods have a strong genetic component.
2.3.2. Fall risk

Fracture risk is not solely determined by density, geometry, and microstructural integrity of bone, but also by fall risk. In elderly women, the functional ability and the tendency to fall predict hip fracture (Cummings et al. 1995). In osteoporotic women, fractures can occur without a major trauma, e.g. by falling from standing height or lower.

The strong role of genetic factors in the regulation of bone health is clear. However, data on the inheritance of neuromuscular functioning and subsequently of the fall risk is not available. The only data available comes from the few candidate gene studies, where the association between muscle strength and candidate genes have been studied (Geusens et al. 1997, Vandevever et al. 1999, Woods et al. 2001).

APOE*4 allele is associated with increased risk of coronary heart disease and Alzheimer’s disease (Wilson et al. 1996, Farrer et al. 1997). Increased risk of sporadic and familiar Alzheimer’s disease due to interaction between the ERα (PvuII and XbaI restriction endonucleases used) and APOE genotypes have been reported (Brandi et al. 1999, Mattila et al. 2000). Thus, these candidate genes may influence fall risk via deterioration of cognitive functions.

2.4. Genetics and medical treatment of osteoporosis

2.4.1. Hormone replacement therapy

Postmenopausal estrogen deficiency is closely related to the risk of osteoporosis whereas HRT prevents or retards postmenopausal bone loss (Lindsay et al. 1976, Riggs & Melton 1986, Komulainen et al. 1999). Additionally, HRT may prevent fractures among postmenopausal women (Lufkin et al. 1992, Komulainen et al. 1998). Postmenopausal women can be classified as fast and slow bone losers, but reasons for the different loss rates are not well understood (Christiansen et al. 1987, Hansen et al. 1991). Although postmenopausal HRT is effective in preventing bone loss, individual
variation exists concerning the bone response to HRT (Hassager et al. 1994, Komulainen et al. 2000).

The response to HRT may at least in part be genetically determined. There have been several studies investigating the possible association between the HRT use, candidate genes, and different bone health describing variables (Han et al. 1997, Kurabayashi et al. 1999, Salamone et al., 2000, Giguère et al. 2000, Ongphiphadhanakul et al. 2001, Gerdes et al. 2001). However, only four randomized HRT trials have investigated the association between the HRT use, candidate genes, and BMD (Deng et al. 1998, Heikkinen et al. 2000, Yamada et al. 2000, Woods et al. 2001). Deng et al. reported that VDR and ERα polymorphisms are associated with HRT use and BMD in 108 elderly women (Deng et al. 1998). In a recent study, the angiotensin-I converting enzyme (ACE) gene polymorphism was associated with BMD response in postmenopausal women treated with HRT (Woods et al. 2001). Interestingly, this polymorphism was also associated with isometric muscle strength. However, the number of women in these trials was small. Yamada et al. investigated the association between HRT/vitamin D use, transforming growth factor β1 (TGF-β) genotype, and BMD in 363 postmenopausal Japanese women (Yamada et al. 2000). The women were divided into three groups: control, vitamin D treatment, and HRT groups. The response of lumbar BMD to HRT was not significantly associated with the TGF-β genotypes. Similarly, in a HRT trial by Heikkinen et al. on 352 early postmenopausal Caucasian women, the APOE genotype did not modify the response of BMD to HRT (Heikkinen et al. 2000).

2.4.2. Other medications

Randomized clinical trials have shown the efficacy of several medications, in addition to HRT, in the treatment of osteoporosis. Bisphosphonates, calcitonin, and raloxifene (selective estrogen receptor modulator) can be used to increase BMD (Liberman et al. 1995, Harris et al. 1999, Ettinger et al. 1999, Chesnut et al. 2000). However, all these medications, including HRT, are antiresorptive. A new potential bone formation-stimulating agent in the treatment of osteoporosis is parathyroid hormone (Neer et al.
2001). In the treatment of osteoporosis it is important to secure sufficient vitamin D and calcium intake, especially among the elderly.

When compared with HRT, even less is known about the possible influence of genetic factors on the response of bone to other osteoporosis medications. In a small study on 24 postmenopausal women, VDR genotype was associated with bone response to etidronate (bisphosphonate) treatment (Marc et al. 1999). In another small study (n=81) VDR genotype was associated with responsiveness to vitamin D supplementation in the elderly (Graafmans et al. 1997). Additionally, Yamada et al. observed an association between vitamin D treatment, TGF-β genotype, and BMD change (Yamada et al. 2000).
3. AIMS OF THE STUDY

The aims of the study were:

The primary hypothesis of this study was that, the ERα gene polymorphism alters the function of the receptor consequently modifying the estrogen responsiveness of target tissues. The validity of this hypothesis was tested in a 5-year long randomized population-based HRT trial of non-osteoporotic postmenopausal women by measuring BMD, fracture risk, fall risk, and muscle strength (I, II, III).

Similarly, the adequacy of the polymorphism in the CYP19 and AR genes as genetic markers for phenotypic differences was evaluated (IV, V).
4. SUBJECTS AND METHODS

The following section presents the subjects and methods briefly. Further details are presented in the original publications I-V.

4.1. Subjects and study design

The study design is shown in Figure 3.

*Figure 3.* The trial profile of subject selection.
The population of the present study was a subgroup of the Kuopio Osteoporosis Risk Factor and Prevention (OSTPRE) Study. In 1989, a postal inquiry was sent to all 14,200 women aged 47 to 56 years of the Kuopio Province, Eastern Finland, to investigate risk factors for osteoporosis among perimenopausal women (Tuppurainen et al. 1993). A total of 13,100 women responded. In 1989-91, BMD was measured in 3,222 women who represented a randomly stratified sample of those women, who were willing to undergo bone densitometry. All 464 postmenopausal volunteers out of those 3,222 women, who had had their last menstrual period within 6-24 months before the study, and who were not osteoporotic, were selected for a 5-year clinical trial (Figure 4). Exclusion criteria were restricted to general contraindications of HRT including a history of estrogen dependent cancer, thromboembolic diseases, and medication-resistant hypertension.

![Figure 4. The longitudinal profile of the trial.](image)

The women were randomized into four treatment groups: an estradiol valerate (E₂Val)/cyproterone acetate (CPA) group, a vitamin D₃ + calcium lactate group, an E₂Val/CPA + vitamin D₃ + calcium lactate group, and a calcium lactate group. As reported previously, the low-dose vitamin D supplementation used alone or in combination with HRT did not influence BMD in non-osteoporotic women (Heikkinen et al. 1997, Komulainen at al. 1997, Komulainen et al. 1999). Therefore, to increase the power of this study, the two hormone groups and the two non-hormone groups were combined. The final study groups were (Table 2):

**HRT group (initially n=232, after drop-outs and exclusions n=151):** Sequential combination of 2 mg E₂Val (days 1-21) and 1 mg CPA (days 12-21), with a treatment-
free interval (days 22-28; Climen®, Schering AG, Berlin, Germany) alone or in combination with vitamin D₃ (100-300 IU) + calcium lactate (D-Calsor®, Orion Corporation, Espoo, Finland), 500 mg/day (equivalent to 93 mg Ca²⁺).

**Non-HRT group** (initially n=232, after drop-outs and exclusions n=180): Calcium lactate (Calcium Lactate®, Rohto Ltd, Tampere, Finland), 500 mg/day, equivalent to 93 mg Ca²⁺ alone or in combination with vitamin D₃, 100-300 IU/day.

The vitamin D₃ dosage was lowered to 100 IU/day after 4 years of treatment, because of adverse changes in the lipid profile noticed during the first year of the trial (Tuppurainen et al. 1995).

Written informed consent was obtained from all participants and the study design was approved by the Ethics Committee of the Kuopio University Hospital. Prospectively defined stopping rules were the same as the exclusion criteria.

### Table 2. Baseline characteristics of 331 postmenopausal women according to the treatment group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HRT group</th>
<th>Non-HRT group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>151</td>
<td>180</td>
</tr>
<tr>
<td>Age (year)</td>
<td>52.6±2.3</td>
<td>52.8±2.2</td>
</tr>
<tr>
<td>Menopause age (year)</td>
<td>51.5±2.3</td>
<td>51.6±2.3</td>
</tr>
<tr>
<td>Time since menopause (year)</td>
<td>1.16±0.47</td>
<td>1.16±0.46</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.1±3.8</td>
<td>26.3±3.8</td>
</tr>
<tr>
<td>Dietary Ca intake (mg/day)</td>
<td>868±410</td>
<td>860±410</td>
</tr>
<tr>
<td>Physically active persons</td>
<td>41 (27.5%)</td>
<td>55 (30.7%)</td>
</tr>
<tr>
<td>Frequency of smokers</td>
<td>35 (23.2%)</td>
<td>39 (21.7%)</td>
</tr>
<tr>
<td>Smoking (pack-years)</td>
<td>7.3±5.5</td>
<td>7.3±7.4</td>
</tr>
<tr>
<td>Alcohol (ethanol g/week)</td>
<td>18.1±42.5</td>
<td>20.0±34.2</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>60.7±28.1</td>
<td>62.1±25.4</td>
</tr>
</tbody>
</table>

Plus-minus values are means ±SD

*Kruskal-Wallis test except in smokers and, physically active persons, χ² test. No statistically significant differences

Physically active person = three or more hours of physical activity reported per week

Smoking = life-time number of cigarettes / 20 x 365
4.2. The 5-year postal inquiry

In May 1994, a 5-year postal inquiry was sent to the entire cohort (n=14,220; Appendix I). The inquiry included questions on falls during the previous 12 months. The fall questions were as follows: 1) How many times did you fall within the preceding year (12 months) including falls which resulted in no injury? (If none, write zero and move to the question 4) ___ times, 2) During which month did you fall last time? Circle the month, and 3) How did you fall last time? I In stairs up, II In stairs down, III Slipping on the same level, IV Stumbling on the same level, and V Otherwise, specify ______. A fall was defined by ICD IX as E880-7.

4.3. Genotype analyses

4.3.1. Estrogen receptor-α gene polymorphism

Genomic DNA was extracted and purified from EDTA blood samples using QIAamp Blood Kit (Qiagen GmbH, Hilden, Germany). The polymerase chain reaction (PCR) product contains parts of intron 1 and exon 2 of the ERα gene. After amplification, the PCR product was digested with PvuII restriction endonuclease (Fermentas, Vilnius, Lithuania) and electrophoresed in 2.0% agarose gel.

4.3.2. Aromatase and androgen receptor gene polymorphisms

The PCR products for the polymorphic TTTA repeat region in intron 4 of the CYP19 gene (Figure 5A) and for the polymorphic CAG repeat region in exon 1 of the AR gene (Figure 5B) were ran on a denaturing gel (ReproGel Long Read, Amersham Pharmacia Biotech, Uppsala, Sweden) by automated fluorescence detection (ALFexpress DNA Analysis System, Amersham Pharmacia Biotech, Uppsala, Sweden). The length of the PCR product was determined using fluorescent-labelled size standards (ALFexpress
Sizer 50 and 150 for the *CYP19* gene; ALFexpress Sizer 250 and 300 for the AR gene), and the data was analyzed using the ALFwin Fragment Analyser 1.00 software package.

![Diagram A](image)

![Diagram B](image)

**Figure 5.** Five exons (ten exons in total) and first four exons (eight exons in total) of genes encoding human aromatase (A.) and AR (B.), respectively. Location of the TTTA and CAG repeat polymorphisms. The numbered boxes indicate exons.

### 4.4. Bone mineral density and grip strength measurements

The BMD of the lumbar spine (L2-4) and the left proximal femur (femoral neck) were measured by trained personnel using dual X-ray absorptiometry (DXA) (Lunar, Madison, WI) at Kuopio University Hospital before and after 5 years of treatment. The short-term reproducibilities (coefficient of variation, CV%) of the spine and femoral neck measurements in our laboratory are 0.9% and 1.5%, respectively. The long-term reproducibility of our DXA instrument during the trial based on weekly repeated phantom measurements was 0.4%. To ensure that bone edges and intervertebral markers in the spine and regions of interest in the proximal femur were set consistently, all scans were reviewed by one investigator without knowing the group allocation.

Grip strength was measured before and after 5 years of treatment with a hand-held dynamometer (Martin vigorimeter, Germany). Means of three measurements were used as grip strength values.
4.5. Electrophysiologic recordings

Nerve conduction velocities were recorded with a VIKING IV-device (Nicolet, Madison, WI, U.S.A.). Both sensory and motor nerve trunks were stimulated with an electrical constant current stimulator. The strength of stimulation was increased in steps until a maximal response was reached and the recordings were made at a strength which was 10-15% above this value to ensure a supramaximal response. Motor responses (the deep peroneal nerve) were measured with surface electrodes above the end plate area of the activated muscle. The motor response of the anterior tibial muscle was recorded with AgAgCl surface cup electrodes, with the active electrode on the upper third of the muscle above the end plate area (with negative onset of the response) and the reference on the distal tibia close to the ankle. The sensory responses (the superficial peroneal nerve) were recorded above the sensory nerve. The conduction latencies were recorded from the onset of the first negative response and the amplitudes of the motor and sensory responses were measured from the onset to the first negative peak (Aminoff 1998). Measurement of the motor and sensory responses was performed on the left side only (except for the motor response of the anterior tibial muscle which was stimulated at the popliteal space bilaterally).

Means of the motor response values of the peroneal nerve, recorded on the anterior tibial muscle, were calculated. Motor conduction velocity of the deep peroneal nerve was calculated with stimulation at the ankle and the popliteal space and recording on the left short toe extensor muscle (Aminoff 1998).

All studies of nerve conduction velocity were done at room temperature (22 to 24 C°). Skin temperatures were measured with a DM 852 (Ellab, Denmark) thermometer at the site of the sensory nerve measurements (Aminoff 1998). Electrophysiological measurements were performed during 1996.

4.6. Validation of fractures

During the follow-up each subject visited the out-patient clinic once a year and information on occurrence of new fractures was collected. Reported symptomatic
fractures were validated by medical and radiographic reports. No attempt was made to exclude high-energetic fractures. However, there were no fractures caused by car accidents or other major trauma.

4.7. Statistical methods

One-way analysis of variance, the non-parametric Kruskal-Wallis test, or the chi-square test was used for comparison of the baseline characteristics. Significance of interaction terms was tested with generalized linear models. Pearson’s correlation test was used for body mass index (BMI) and E2 levels.

One-way analysis of variance or t test (two groups) was used to test the significances of differences in continuous variables between the candidate gene groups at the baseline, after 5 years, and the BMD changes. Fracture data were analyzed with the Cox proportional hazards model. In the analysis, the period of time to the first fracture was used as the dependent variable.

Fall data were analyzed with logistic regression. Grip strength values were evaluated using the Kruskal-Wallis test. Some of the parameters in the electrophysiologic studies were not normally distributed, but analysis of variance was used. This was done because the electrophysiologic measurements were controlled for confounding factors (analysis of covariance).

Statistical analyses were carried out using the SPSS for Windows statistical package (SPSS Inc., Chicago, IL, U.S.A.). P-value of less than 0.05 was considered statistically significant.

Regarding the ERα gene polymorphism, an additive model was adopted based on the bone loss data. Apparently, the CAG repeat length variation of the AR gene influences the structure and function of the receptor. Longer polyglutamine chains in the AR protein are associated with a lower ability to activate transcription (Chamberlain et al. 1994, Tut et al. 1997, Irvine et al. 2000). Thus, an additive model was adopted. Similarly, an additive model was assumed for the TTTA repeat polymorphism of the CYP19 gene.
5. RESULTS

The following section presents the main results of each study. Further details are presented in the original publications I-V.

5.1. Effect of estrogen receptor-α genotype and hormone replacement therapy on bone mineral density (I)

At the baseline, the distribution of the PvulI genotypes (PP 17.2%, Pp 49.5%, and pp 33.2%) was similar to those reported earlier in different populations (Han et al. 1997, Kobayashi et al. 1996, Willing et al. 1998) and in Hardy-Weinberg equilibrium. There was a significant difference between women with the different ER genotypes in the variable time since menopause. However, the variable age at menopause did not differ significantly between the genotype groups (Study I). During the 5-year follow-up, the relative weight increases were similar (1.9-4.8%) between the different treatment groups and the ER genotypes (data not shown).

At the baseline, there were no significant differences in the lumbar or femoral neck BMDs between the three ERα PvulI genotype groups (PP, Pp, pp) (Study I). The ERα genotype did not modulate the femoral neck BMD change during the follow-up (Study I). In contrast, in the non-HRT-group the lumbar spine BMD decreased more in subjects with the ERα genotypes PP (6.4%) and Pp (5.2%) than in subjects with the pp genotype (2.9%) (p<0.01) (Figure 6). In the HRT group, the relative changes of the lumbar spine BMD were similar in all three ERα genotype groups (Figure 6). Adjustment for the time since menopause did not alter the BMD change results.
5.2. Estrogen receptor-α genotype and the risk of fractures in early postmenopausal women (II)

At baseline, there were significant differences between women with different ERα genotypes and HRT use in the variables FSH, estradiol (E₂), 1-year FSH change, and 1-year estradiol change (Study II).

In all, 28 women sustained 33 fractures during the approximately 5.1-year follow-up. In the HRT group, the ERα genotype (PP, Pp, and pp) was not significantly associated with fracture risk (p=0.14, Cox proportional hazards model) (Study II). When the genotype was dichotomized (PP+Pp vs. pp), the incidence of new fractures in the HRT group was significantly reduced in women with the P allele (p<0.05) with the relative risk (HR) of 0.25 (95% CI, 0.07-0.98), in comparison with the non-P allele group (Figure 7). After adjustment for time since menopause and previous fracture, the association between the dichotomous genotype and fracture risk persisted with HR of
0.24 (95% CI, 0.06-0.95, \(p=0.04\)) (Study II). In the non-HRT group, the ER\(\alpha\) genotype was not significantly associated with fracture risk (study II).

**Figure 7.** Cumulative fracture-free survival as a function of the period of time to the first fracture. In the HRT group the reduction in fracture risk of was significant in women with the \(P\) allele \((p<0.05)\). In the non-HRT group the ER\(\alpha\) genotype was not significantly associated with fracture risk \((p=0.40\), Cox proportional hazards model).”

5.3. **Relation of estrogen receptor-\(\alpha\) genotype and hormone replacement therapy to fall risk and muscle strength (III)**

A total of 97 women out of 331 reported falls. Half of those (56%) were slip falls. Nearly all slip falls (93%), but only half of the non-slip falls (50%) occurred during the winter season (from November to April).

In the HRT group, the ER\(\alpha\) genotype was associated with fall risk \((p=0.002\), logistic regression) (Study III). The risk of falls (RR) was higher in women with the \(PP\) genotype than in those with the \(Pp\) \((\text{RR}=5.3, 95\% \text{ CI} 2.0-13.9, p<0.01)\) or the \(pp\) \((\text{RR}=3.8, 95\% \text{ CI} 1.5-10.1, p<0.01)\) genotype (Figure 8). When the falls were divided into slip (environment-related) and non-slip (endogenous) falls, the non-slip falls were associated with the genotype \((p<0.01)\), but the slip falls were not so clearly \((p=0.06)\).
(Study III). When all falls and non-slip falls were adjusted to the number of chronic health disorders and the variable time-since-menopause, the difference between the genotypes persisted ($p<0.01$ and $p=0.01$, respectively). In the non-HRT group, the ER$\alpha$ genotype was not associated with fall risk (Figure 8). The baseline or the 5-year grip strength values were not influenced by the ER$\alpha$ genotype.

In a subgroup of women ($n=70$), the mean motor amplitude of the right and left anterior tibial muscle after stimulation of the deep peroneal nerve at the popliteal space showed a significant reduction in the HRT group compared with the placebo group ($p=0.02$). The sensory conduction velocity of the superficial sensory nerve was significantly decreased in the HRT group compared with the placebo group ($p=0.02$).

**Figure 8.** Prevalence of falls in the HRT and non-HRT groups by ER$\alpha$ genotypes as determined by the use of restriction enzyme $PvuII$. *$p=0.01$ compared with $Pp$ in the non-HRT group. **$p<0.01$ compared with $PP$ in the non-HRT group ($\chi^2$ test; n=number of women with falls).

5.4. Influence of aromatase genotype on serum estradiol levels, bone mineral density, and fracture risk (IV)

For statistical analysis the subjects were divided into three repeat groups according to $CYP19$ genotype: short (length of 7 or 8 in both alleles; n=135), long (length of 11 or higher in both alleles; n=47), and medium (rest of the values; n=149).
At the baseline, no significant differences in lumbar or femoral neck BMDs between the genotype groups were observed (Study IV). Similarly, serum E₂ concentration was not associated with the CYP19 genotype groups. There was a significant difference between women with the different CYP19 genotype groups in physical activity and a borderline significance with previous fractures (Study IV). During the 5-year follow-up, the relative weight increases were similar (3.3-4.9%) between the different treatment groups and the CYP19 genotype groups (data not shown).

In the HRT or non-HRT groups, the 5-year serum E₂ change was not associated with CYP19 polymorphism (p = 0.87 and 0.74, respectively). Further, the polymorphism did not influence the calculated annual changes of lumbar or femoral neck BMD during the 5-year follow-up in the HRT (p = 0.60 and 0.17, respectively) or non-HRT (p = 0.92 and 0.80, respectively) groups (Study IV).

The CYP19 polymorphism was not significantly associated with fracture risk (p = 0.89 and 0.23 respectively; Cox proportional hazards model) in the HRT or non-HRT groups (Figure 9).

**Figure 9.** Cumulative fracture-free survival as a function of the period of time to the first fracture. In the HRT group the CYP19 genotype groups were not significantly associated with fracture risk (p = 0.89). Similarly, in the non-HRT group the genotype groups were not significantly associated with fracture risk (p = 0.23, Cox proportional hazards model).
5.5. Relation of androgen receptor genotype on grip strength values, bone mineral density, and fracture risk (V)

For statistical analysis all alleles were divided into two repeat groups of approximately equal size: those with 18 or fewer repeats were defined as short (S) and those with more than 18 repeats were defined as long (L). Consequently, the subjects could be divided into three repeat groups according to AR alleles: short & short allele (SS), short & long allele (SL), and long & long allele (LL). Analyses were also performed using only two groups; SS and LL. Reason behind the exclusion of the SL group is the fact that the AR gene is located in the X-chromosome and this results in a random inactivation of one of the two alleles in women (Lyon 1999). Thus, we were able to evaluate the importance of having an active short or long allele.

At the baseline, no significant differences in lumbar or femoral neck BMDs between the allele groups were observed (Study V). Similarly, grip strength values were not associated with the AR allele groups. During the 5-year follow-up, the relative weight increases were similar (2.8-5.2%) between the different treatment groups and the AR allele groups (data not shown). When only SS and LL allele groups were taken into analysis, there was no association between the AR allele groups and baseline BMDs or grip strength values (data not shown).

The polymorphism did not influence the calculated annual changes of lumbar or femoral neck BMD during the 5-year follow-up in the HRT ($p = 0.93$ and 0.15, respectively) or non-HRT ($p = 0.82$ and 0.92, respectively) groups. Similarly, the grip strength values were not influenced by the AR genotype (Study V).

The AR polymorphism was not significantly associated with fracture risk ($p = 0.63$ and 0.46 respectively; Cox proportional hazards model) in the HRT or non-HRT groups (Study V).

When only SS and LL allele groups were taken into analysis, there was no association between the AR allele groups and 5-year grip strength values, 5-year BMDs, the annual BMD change, or fracture risk (data not shown).
5.6. Interaction results

In this study we have analyzed ERα (PvuII restriction enzyme site in intron 1), CYP19 (TTTA repeat region in intron 4), and AR (CAG repeat region in exon 1) gene polymorphisms in the sex steroid pathway. In the non-HRT group, interaction was not detected between the ERα genotype effect on lumbar bone loss and CYP19 and/or AR gene polymorphisms (Table 3).

Table 3. Interaction between the ERα genotype effect on lumbar bone loss and CYP19 and/or AR gene polymorphisms in the non-HRT group

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>p-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα * AR</td>
<td>0.71</td>
</tr>
<tr>
<td>ERα * CYP19</td>
<td>0.57</td>
</tr>
<tr>
<td>ERα * AR * CYP19</td>
<td>0.42</td>
</tr>
</tbody>
</table>

aGeneralized linear models
6. DISCUSSION

6.1. Discussion on the subjects

In this prospective study we investigated the influence of the ERα genotype on bone loss rates, fracture risk, fall risk, muscle strength, and responsiveness to HRT in a 5-year trial with HRT in a placebo-controlled, population-based, randomized group of nonosteoporotic early postmenopausal Finnish women. Additionally, we evaluated whether the CYP19 and AR gene polymorphisms influence serum E2 levels, grip strength values, BMD values, and fracture risk. This study had the advantage of being a long-term, population-based trial with a homogenous group of nonosteoporotic early postmenopausal women with a good compliance rate compared with other HRT trials.

Combination of the study groups (HRT group and HRT+vitamin D group to HRT group, vitamin D and placebo group to non-HRT group) was done to increase the statistical power of the study. The used low-dose vitamin D supplementation alone or in combination with HRT does not influence BMD in this study population as described previously (Heikkinen et al. 1997, Komulainen at al. 1997, Komulainen et al. 1999). It is likely that the low dose vitamin D supplementation was ineffective in prevention of bone loss, because our study subjects may not have been deficient with respect to vitamin D. This suggestion is further supported by the results of a 1-year substudy of this trial in which vitamin D supplementation increased circulating 25-OHD concentrations significantly, but did not affect 1,25-(OH)2D levels (Heikkinen et al., 1998). Previous randomized trials of 1.5- to 2-year treatment with vitamin D (400-800 IU/day) have shown some positive effect on femoral BMD in the elderly (mean age over 80 years) (Chapuy et al. 1992, Ooms et al. 1995). The subjects of our study population were early postmenopausal, non-osteoporotic and generally healthy. Consequently, in our opinion, the used very low dose (100-300 IU/day) vitamin D supplementation did not influence the BMD results obtained.
6.2. Discussion on the results

6.2.1. Influence of the estrogen receptor-α genotype in nonosteoporotic early postmenopausal women

6.2.1.1. Bone mineral density

The results of the present study indicated that the ERα PvuII genotype is associated with the magnitude of long-term lumbar BMD changes in the Caucasian early postmenopausal women who did not use HRT. In the HRT group, the increment of the lumbar BMD was similar between the different genotype groups.

The mechanism of estrogen action in the skeleton is not entirely clear (Riggs et al. 2002). Especially on molecular level, it is still unclear how the whole system works, although the function of the different cytokines is becoming clear (Riggs et al. 2002). Also the mechanism by which the different ERα alleles affect bone loss rate is not known. Recently, it was discovered that the P allele of the PvuII polymorphism produces a potential binding site for the myb family of transcription factors (Herrington et al. 2002b). Results from two studies investigating association between the ERα gene polymorphisms and transcription activity, suggest that the polymorphic variants of ERα gene regulate transcription (Maruyama et al. 2000, Herrington et al. 2002b). Possibly influencing quantity or quality of ERα transcripts or protein. In addition, an unknown gene in linkage with the locus for ERα may be responsible for the correlation of specific ERα alleles and bone loss rate, although that is unlikely.

At the baseline, we found no significant association between the ERα genotype and the lumbar or femoral neck BMD. Our results are similar to other studies in postmenopausal women (Kobayashi et al. 1996, Han et al. 1995, Mizunuma et al. 1995, Gennari et al. 1998, Vandevyver et al. 1999, Becherini et al. 2000, Albagha et al. 2001, Brown et al. 2001).

In the non-HRT group, we found that the ERα genotype was associated with long-term lumbar BMD changes. Deng et al. reported similar results (Deng et al. 1998). Mizunuma et al., however, found no association between PvuII RFLP and the change in
lumbar BMD in Japanese postmenopausal women (Mizunuma et al. 1995). In their study, however, the number of postmenopausal women was smaller \((n=50+50, \text{ the group of women was divided into early and late postmenopausal groups})\) and the follow-up time was shorter (one year), both factors possibly explaining differences between the results. In Caucasian women bone loss rates have not been associated with \(PvuII\) RFLP in late postmenopausal women (Bagger YZ et al. 2000, Brown MA et al. 2001). The femoral neck BMD was not associated with the \(PvuII\) polymorphism. One possible reason for the difference between the measurement sites is the fact that lumbar spine contains more metabolically active trabecular bone than femoral neck. Furthermore, we have previously shown that femoral neck BMD does not respond as well to HRT as lumbar spine BMD (Komulainen et al. 1999). Also in population-based studies where both \(ER_\alpha\) and VDR polymorphisms have been examined, a differential genetic effect on lumbar and femoral BMD has been observed previously (Willing et al. 1998).

In the sex steroid pathway \(ER_\alpha\) gene polymorphism is downstream from \(CYP19\) and AR gene polymorphisms. Thus, theoretically \(CYP19\) and AR gene polymorphisms could modify the \(ER_\alpha\) genotype effect on bone health. However, we did not detect any interaction.

In the HRT group, there were no significant associations between the lumbar BMD changes and the \(PvuII\) polymorphism, which is in agreement with the results from previous studies (Han et al. 1997, Deng et al. 1998, Kurabayashi et al. 1999).

Previously, \(ER_\alpha\) gene polymorphisms (\(XbaI\) and/or \(PvuII\) polymorphisms used) have been associated with age of menarche (inbreeding in the study population) and onset of natural menopause (Weel et al. 1999, Stavrou et al. 2002). In our study, the \(ER_\alpha\) polymorphism was associated with the variable time-since-menopause. The menopause age, however, was not associated with the \(ER_\alpha\) polymorphism (Study I). It is possible, that the size of our study population was inadequate to detect the possible association between the \(PvuII\) polymorphism and menopause age, which was observed by Weel and co-workers (Weel et al. 1999). When we used the variable time-since-menopause as a covariate in the statistical model, the significance in the 5-year lumbar
BMD changes remained ($p<0.01$). Therefore, this baseline difference most likely did not confound our results.

To our knowledge this is the largest and longest clinical trial investigating the association between the ERα polymorphism, the BMD, and HRT responsiveness. Based on our findings, it can be estimated that subjects with the ERα PvuII genotypes PP and Pp may have a greater risk of relatively fast bone loss after menopause than those with the pp genotype and that they may preferentially derive benefit from HRT.

6.2.1.2. Fracture risk

The results of our longitudinal study show for the first time that the ERα gene allelic variation is associated with fracture risk in non-osteoporotic early post-menopausal Caucasian women who use HRT.

Results of studies concerning the relationship between ERα and BMD changes in postmenopausal women have not been consistent (Mizunuma et al. 1997, Deng et al. 1998, Bagger et al. 2000, Brown et al. 2001, Study I). Our study showed that ERα gene polymorphism (PvuII) is associated with the magnitude of long-term lumbar BMD changes (Study I). The effect of COLIA1 gene polymorphism on fracture risk has been studied earlier (Uitterlinden et al. 1998, Langdahl et al. 1998, Keen et al. 1999, Mann et al. 2001, Efstathiadou et al. 2001b, Uitterlinden et al. 2001) and the results suggest that this polymorphism is associated with BMD and fracture risk. Two case control studies have investigated the effect of the ERα gene polymorphisms on prevalence of fractures (Aerssens et al. 2000, Langdahl et al. 2000b). Also one study has investigated the association between ERα gene polymorphisms and prevalent vertebral fractures (based entirely on radiographs) (Becherine et al. 2000). However, there have not been previous prospective studies concerning ERα genotype and incident fractures. In addition, there have not been studies concerning ERα gene polymorphism and the HRT effect on fracture risk. The positive influence of postmenopausal HRT on BMD is well established and its antifracture effect is widely accepted even though it is based mostly on observational studies. There have been only two randomized trials showing the positive effect of HRT on fracture risk (Luiskin et al. 1992, Komulainen et al. 1998). Our
5-year trial showed that HRT prevents non-vertebral fractures in this population (Komulainen et al. 1998). However, it seems that densitometric non-responders to HRT exist (Hassager et al. 1994, Komulainen et al. 2000). The result of this study, showing that the ERα genotype may modify the HRT effect on fracture risk, may give an explanation why some women do not have favorable bone response to HRT.

At the baseline, serum FSH and E2 concentrations were associated with ERα genotype and HRT use. Especially in the non-HRT group, the women with the pp genotype had the longest time since menopause, highest serum FSH, and lowest serum E2 values. The situation with the PP genotype was vice versa. The reason why the pp genotype had lowest serum E2 values may result from the time since menopause. The level of serum E2 values drops dramatically after the menopause and simultaneously the level of serum FSH starts to rise. The statistical difference in the 1-year FSH and estradiol changes are caused by HRT use and with the annual FSH measurements compliance to HRT can be estimated. In the HRT group, the annual FSH levels and the FSH changes during the 5-year follow-up were similar between those women who sustained a fracture compared with those who did not, showing that compliance was good (data not shown).

From the BMD results we concluded that women with the P allele will derive more benefit from long-term HRT than women without the P allele. In the present study, however, the numbers of fractures were relatively small, probably because the subjects were relatively young and non-osteoporotic. In the non-HRT group, the statistical power of our study was not strong enough to show the possible influence of the ERα genotype on fracture risk during the natural menopause. However, a new and clinically important finding was that women with the P allele appear to benefit from the fracture preventive effect of HRT, whereas women without the P allele do not seem to benefit similarly from HRT.

6.2.1.3. Fall risk and muscle strength

The ERα genotype-related differences in the fracture risk appear to be only partly dependent on BMD. It has been previously reported that hip BMD, heel broadband
ultrasound attenuation (BUA), and bone resorption markers are independent predictors of hip fracture in elderly women (Hans et al. 1996, Garnero et al. 1996b). Thus, this genotype-related difference in fracture risk may, in addition, also be due to increased bone resorption, different architectural characteristics of the trabecular bone, fall risk, decreased muscle strength, or a mixture of them. An association between ERα genotypes (PvuII and XbaI restriction endonucleases used) and bone turnover markers in postmenopausal women has not been observed (Kobayashi et al. 1996, Han et al. 1999b). However, an association between candidate genes and fall risk has not been studied.

The results of this study indicated that ERα gene polymorphism is associated with the risk of falls in non-osteoporotic early postmenopausal Finnish women who use HRT. Why did women with the PP genotype fall more often during the HRT than women with the Pp or pp genotypes? Physical activity is a potential confounding factor, but we did not observe any association between the ERα genotype and the physical activity or the physical loading at work. An increased risk of falls might be mediated via decreased muscle strength, but we did not observe any association between the ERα genotype and the grip strength values. Vandevyver et al. have reported similar results in the elderly (Vandevyver et al. 1999). Whether the functioning of the peripheral (e.g. myelin sheath) or central nervous system differs in women with the different ERα genotypes, is presently not known. Interestingly, Brandi and coworkers observed an increased prevalence of the PP genotype in individuals affected with sporadic Alzheimer’s disease (Brandi et al. 1999). As with the nervous system, the mechanism by which the different ERα alleles affect bone loss rate is not known, but the results according to which the BMD difference between the women who used HRT and those who did not was greatest during the 5-year follow-up in the P allele group suggest that women with the P allele are biologically more estrogen sensitive than women without the P allele. The fall risk results point to the same direction. Weel and co-workers reported, that women with the P allele have an earlier onset of natural menopause and a higher prevalence of hysterectomy due to uterus myomatosis and menometrorrhagia (Weel et al. 1999). Suggesting higher responsiveness of P allele to estrogen. Additionally, in postmenopausal women with coronary disease P allele was associated
with augmented response of high-density lipoprotein (HDL) cholesterol to HRT (Herrington et al. 2002a). Thus, based on all of these results from various tissues, it appears that the sensitivity of the P allele for the estrogen effect remains in different tissues. However, the direction of the HRT effect (between different genotypes) on fracture risk via BMD or on fall risk via unknown mechanism may vary in different tissues (see below).

Based on our BMD and fracture risk results, the hypothesis of this study was initially that women with the P allele may have had a decreased susceptibility to falls when using HRT (Study I, Study II). This was based on the assumption that the functioning of the ERα gene and the effects of ERα gene polymorphism are similar in different tissues. Today it is known that the distribution of ERα and ERβ isoforms differ quite markedly in different tissues of the human body (McEwen & Alves 1999). There are several isoforms of ERβ and, when expressed in the same cells, ERα and ERβ1 can form heterodimers (McEwen & Alves 1999). In rats, ERβ2 appears to act as a negative regulator of estrogen action (Maruyama et al. 1998). In humans, ERβcx has been found to inhibit ERα-induced transcription (Ogawa et al. 1998). These findings exemplify the diversity of estrogen actions, suggesting that the biological effects of ERα polymorphism may differ in different tissues. Based on previous studies on humans and animals indicating that estrogen is a potent neuroprotective and neurotrophic factor in the adult central nervous system, our hypothesis was that estrogen would also have a positive effect in the peripheral nervous system (Fugger et al. 2000, Wise et al. 2001). However, we observed a minor (subclinical) but significant decrease in the sensory nerve conduction velocity of the superficial peroneal nerve and a diminution of the motor response amplitude in the anterior tibial muscle in women who used HRT compared with the placebo group. Considering the results from two recent studies on postmenopausal women, where estrogen administration has been found to be associated with decreased muscle sympathetic activity and sympathetic nerve discharge, our results are not surprising (Weitz et al. 2001, Vongpatanasin et al. 2001). The number of women in this subgroup (n=70) was too small to evaluate the influence of ERα polymorphism on the peripheral nerve functions.
Falls generally predispose to fractures. Our previous results have shown that, when using HRT, women with the P allele have decreased fracture risk in comparison with women without the P allele (pp genotype) (Study II). Here we show that, when using HRT, the women with the PP genotype had actually an increased risk of falls which may relate to an altered response to the HRT effect in different tissues, although the sensitivity of the P allele for the HRT effect remains in different tissues. The lack of correlation between the risk of falls in women, who did not use HRT postmenopausally, may be due to lower circulating estrogen levels, i.e., the risk of falls may be estrogen-related in general. At the baseline, there were differences in lumbar BMD between the ERα genotypes in the HRT group (p=0.039), but these differences weakened after adjustment to the variable time-since-menopause (p=0.052) (Study I). Thus, these baseline differences in the lumbar BMD may, in part, explain why the increased risk of falls did not result in increased fracture risk. Additionally, all women of this study were early postmenopausal, nonosteoporotic, and relatively healthy. Therefore, they were not susceptible to fractures.

The limitation of this study was that falls were a secondary end-point inquired only once. Thus, recalling falls may have been a problem. Also, we do not have data on balance or gait, which would have allowed us to study neuromuscular functioning more extensively.

This is, to our knowledge, the first study concerning the association between ERα gene polymorphism, HRT use, and the risk of falls. The new finding was that women, who used HRT and had the PP genotype, tended to fall most often. This effect was especially seen with (endogenous) non-slip falls. Based on our previous findings, we suggest that the influence of ERα polymorphism depends on the target tissue (bone vs. nervous tissue). Further, in these early postmenopausal, non-osteoporotic, and relatively healthy women, the increased risk of falls related to the PP genotype was not associated with increased fracture risk.
6.2.2. Aromatase polymorphism

Conversion of C19 steroids to estrogens is catalyzed by the enzyme aromatase. Before menopause, the ovary contains substantial amounts of aromatase and produces estrogen that acts on target tissues. After the menopause, extraglandular estrogen synthesis predominates, mainly taking place in adipose tissue (Speroff et al. 1999). However, aromatization activity has been detected in almost every tissue tested (Speroff et al. 1999). Estrogen produced in these peripheral tissues can enter circulation and act at distant target tissues.

Due to the rarity of aromatase deficiency (caused by different mutations of the \textit{CYP19} gene) only a few well-documented cases have been described (Deladoëy et al. 1999, Grumbach & Auchus 1999). There have been reports on adult males, a boy, and girls (Deladoëy et al. 1999, Grumbach & Auchus 1999). The two adult men were tall and presented continued linear growth with a decrease in BMD.

There has been a preliminary report on the association of \textit{CYP19} polymorphism with bone health in aged men (Gennari et al. 2000). Men with a lower number of TTTA repeats (less than 10) showed lower BMD values at the lumbar spine and Ward’s triangle, lower ultrasound parameters at the phalanx, higher rates of bone loss, lower circulating E2, and higher bone alkaline phosphatase levels than men with a high number of repeats (more than 11). In a recent study by Masi et al., the number of TTTA repeats was found to be associated with lumbar BMD and spine fracture incidence in postmenopausal Italian women (Masi et al. 2001). Women with a lower number of TTTA repeats (8-11) had lower lumbar BMD than women with a high number of repeats. However, their study group was consisted of both osteoporotic and nonosteoporotic women, whereas in our trial the osteoporotic women were excluded at the baseline. Additionally, in the study of Masi et al. the age of the osteoporotic women was 60±2 years, whereas in our trial the baseline age of the women was 52.7±2.3 years. It is possible that the polymorphism association reported by Masi et al. was not due to the \textit{CYP19} gene itself, but to a second linked gene, although that is unlikely. If this is the case, it may be that in our Finnish population the polymorphism is not linked in the same way as in the Italian population. These differences in experimental design and the
different genetic backgrounds of the study populations may explain the different results in these two studies.

After menopause most of the estrogen is derived by aromatase from extraovarian and extraglandular production of estrone (E₁). Thus, E₁ becomes the principal circulating estrogen. The amount of adipose tissue influences the quantity of E₁ produced by aromatase mainly from androstenedione. We used BMI as a measure of the amount of adipose tissue in the women. At the baseline or at the 5-year measurement, the BMI and E₂ values did not correlate. The women of our study were early postmenopausal and possibly a plateau of postmenopausal estrogen production was not yet fully established during the follow-up. Furthermore, BMI only roughly represents the total amount of adipose tissue and does not indicate its distribution. It appears that the location of the adipose cells influences their activity in this respect, since women with central obesity produce more androgens (Kirschner et al. 1990). We only measured serum E₂ levels and not E₁ levels. However, most of the circulating E₂ is derived from peripheral conversion of E₁ (Speroff et al. 1999) and presumably alterations in E₁ levels by CYP19 polymorphism would result in altered serum E₂ levels. Measuring E₁ might have provided a more accurate picture of the possible effect of CYP19 polymorphism on aromatase function, yet E₂ is a more potent estrogen (E₁ is 4-fold weaker) causing stronger biological effects.

We divided CYP19 polymorphism into three repeat groups: short (length of 7 or 8 in both alleles), long (length of 11 or higher in both alleles), and medium (rest of the values). The division was made in this way on functional basis. The function of the gene with two short alleles may differ most from the gene with two long alleles, assuming that this polymorphism has an effect.

The hypothesis of this study was that CYP19 polymorphism may alter estrogen levels in early postmenopausal women, consequently influencing BMD and fracture risk. The effect was expected to become apparent during the 5-year follow-up. However, the 5-year serum E₂ levels or the 5-year E₂ changes were not associated with CYP19 polymorphism. Furthermore, the 5-year BMD values, the 5-year BMD change, and the fracture risk were not associated with this polymorphism. It is possible that our follow-up period was too short, i.e., the effect of the polymorphism may become
apparent in older age groups, assuming that CYP19 polymorphism alters the ability of aromatase to convert androgens to estrogens.

6.2.3. Androgen receptor polymorphism

In men androgen deficiency is clearly associated with decreased BMD (Greenspan et al. 1986 Finkelstein et al. 1987, Katznelson et al. 1996) and with testosterone treatment it is possible to increase BMD (Katznelson et al. 1996). However, in women the situation is not so clear (Miller et al. 2001). Several prospective trials have investigated the effect of androgen replacement therapy on bone health (Davis et al. 1995, Watts et al. 1995, Raisz et al. 1996, Barrett-Connor et al. 1999). Three studies showed increase in bone formation markers or BMD when androgen replacement therapy was added to the estrogen replacement therapy (ERT) (Davis et al. 1995, Raisz et al. 1996, Barrett-Connor et al. 1999). These studies were performed on postmenopausal or surgically menopausal women. However, in a study by Watts et al. adding androgen replacement therapy to ERT did not improve BMD in oophorectomized women (Watts et al. 1995). In women serum testosterone and free testosterone levels have been found to correlate with BMD (Slemenda et al. 1996, Greendale et al. 1997). In a recent study by Westberg et al., serum testosterone was associated with AR gene polymorphism (Westberg et al. 2001). All these results suggest that androgens have an effect on bone in women, but it is unclear how much of the androgen effect on bone is direct via AR. Abu et al. demonstrated that ARs were expressed in the majority of osteoblasts at bone modelling and remodelling sites (Abu et al. 1997). Additionally, in a small study on women with androgen insensitivity syndrome (AIS), a decrease in BMD z-score (-0.75 SD) was observed even with good compliance to estrogen treatment (Marcus et al. 2000). However, indirect effects via aromatization to estrogens and/or through changes in muscle strength may mediate the skeletal actions of androgens. We did not observe any association between grip strength values and the AR gene polymorphism.

The trinucleotide repeat length variation of the AR gene appears to influence the structure and function of the receptor (Chamberlain et al. 1994, Tu et al. 1997, Irvine et al. 2000). Longer polyglutamine chains in AR protein are associated with its lower ability to activate transcription (Chamberlain et al. 1994, Tu et al. 1997, Irvine et al.
2000). There is only one previous candidate gene study investigating the possible association between the AR gene polymorphism and BMD. The study group consisted of 261 pre- and perimenopausal women, aged 28-48 years at baseline (mean age 37 years) (Sowers et al. 1999). Sowers et al. reported that the AR genotype distribution was different at the lumbar and femoral neck BMD groups. However, significant associations between the AR genotypes and the 3-year BMD changes were not reported (Sowers et al. 1999). It is then possible that the AR polymorphism affects attainment of peak bone mass, but during the phase of bone loss it does not have a significant role. This would explain why we did not observe an association between the AR gene polymorphism and the baseline BMD or the 5-year BMD change in early postmenopausal women. In a linkage study by Duncan et al., the AR gene was not linked with BMD (Duncan et al. 1999). The mean age of subjects in this study was 50 years (range 16-90 years).

To our knowledge, this is the first study investigating the association between AR polymorphism, grip strength values, BMD values, and fracture risk in postmenopausal women. Although there is an increasing amount of data suggesting that androgens and AR influence bone health in women (direct and/or indirect effect), the results of our study indicate that the AR repeat polymorphism (CAG) in exon 1 does not influence grip strength values, BMD, or fracture risk in early postmenopausal Finnish women.
7. SUMMARY AND CONCLUSIONS

The results of this randomized trial show that the ERα gene allelic variation is associated with the magnitude of long-term lumbar BMD changes. Women with the P allele seem to show fast bone loss, the PP genotype indicating the greatest risk. With HRT, however, it is possible to prevent the influence of the different ERα genotypes on bone loss. Therefore, it can be estimated that the women with the PP and Pp genotypes derived more benefit from HRT than those with the pp genotype. Additionally, the ERα genotype appears to modify the HRT effect on fracture risk. This effect of the ERα gene polymorphism may, at least in part, provide an explanation why some women do not have a favorable bone response to HRT.

In this prospective study we also evaluated whether ERα gene polymorphism influences fall risk and muscle strength. The women, who used HRT and had the PP genotype, tended to fall most often. This effect was especially seen with (endogenous) non-slip falls. We did not observe any association between the ERα genotype and the grip strength values.

Our findings do not support the hypothesis that the aromatase gene polymorphism alters estrogen levels in early postmenopausal women, consequently having an influence on BMD and fracture risk. Additionally, despite increasing amount of data suggests that androgens and androgen receptor influence bone health in women, our results indicate that, during the phase of rapid bone loss, the androgen receptor gene polymorphism does not have a significant role in this respect. The results of our study show that the polymorphic TTTA repeat region in intron 4 of the CYP19 gene and the polymorphic CAG repeat region in exon 1 of the AR gene do not influence the circulating E2 levels, grip strength values, BMD values, or fracture risk in early postmenopausal Finnish women.

This is the largest and longest clinical trial investigating the association between the ERα polymorphism, BMD, fracture risk, fall risk, muscle strength, and HRT responsiveness. A new and clinically potentially important finding was that the ERα genotype appears to modulate bone loss rates, fracture risk, and the responsiveness to
HRT. The results suggest that women with the $P$ allele are biologically more estrogen sensitive than women without the $P$ allele.

In a multifactorial disease like osteoporosis, generalizations on the basis of genetic results from different populations and environments are problematic. Thus, the amount of current data on genetically determined variation in bone loss rates and HRT responsiveness in Finnish women is inadequate. Most importantly, it is not known whether women with a favourable HRT response will also have a higher risk of adverse effects. Additionally, the availability of genetic tests is limited at present. In conclusion, the clinical usability of this genetic marker to detect women with high risk of developing osteoporosis and to predict non-responders to HRT in large scale screenings remains to be determined.

Based on the findings of the present study, the following conclusions were drawn:

- The $PvuII$ polymorphism of the ER$\alpha$ gene is a potential genetic marker for detecting women with increased bone loss rates and fracture risk during the early, accelerated phase of bone loss.
- In pharmacogenomics, this polymorphism is a potential genetic marker for HRT responsiveness.
- The aromatase and androgen receptor polymorphisms were not associated with circulating $E_2$ levels, grip strength values, BMD values, or fracture risk.
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9. ORIGINAL PUBLICATIONS (I-V)