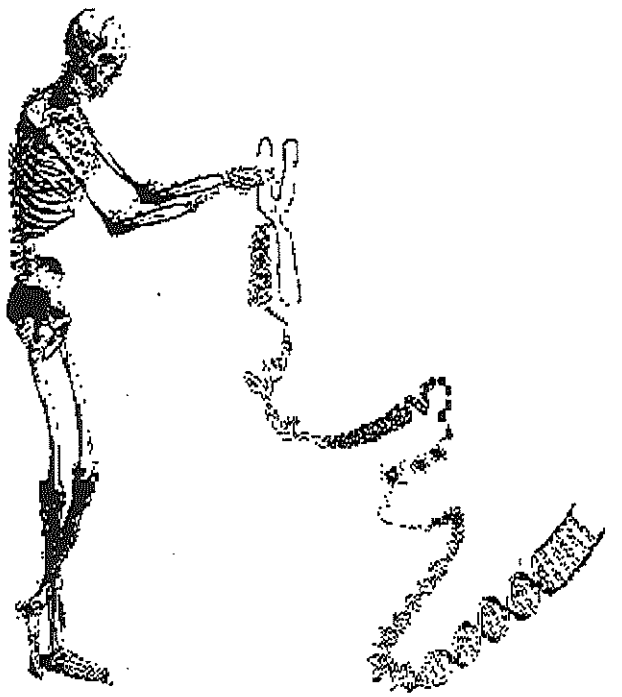
SKELETAL AND GENETIC DETERMINANTS OF OSTEOPOROSIS



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SKELETAL AND GENETIC DETERMINANTS OF OSTEOPOROSIS

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Voor Michael

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Chapter 2.1
Weel AEAM, Burger H, de Laet CEDH , Hofman A, van Leeuwen JPTM, Pols HAP. Fractures in Elderly Men and Women:Incidence and Association with Bone Mineral Density. *Submitted*

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Chapter 3.1.1
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Chapter 3.2.2

Colin EM, Weel AEAM, Uitterlinden AG, van Leeuwen JPTM, Pols HAP. Consequences of vitamin D receptor gene polymorphisms for growth inhibibition of cultured peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D3. *Clin Endocrinology 2000;.52(2): 211-216*

Chapter 3.3

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1

Introduction

INTRODUCTION

BACKGROUND

Osteoporosis, one of the critical diseases facing the ageing population and along with cardiovascular disease, diabetes and cancer, is a major concern for public health in western countries. Fractures, the clinical endpoint of osteoporosis, contribute considerably to overall morbidity, mortality and healthcare costs. It has been estimated that the lifetime risk to suffer a fracture is 40% for a 50-year-old white woman and 15% for men.[1] Of those who have suffered a fracture of the hip, 50% are subsequently unable to walk unassisted and 20 % die within the first year after the hip fracture occurred.[2] The total health care expenditures attributable to osteoporotic fractures in the United States was estimated at US$ 13.8 billion in 1995.[3] In the Netherlands direct medical cost of osteoporosis-related fractures was estimated to be over 400 million guilders each year.[4] Due to ageing, fractures from osteoporosis occurring each year are projected to increase world-wide from 1.7 million in 1990 to 6.3 million in 2050.[5]

DEFINITION

Osteoporosis is defined as a systemic skeletal disease, characterised by low bone mass and microarchitectural detoriation of bone tissue with a consequent increase in bone fragility and susceptibility to fracture.[6]

BMD), as a reflection of the amount of bone mass present, is currently the sole factor that can be measured with sufficient accuracy and it is currently the best available indicator of fracture risk.[7] Therefore, the diagnosis of osteoporosis is primarily based on BMD measurement. BMD is a continuous variable with a normal distribution in the population. Thus, cut-off or threshold levels have to be decided upon for diagnostic purposes. Diagnostic criteria for osteoporosis that have been accepted by the World Health organisation (WHO, 1994) are given in Table 1.1.

Currently several non-invasive techniques are available to measure bone fragility such as single photon absorptiometry (SPA), dual energy X-ray absorptiometry (DEXA), computed tomography (CT) and ultrasound, who showed large differences in precision, accuracy and

complexity. In this thesis we measured BMD by using DEXA, which is widely viewed as the preferred method to assess bone mineral density because of its speed, precision, and minimal radiation exposure, and the availability of reference data.[8]

Table 1.1 Diagnostic criteria for osteoporosis

Diagnosis	**Criteria**
Normal	A value for BMD not more than 1 SD below the average value of young adults (T-score > -1)
Osteopenia	A value for BMD more than 1 SD below the young adult average, but not more than 2.5 SD below (T-score > –2.5 and < -1)
Osteoporosis	A value for BMD more than 2.5 SD below the young adult average value (T-score < - 2.5)
Established Osteoporosis	A value for BMD more than 2.5 SD below the young adult average value and the presence of one or more fragility fractures

T-score; number of standard deviation difference in bone mineral density from the young adult average (premenopausal mean)

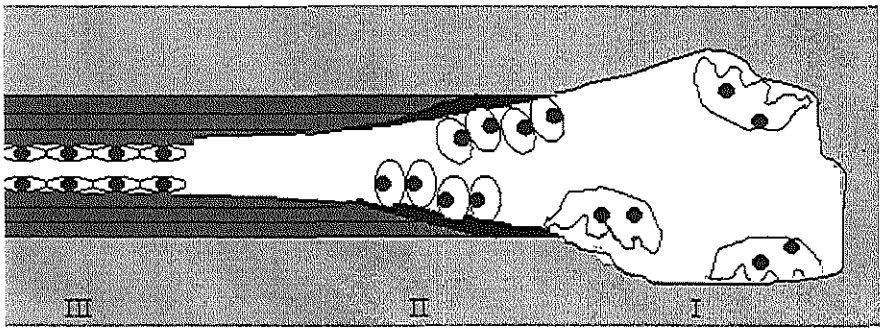
BONE METABOLISM

There are two types of bone: rigid cortical bone, which is composed of densely packed mineralised collagen, and more elastic trabecular bone which is more spongy but also consist of mineralised collagen. The mineralised matrix is composed of collagenous (90% is collagen type I) and non-collagenous proteins and contains a variety of locally produced growth factors.

Bone is a dynamic tissue that is continuously remodelled throughout life (Figure 1.1). In physiologic situations there is a balance between bone formation and bone resorption. But under pathophysiologic circumstances both the degree of remodelling balance within a bone-remodelling unit and the frequency at which those remodelling-units are activated determine the rate of bone gain or loss.[9] In bone there are three major types of cells that are relevant, the osteoclast, osteoblast, and osteocyte. The osteoclast precursors recruit to the site that is to be remodelled. There they differentiate into mature osteoclasts, which resorb a certain amount of

bone. After resorption is completed, osteoclasts move away and/or undergo apoptosis and bone formation begins. Osteoblast form new bone by synthesising bone matrix, which subsequently mineralises. Osteocytes are osteoblasts that have been trapped in mineralised matrix and changed their function and morphology. They probably have a mechanosensor function and are in contact with each other through lacunae.[10]

Figure 1.1 Schematic diagram of a bone-remodelling unit



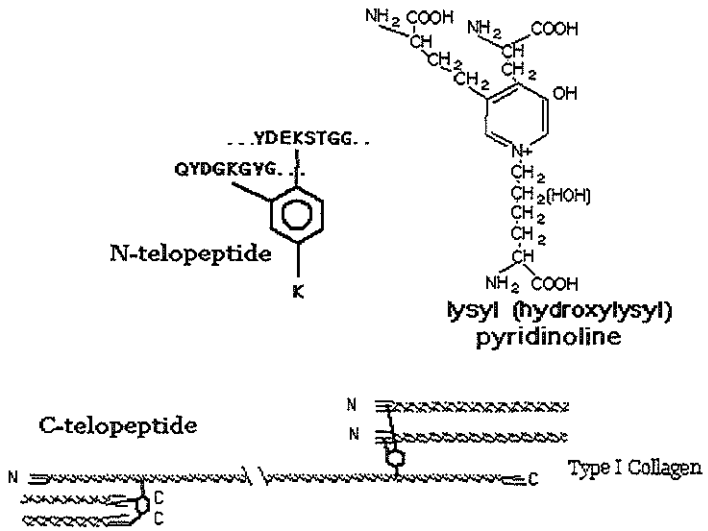
I	Resorption phase: Osteoclast resorb bone
II	Formation phase: Osteoblast synthesise an osteoid matrix
III	Resting phase: Lining cells are inactive osteoblasts

In osteoporosis there is both an increased bone turnover and an imbalance between bone formation and bone resorption, favouring bone resorption. The rate of bone turnover can be estimated by measuring bone matrix components released into the circulation. Bone turnover markers have the advantage of assessing bone turnover in the whole body metabolism, being non-invasive and being relatively cheap. Biochemical markers of bone turnover represent either the enzymes involved in bone formation (osteoblast) and bone resorption (osteoclast) or the formation and degradation products of bone matrix metabolism (primarily type I collagen).

Bone formation activity is measured by the enzyme bone alkaline phosphatase (BAP), secretion of osteocalcin, and fragments of type I collagen: procollagen type I carboxy-propeptide (PICP) and procollagen type I amino propepetide (PINP).[11,12] The function of BAP remains unclear although the levels increase in high bone turnover states such as Paget's disease. Type I procollagen is secreted by osteoblast and during formation of the mature

collagen type I, the PICP and PINP fragments are cleaved off and released in the circulation. Osteocalcin is synthesised by osteoblast and subsequently undergoes a vitamin K dependent gamma-carboxylation. It represents 1% of the organic bone matrix, where it is closely associated with hydroxyapetite crystals. Its precise function remains unclear but appears to relate to osteoblast synthetic activity, with a possible role as a messenger in the coupling process of osteoblast and osteoclast activity.[13]

Figure 1.2 **Structure of collagen type I**



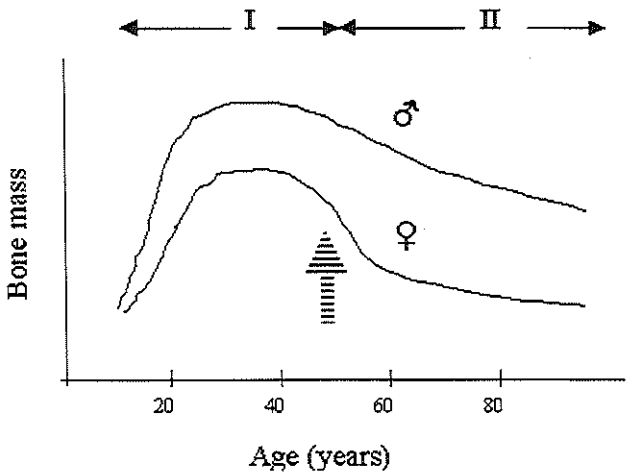
Bone resorption markers include the amino acids hydroxyproline (Hyp) and hydroxylysine (Hyl) and the isoenzyme tartrate resistant acid phosphatase (TRAP). During osteoclastic resorption breakdown products of collagen type I (Figure 1.2), pyridinoline (PYD) and deoxypyridinoline (DPD) are secreted in urine as peptide bound (60%) and free peptides (40%).[14,15] DPD appears to be a more specific marker of bone resorption since PYD is presented in many tissues including cartilage and skeletal muscles. PYD and DPD-containing peptides have also been isolated; these are known as the N-terminal cross-linking telopeptide (Ntx) and the C-terminal cross-linking telopeptide (Ctx) and the type I collagen C-telopeptide assay (ICTP). Ntx and Ctx show a significant response to anti-resorptive therapy.

The activity of bone cells and the rate of bone remodelling are influenced by many factors, including the important systemic hormones, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and sex steroids (17β-estradiol (E_2)).[16] The effects of 1,25-$(OH)_2D_3$ and E_2 are primarily mediated by their receptors, the vitamin D receptor (VDR) and the estrogen receptors α and β (ERα and ERβ, respectively), which are both members of the nuclear receptor superfamily.[17-19] VDR, ERα and ERβ are expressed in a wide variety of cells and tissues including, osteoblast, osteocytes and osteoclast precursors.[19-22]

RISK FACTORS

The two most important factors that determine the amount of bone are the peak bone mass (I) attained in early adulthood and the amount of bone lost (II) with ageing (Figure 1.3).

Figure 1.3 Bone mass as a function of age in men and women



Phase I; peak bone mass attained in early adulthood

Phase II; bone loss with ageing

Vertical arrow; accelerated bone loss after menopause

During childhood and adolescence and up to the acquisition of adult stature, two phenomena occur simultaneously: the synthesis of new bone from cartilage due to the process of endochondral ossification, and the modelling-remodelling of previously synthesised bone. The final result of this processes is expressed as peak bone mass. After peak bone mass has been reached, both men and women experience age-related bone loss. The cellular mechanism underlying age-related bone loss is generally accepted to be a decline in osteoblast function leading to an imbalance of bone remodelling, which produce lower amounts of mineralization matrix and subsequently lead to thinning of trabeculae. Superimposed upon the age-related bone loss, women experience a period of accelerated bone loss after menopause (vertical arrow). The cellular mechanism underlying postmenopausal bone loss is related to loss of estrogen production by the ovaries resulting in an increased activity and/or number of osteoclasts, which subsequently produce deeper resorption cavities with ultimately removal of complete trabecular plates.[23,24]

Both peak bone mass and age-related bone loss are influenced by a number of factors. An overview of these factors is given in Table 1.2. However, models incorporating all of these factors together explain only 20-30% of the variance in BMD.[25] Moreover, bone mineral density measurement reflects only the quantitative amount of bone that account for 70-80% of the capability of bone to resist force,[26] while the other 20-30% can be described to factors other than BMD. Those factors may be other skeletal factors like bone quality expressed in bone turnover rate, bone architecture and bone matrix components[27,28] or extraskeletal, like falling, alcohol and nutritional factors.[29-33] Interestingly, recent published reports have shown that considerable hereditary components contribute to the both quantitative (meaning BMD) and qualitative aspects of bone like ultrasound and bone turnover indices.

Table 1.2 Factors influencing peak bone mass and bone loss that determine BMD

Peak bone mass	*Bone loss*
Genetic	**Genetic**
Nutritional	***Nutritional***
Calcium intake (↑)	Calcium intake (↓)
Vitamin D intake (↑)	***Vitamin D intake (↓)***
Malnutrition (↓)	***Alcohol (↑)***
Hormonal factors	**Hormonal factors**
Delayed puberty (↓)	Premenopausal oophorectomy (↑)
Primary and secondary gonadal failure (↓)	***Premenopausal amenohorrea (↑)***
Lactation (↑)	**Life style**
Multiparity (↓)	**Body weight (↓)**
Oral anticonceptives (↑)	Physical activity (↓)
Life style	Smoking (↑)
Physical activity (↑)	**Other**
Smoking (↓)	Non-insulin-dependent Diabetes (↑)
	Chronic corticosteroid use (↑)
	Age (↑)

(↑) Increased exposure is associated with a increase in peak bone mass and/or increase in bone loss
(↓) Increased exposure is associated with a decrease in peak bone mass and/or decrease in bone loss

GENETIC FACTORS

Osteoporosis can be described as a complex trait, meaning a common disease that usually has a late onset, a multifactorial nature (environmental and genetic conditions interact), and is polygenetic (more than one gene).[34] The genetic nature of osteoporosis became apparent from studies in individuals with different racial background and from twin- and family studies.

Racial variation

Blacks have a significantly higher BMD and lower frequency of osteoporotic fractures compared to Caucasians.[35-39] By contrast, Asians tend to have lower BMD values but the incidence of fractures is generally lower compared to Caucasians indicating that not only BMD explains the fracture risk.[40,41]This racial difference in BMD and the frequency of fractures suggests a genetic component in the aetiology of osteoporotic fractures.

Twin and family studies

In the last decades, twin- and family studies have shown the importance of genetic factors in the pathogenesis of osteoporosis. Monozygous (MZ) and dizygous (DZ) twins share 100% and 50% of their genome, respectively. Thus, if a trait is strongly influenced by genetic factors one expects the difference between the two members of a MZ twin pair to be smaller than between members of a DZ twin pair. This difference in variance between the two twin types can be expressed as a heritability score between 0-100%. For BMD the heritability has been estimated to be from 50% up to 80%.[42,43] This means that up to 80% of the variance in BMD values can be explained by genetic factors while the remaining 20% could be due to environmental factors. Twin studies, however, do not necessarily give an accurate estimate of heritability measures due to overestimation of the phenotypic similarity in twins such as similar environmental factors, which already exist before birth. Nevertheless, although the precise value of this figure is subject to discussion it can be concluded that BMD has a strong genetic basis. Also other types of studies have provided evidence for the genetic basis of risk factors for osteoporosis. For example, daughters of mothers with osteoporosis and first degree relatives of osteoporotic patients have a reduced BMD compared to relatives of individuals with normal BMD values.[44-46] The polygenetic control of BMD, has been shown in segregation analysis in families.[47,48]

As mentioned in the previous paragraph, not only BMD is important as a risk factor for fractures. Therefore, besides bone mineral density also other skeletal factors as measured by ultrasound, bone turnover parameters and hip axis length have been reported to be under genetic control.[49-51] Estimates of heritability based on twin studies for quantitative ultrasound parameters are around 75%, for bone turnover indices this is about 65% and 60% for hip axis length.

DNA Sequence variation

In the last couple of years, part of osteoporotic research has focused on molecular genetics that aims to explain the genetic variation of the disease. Crucial in any genetic analysis is the detection of variations in the basepair sequence of the genomic or mitochondrial DNA between individuals with and without the disease of interest. Sequence analysis of a gene in a number of different individuals will identify basepairs at certain positions that vary between individuals.

Such sequence variants can be classified by definition as "mutations" when they occur at a frequency of less than 1% of the population and as "polymorphisms" when they occur at a frequency of at least 1%. There are two main types of DNA sequence variations, **single nucleotide polymorphism (SNP)** and the **tandem repeats** of short sequence motifs in genetic studies.

The simplest DNA sequence polymorphism is the **SNP**. The human genome is estimated to contain roughly 30.000 genes, with about 60.000 SNPs to be situated in the coding regions of the genes.[52] The currently most widely applied method to detect SNPs is by using restriction fragment length polymorphism (RFLP).

A special case is presented when several polymorphisms of interest are located adjacent to each other. Although each can be analysed separately, analysing them in relation to each other can increase the information of the association with the phenotype of interest. For example, a detected SNP could represent; (1) the main functional polymorphism in the association, but (2) it could also serve as a marker for yet unknown molecular variants or both (3) the detected and unknown variants could be causally implicated.

Analysing such polymorphisms in relation to each other can be accomplished by determining which alleles of each of the polymorphic sites are on the same chromosome. Such a combination of alleles of adjacent loci is called a haplotype. At large distances (> 10 kb) pedigree studies are necessary to identify haplotypes. At smaller distances (< 10kb) however, a molecular method exist to directly identify haplotypes. An example is the method to detect three anonymous polymorphic restriction enzyme recognition sites at the 3' end of the VDR gene, *BsmI*, *ApaI*, and *TaqI* developed in our laboratory.[53]

A second type of DNA sequence variation involves **tandem repeats** of short sequence motifs. Depending on their length they are arbitrarily referred to as microsatellites (motif length < 6 bp) or minisatellites (motif length > 6 bp). Due to the polymorphic variation in number of repeat units these sequences are termed variable number of tandem repeats (VNTR). Microsatellites are used in genome searches since they are present in an estimated 100.000 copies per haploid genome and they are spread throughout the chromosomes.

Functional versus anonymous polymorphisms

For interpretation of a given association between a genetic marker and a phenotype of interest, it is important to understand the consequences of such a genetic variation. In this respect polymorphisms have been divided in so-called '**functional**' and '**non-functional** or **anonymous**' polymorphisms.

Functional polymorphisms can include, e.g., sequence variations leading to alterations in the amino acid composition of the protein, changes in the 5' promotor region leading to differences in expression, and/or polymorphisms in the 3' region leading to differences in mRNA degradation. The functional polymorphisms are of prime interest to further test in association analyses to evaluate if the candidate gene is a true osteoporosis gene or not. Because functional polymorphisms lead to meaningful biological differences in function of the encoded protein this also makes the interpretation of association analyses using these variants quite straightforward. For functional polymorphisms it is expected that the same allele will be associated with the same phenotype in different populations.

However, since it is clear that there is considerable work involved before such functional variations are identified as such also non-functional or anonymous polymorphisms are of interest. The **anonymous** polymorphisms are used as markers whereby the alleles "flag" different DNA sequence variants of (parts of) chromosomes between individuals rather than just the gene in or near which they are present. In the case of a positive association of one of the marker alleles with the phenotype of interest, one supposes then that the marker allele is in linkage disequilibrium (LD) with a truly functional polymorphism elsewhere in the gene. Usually it cannot be excluded that the LD extends into another nearby gene. In normal outbred populations this means that the other gene has to be in a region of approximately <<<1 Mbp, usually 10-100 kb near the marker. However, in more inbred populations (isolated populations) this area can be much larger. Recent modelling studies using population simulation to estimate the extent of LD surrounding common gene variants showed that "useful" association does not extend beyond 3 kb.[54] In genetic terms this is extremely close and has considerable consequences, for example, for genome wide genome searches using SNPs.[55] This estimate, however, is contradicted by other studies showing much larger distances (up to several cM) over which LD can extend.[56] To complicate LD-matters further,

dozens of genes can be present in an area of 500 kb while functionally related genes also have a tendency to be localised near each other.

Analytical approaches

Two approaches are usually used to determine the genetic contribution to complex diseases; i.e. **Genome searches** and the **Candidate gene** approach. **Genome searches** are based on the assumption that relatives who share a certain phenotype will also share one or more chromosomal areas identical by descent (IBD) containing one or more gene variants causing (to a certain extent) the phenotype of interest (e.g. low BMD). DNA markers are used to flag a certain chromosomal region that is supposed to be linked with the phenotype of interest. Upon positive linkage, subsequent research will zoom in on identifying which one of the dozens of genes in the chromosomal area is the one involved in bone metabolism and then identify the particular sequence variant giving rise to aspects of a complex trait such as osteoporosis. The genome search approach is highly powerful for pedigree based research but may be of less value in osteoporosis because osteoporosis is a late onset disease and, therefore, it is difficult to study a large number of pedigrees with reliable disease diagnosis. Furthermore, osteoporosis is probably a multigenetic disease with partial contributions of different genes involved. Therefore, to observe statistical significant effects of each single gene, large numbers of sib pairs are necessary.[57]

The second approach, **candidate gene** association studies, investigates the contribution of sequence variants in or outside already known genes to the different phenotypic endpoints of the disease. The choice of a candidate gene is primarily guided by selection of genes coding for factors that regulate bone turnover and proteins that make up normal bone matrix. In this respect, several lines of evidence can be followed. For example, a gene mutation responsible for a Mendelian bone disorder is a potential candidate to screen, whereby their polymorphisms can subsequently be evaluated in association analysis. The involvement in bone biology can also be established when the gene-of-interest is knocked-out in mice and a bone-phenotype occurs. Or, simply when the gene product is specifically expressed and/or functional in bone tissue. Thus, an osteoporotic candidate gene will have characteristics all more or less in line with at least one of these considerations. In view of this rather wide definition it can be expected that there are many potential osteoporosis candidate genes. In Table 1.3 a list is provided of such osteoporotic

candidate genes, together with some characteristics. A few particular candidate genes will be discussed in more detail because of their central role in bone metabolism and the knowledge on their genetic contribution currently available.

Table 1.3 Osteoporotic candidate genes grouped by nature of the polymorphic variants

GENE	SYMBOL	LOCATION	POLYMORPHISM
Protein isoform			
Methylenetetrahydrofolate Reductase	MTHFR	1p36.1	C→T: Ala222Val[
Bone Alkaline phosphatase	ALPL	1p36.1	T→C: His246Tyr
α2 HS Glycoprotein	AHSG	3q27	*1, *2 protein isoforms
Vitamin D binding protein	GC/DBP	4q12	1S, 1F, 2 protein isoforms
Glucocortoicoid Receptor	GR/NR3C1	5q31	Asn363Ser
Calcitonin Receptor	CALCR/CTR	7q21	Pro447Leu
β3Adrenergic Receptor	ADRB3	8p12	Trp64Arg
Vitamin D Receptor	VDR	12q13	T→C 3 AA deletion at translation start
Transforming Growth Factor β1	TGFβ1	19q13	Leu10Pro + Arg25Pro
Apolipoprotein E	ApoE	19q13.2	Cys112Arg + Arg158Cys
Regulatory			
Interleukin-4	IL4	5q31	C→T -524 promotor
Tumour Necrosis Factor-α	TNFα	6p21	C→A –863 promotor
Interleukin 6	IL6	7p21	G→C –174 promotor
Osteoprotegerin	TNFRSF11B	8q24	T→C –950 promotor
Vitamin D Receptor	VDR	12q13	G→A promotor (Cdx2)
Collagen Type Iα1	COLIA1	17q21	G→T intron 1 (Sp1)
Anonymous			
Estrogen Receptor	ER1	6q25	C→T intron 1 (PvuII RFLP)
			G→A intron 1 (XbaI RLFP)
Vitamin D Receptor	VDR	12q13	3'UTR haplotype
Collagen type IIα1	COL2A1	12q13	3' VNTR
			exon 5B
			intron 33
Insulin-like Growth Factor-I	IGF-I	12q24	CA-repeat promotor

The vitamin D receptor gene polymorphism

The candidate gene that actually initiated the "molecular genetics of osteoporosis" is the vitamin D receptor gene (VDR) that is located on chromosome 12q13-14 (Figure 1.4).[58] The VDR gene codes for the vitamin D receptor that binds the hormonally active form of vitamin D ($1,25\text{-}(OH)_2D_3$) and exerts the hormonal effect. $1,25\text{-}(OH)_2D_3$ plays a central role in bone metabolism by regulating differentiation and activity of bone cells like osteoblasts and osteoclast. In line with this, substitution of vitamin D reduces the incidence of fractures in the elderly, although conflicting results have been reported.[59-61] A plausible explanation for this divergent effect of vitamin D substitution might be genetic variations of the VDR. Therefore, the VDR gene is a potential candidate gene in the aetiology of osteoporosis.

Three adjacent RFLPs, i.e., for *Bsm*I, *Apa*I and *Taq*I, respectively, in intron 8/exon 9 at the 3' end of the gene, are most frequently studied. Morrison et al. reported that the VDR allele of the *Bsm*I RFLP was related to increased serum osteocalcin concentration and subsequently showed this allele to be associated to low BMD in a twin study and in postmenopausal women.[62] Although the initial observations in the twin study have been withdrawn, dozens of reports on the association with BMD have been published. The largest study published sofar, in 1782 Dutch elderly men and women from the Rotterdam study, reported no effect of single RFLPs on BMD. However a small significant effect was detected employing haplotypes constructed of the 3 adjacent 3' RFLPs.[53] In line with this, a meta-analysis of 29 studies (excluding the Dutch study) on the relationship of VDR genotype with BMD concluded that VDR genotype is associated with BMD in elderly subjects but with only 1-2% difference between extreme genotypes.[63]

In view of the genome-wide observed frequency of SNPs one can expect over 100 polymorphisms to be present in the VDR gene area alone, including in areas which are functionally relevant such as the promotor region. Indeed, a recent study from our laboratory has identified more than 25 different polymorphisms at the VDR locus, so far mostly near the 3' end of the gene (see Figure 2).(Fang et al. manuscript in preparation) Also towards the 5' end of the gene a sequence variation has been reported showing a substitution (T to C) at exon 2 that eliminates the first ATG translation inition site and allows a second one 9 bp down stream to be used. Thus, two variant forms of the VDR protein can be translated which differ

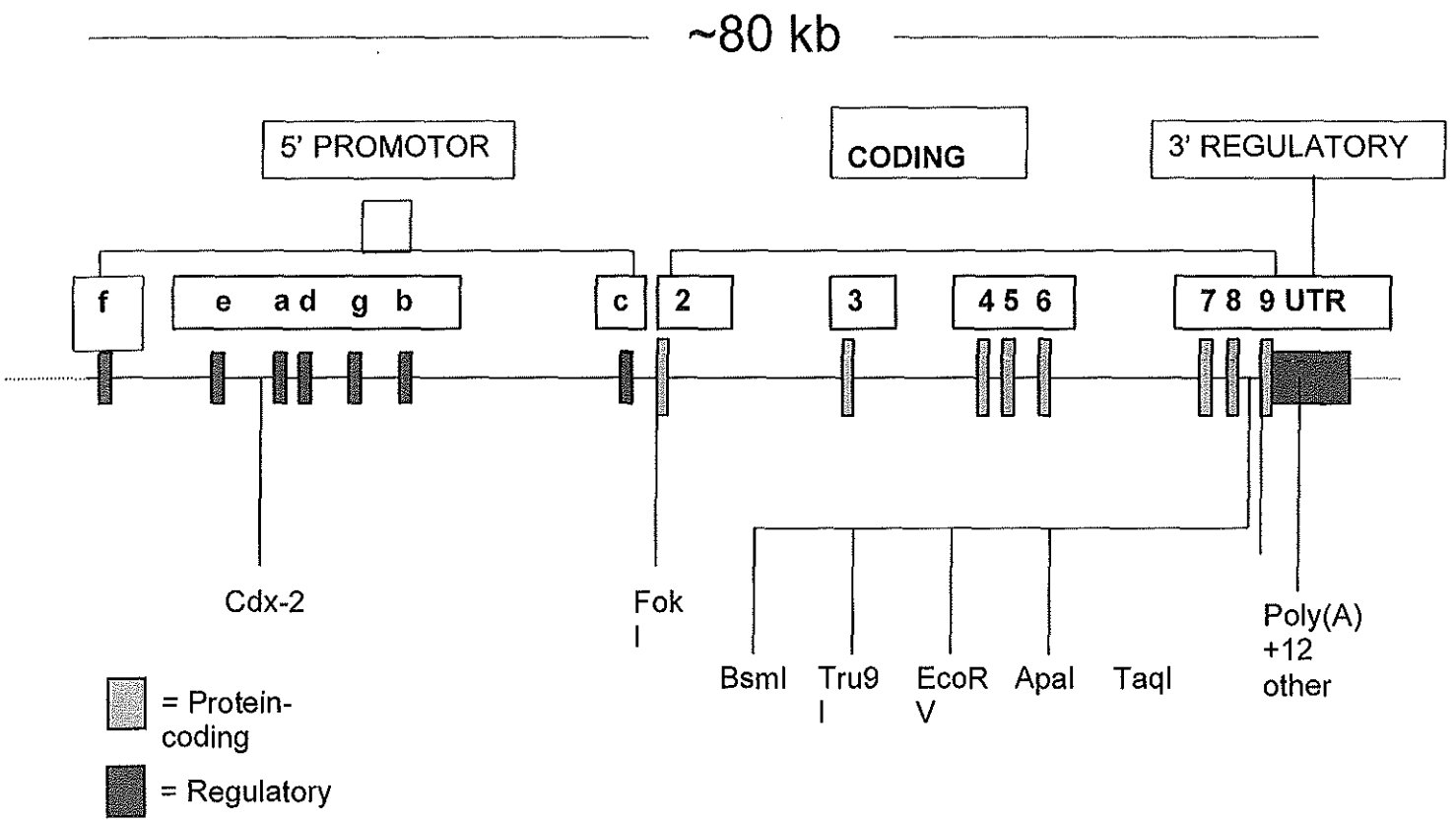
by 3 amino acids resulting in proteins of 427 (M1) and 424 (M4) amino acids. The shorter form was found to give greater transcriptional activation.[64,65] The presence of the 5'-ATG-site results in recognition sequence for the *FokI* restriction enzyme. The sequence change can be detected as a *Fok*I RFLP and the "f" allele (corresponding to M1) has been found associated with low BMD in some study populations,[64,66,67] but others did not.[68,69]

Although the identification of functional polymorphisms in the 3' end of the VDR gene is still eagerly awaited, several investigators have – nevertheless - analysed multiple bio-response parameters using the anonymous polymorphisms, including the *Bsm*I, and *Bsm-Apa-Taq* haplotypes. These studies include *in vitro* cell biological and molecular biological studies, and *in vivo* studies on measurements of biochemical markers and response to treatments with vitamin D, calcium, and even HRT or bisphosphonates. These studies have not shown one allele being consistently associated with all of the different parameters.[70-74]

The estrogen receptor gene polymorphism

Estrogens (E_2) and their receptors play an important role in bone metabolism. This is illustrated by the need for estrogen for initiation of adolescent growth and bone acquisition and by the bone loss induced by estrogen deficiency, occurring in the menopausal phase, with the subsequent increase in bone fragility.[75,76] Not only in women but also in men E_2 influences bone metabolism. This is nicely illustrated by the fact that a man lacking the aromatase cytochrome P_{450} enzyme also has a low BMD.[77] This enzyme was lacking due to a gene mutation and, therefore, androgens could not be converted into E_2. The ER gene codes for the estrogen receptor that binds E_2 and mediates the effect. The ER exists as two subtypes ERα and ERβ. In humans the ERα is located on chromosome 6q25-27 (Figure 1.5) and exists of eight exons and spans more than 400 kb.[78] ERβ is encoded on chromosome 14q22-24, comprises eight exons and spans approximately 40 kb.[79]

Figure 1.4 The vitamin D receptor gene locus and polymorphism



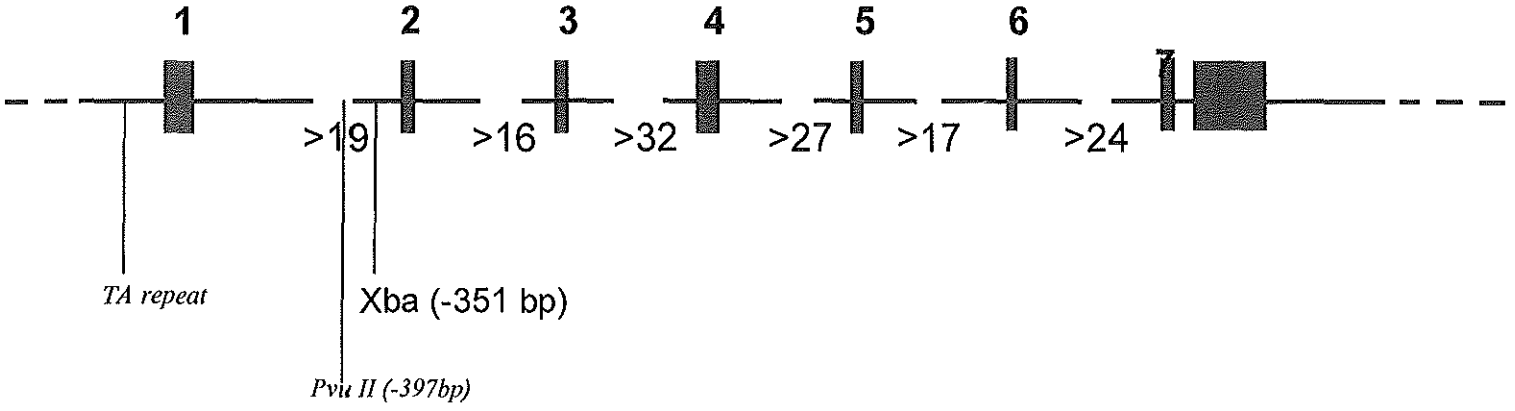
Several studies on the ERα polymorphisms in humans have recently been published. In the ERα gene a TA-repeat polymorphism has been identified in the promotor region 1174 bp upstream from the first exon.[80] This polymorphism has been found to be associated with several diseases including familiar premature ovarian failure and osteoporosis.[81] Interestingly, the variation in TA-repeats has been suggested to be associated with differences in expression levels of the ERα mRNA.[82] Although differences in mRNA levels are likely to be small and therefore difficult to measure in *in vitro* studies, over a lifetime they may have important consequences.

Two other polymorphisms in the ERα gene have been identified in the first intron, i.e. a T to C substitution 397 bp upstream from exon 2 (e2-397) and an A to G substitution 350 bp upstream from exon 2 (e2-347).[83] These polymorphisms have been shown to be associated with osteoporotic phenotypes and response to hormone replacement therapy but other studies could not confirm these results.[84-89] No functional studies have been performed yet to clarify how these intronic polymorphisms could effect the estrogen endocrine system.

The collagen type I α1 gene polymorphism

Mutations in the genes encoding collagen type Iα1 (COLIA1) and collagen type Iα2 cause the Mendelian disease Osteogenesis Imperfecta. In an early phase of the genetic dissection of osteoporosis these genes have already been considered as candidate genes for osteoporosis. While no frequent allelic variants could be found in the coding region of these genes,[90] Grant et al. found a G to T substitution in intron 1 of the COLIA1 gene (17q21) at a potential binding site for the transcription factor Sp1 (Figure 1.6).[91] The "T" allele was shown to have a population frequency of about 18% making this a polymorphism of potential functional significance. In an analysis of 205 predominantly postmenopausal British women Grant et al. reported decreased BMD for carriers of the T allele and an increased fracture risk.[91] In a larger cohort of 1,778 Dutch Caucasian elderly women from the Rotterdam Study the

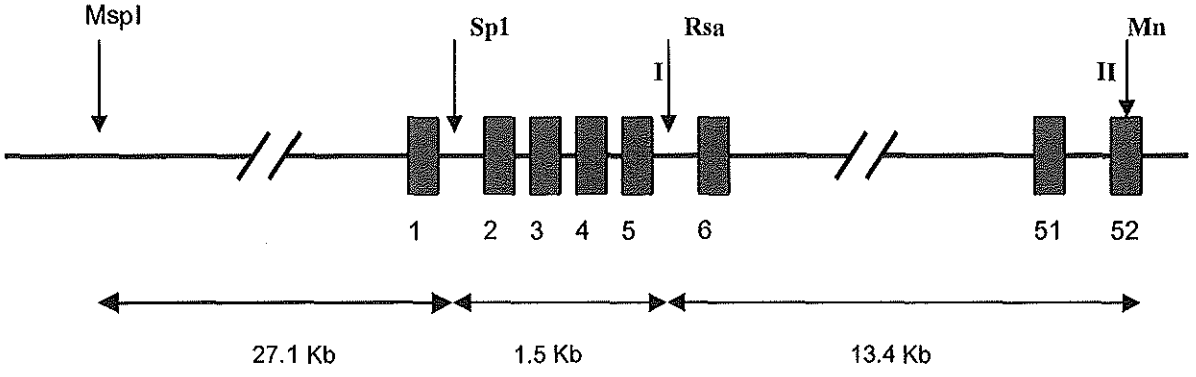
Figure 1.5 The Estrogen receptor α gene



associations of the T-allele with decreased BMD and increased fracture risk could be confirmed with evidence for a gene dose effect.[92] The COLIA1 genotype-dependent BMD differences in this large cohort of elderly women were found to increase with age, suggesting a relation with rates of bone loss. This notion is supported by the observation of increased rates of bone loss for subjects carrying the "T" allele, in a 5-year follow-up analysis of 243 American men and women of 65 years and older.[93] Although some studies have not been able to demonstrate a relationship between this polymorphism and aspects of osteoporosis,[94-97] the association of the "T" allele with decreased BMD and/or increased risk for fracture has been confirmed in several other studies.[98-100]

Importantly, there is evidence to suggest that the COLIA1 "T" allele has direct biological effects which could explain the observed associations. The putative Sp1 binding site containing the 'T' allele binds the Sp1 transcription factor protein two-fold stronger and causes a 3-fold higher COLIα1 mRNA level and increased COLIα1 protein expression levels.[101,102] In cultured osteoblasts such differences lead to altered COLIα1/COLIα2 protein ratios, comparable to what is seen for null mutations (allelic "knock-outs") in Osteogenesis Imperfecta patients but to a much milder extent. On the basis of these so-called null-mutations in OI patients, it can be speculated that an increased proportion of the homotrimers, such as could be the case in GT and TT subjects, would lead to a more fragile bone. This notion is strongly supported by the observation that the "T" allele was found associated with decreased bone strength in that the yield strength of bone taken from the femoral neck was about half in "GT" heterozygotes compared to that of "GG" homozygotes.[102] This explanation of the COLIA1 Sp1 genotype effect is further supported by what is seen in the *oim/oim* mouse. In this naturally occurring mutant mouse strain a COLIA1 homotrimer is produced due to a non-sense mutation in the COLIA2 gene. The phenotype of homozygous *oim* mice includes skeletal fractures, generalised osteopenia and small body size,[103] aspects of osteoporosis which are also observed in human TT homozygotes

Figure 1.6 Collagen type 1 α1 polymorphism



AIM OF THIS THESIS

Due to ageing, in the coming 50 years a 4-fold increase in the world-wide incidence of osteoporotic fractures has been expected.[5] To control and limit this exponential increase in fracture rate, over the last decade a growing number of studies have focused on prevention of osteoporosis. There has been a considerable increase in knowledge of incidence rates of osteoporosis and the role of the important quantitative measure of bone, i.e. BMD, in osteoporosis. However, most studies were performed in women only and focused on hip fractures. Therefore, in order to get a more precise insight in the magnitude of the problem we aimed to study the incidence rates of all fracture types and its relation with BMD in both genders. Furthermore, since only 70-80% of the bone fragility can be explained BMD. We investigated whether qualitative bone measures, i.e. biochemical markers of bone turnover, are associated with fractures.

Recently, both quantitative and qualitative aspects of bone have been found to be genetically determined but the genes involved are ill defined. Because lately more knowledge has become available on gene structures and, in particular, on the presence of genomic polymorphisms, it is possible to study the genetics of osteoporosis.

In view of these considerations we aimed to get more insight in skeletal and genetic determinants of osteoporosis not only in women, but also in men.

OUTLINE OF THE DISSERTATION

Since all studies have been performed within the Rotterdam Study, a short overview of this study is presented below. Firstly, we analysed the magnitude of osteoporosis by calculating the incidence rates of fractures in both men and women (Chapter 2.1), and we determined skeletal risk factors of osteoporosis such as BMD (Chapter 2.1) and the rate of bone resorption assessed by biochemical indices (Chapter 2.2). The third chapter covers the genetic studies performed. ERα polymorphism was found to be associated with age at menopause, as a determinant of osteoporosis, and with fractures as clinical endpoint of osteoporosis (chapter 3.1.1 and 3.1.2). Chapter 3.2.1 describes a pilot study conducted to investigate whether divergent rates of bone turnover among women with either a low or high BMD could be explained by genetic variations of the VDR. Furthermore, we examined whether VDR gene polymorphism differed in biochemical response to short-term substitution of 1,25-$(OH)_2D_3$ in

both BMD groups. In addition, hypotheses about functional consequences of the VDR were also studied *in vitro* (chapter 3.2.2). In chapter 3.3 we studied potential interactions between the VDR and ColIA1 gene variations in relation to fractures. Finally, chapter 4 comprises a general discussion about the results of the foregoing chapters. Some mechanisms that potentially underlie osteoporosis are considered and recommendations for future etiologic studies on osteoporosis are given.

SOURCE POPULATION

The research has been conducted as part of the Rotterdam study; a prospective population based study among elderly people living in Ommoord, a district of Rotterdam the Netherlands. The Rotterdam study focuses on the aetiology of chronic disabling diseases by investigating the incidence and determinants of, in particular, neurogeriatric, cardiovascular, locomotor and ophthalmologic diseases.[104] The prospective design of the Rotterdam study included a baseline visit, follow-up visits and during follow-up data on morbidity including non-vertebral fractures and mortality were captured.

All 10,275 men and women, aged 55 year and over, were invited for this study between August 1990 and June 1993. From those, 7,983 (3,105 men) agreed to participate, bringing the overall response rate to 78%. All participants were extensively examined during a home interview and during two visits at the clinical research centre. The baseline assessments concerning locomotor diseases included medical history (fractures during past five years), dietary history (calcium- and vitamin D intake), history of pharmacotherapy (drugs known to be influencing bone metabolism), physical examination (height, weight and body mass index), laboratory examination (overnight fastening urine and blood samples), BMD measurement was performed by dual energy X-ray absorptiometry (DXA, Lunar DPX-L densitometer) and radiographs of the spine were achieved.

A first re-examination took place between 1993-1994 and a second between 1997-1999. For this thesis we used only spinal radiographs of the second survey. All follow-up radiographs were scored for the presence of vertebral fracture by the McCloskey/Kanis method.[105] If a vertebral fracture was present, the baseline photo was scored as well, to ascertain whether a fracture was incident or prevalent.

Follow-up started either at 1st of January 1991 or, when later, at the time of inclusion into the study. For this analysis follow-up ended either at March 31, 1997, or, when earlier, at the participants' death. The general practitioners of the participants provided data on morbidity including non-vertebral fractures and mortality. For approximately 80 percent of the study population, medical events were reported through computerised general practitioner diagnosis registers. For the remaining 20%, research physicians collected data from the general practitioners' medical records of the study participants. All collected fractures were verified by reviewing discharge reports and letters from medical specialists. Fracture events were coded independently by two research physicians according to the International Classification of Diseases, 10th revision (ICD-10).[106] In case of discrepancy, consensus was attained in a separate session. A medical expert in the field reviewed all coded events for final classification. Data on all non-vertebral fractures were at the time of analysis available till 31st march 1997.

The Rotterdam Study has been approved by the Medical Ethics Committee of Erasmus University Medical School and participants provided written informed consent.

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2

Skeletal risk factors of osteoporosis

2.1

Fractures in elderly men and women: Incidence and association with bone mineral density

ABSTRACT

Most studies on osteoporosis focus on hip fracture and included women only. The objective of this study was to estimate the incidence of all non-vertebral fracture and its association with bone mineral density (BMD) in both men and women.

During 4.6 year of follow-up, 7,928 (3,086 men) individuals who participated in the Rotterdam Study, a population-based cohort study of individuals aged 55 years and over living in the Netherlands, were studied. Non-vertebral fracture incidence was 8.8 (95% confidence interval (CI) 7.4-10.5) per 1,000 person-years in men and 24.6 (95% CI 22.6-26.7) in women. In both genders, hip, wrist and upper humerus fractures were the most frequent fractures and most strongly related to BMD. The relative risk for hip fracture per SD decrease in BMD was 3.4 (2.1-5.3) and 2.3 (1.8-3.0), in men and women respectively. For wrist fracture those relative risks were 2.4 (1.1-5.0) and 1.5 (1.2-1.9) and for upper humerus fractures those were 2.3 (1.0-5.2) and 1.6 (1.1-2.3).

We conclude that among all non-vertebral fractures those of the hip, wrist and upper humerus are predominant and most strongly related to low bone mineral density not only in women, but also in men. We further conclude that bone mineral density is a similarly strong risk factor in men and women.

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INTRODUCTION

Among all types of fractures, those of the hip, vertebrae and wrist are usually considered as 'osteoporotic' fractures.[1-4] The lifetime risk for a 45 year old woman of any of these fractures was 47.3% and 23.8% in women and men, respectively.[5] Hip fractures in particular are associated with considerable morbidity, mortality and costs in the elderly. It was estimated that 20 % of hip fracture patients die within the first year after the hip fracture occurred.[6] A recent study on health care expenditures in the US estimated that out of US$ 13.8 billion for all types of fractures 62% could be attributed to hip fractures, but that another 38 % could be attributed to other types of fractures.[7] As the typical 'osteoporotic' fractures have been the focus of most epidemiological studies, the incidence of other fractures is largely unknown.

Already in 1842 Antley Cooper [8] noticed that the incidence of fractures increased with thinning of bone. Much later, several studies have shown that independent of age, the likelihood of a fracture increases with decreasing bone mineral density (BMD).[9-11] Again, the majority of these studies were on 'osteoporotic' fractures and only included women. Consequently, the relation between BMD and non-osteoporotic fractures is largely unknown. Furthermore, these studies did not compare the risk of fractures between men and women.

The objective of this study is to describe the incidence of non-vertebral fractures in both men and women according to age, fracture site, and BMD.

MATERIAL AND METHODS

Study population

The Rotterdam Study is a prospective cohort study of the occurrence and determinants of disease and disability in the elderly. Rationale and study design have been described previously.[12] All 10,275 men and women, aged 55 year and over living in Ommoord, a district of Rotterdam the Netherlands, were invited for this study between August 1990 and June 1993. From those, 7,983 agreed to participate, bringing the overall response rate to 78%. The baseline assessments included the measurement of femoral neck BMD (expressed in g/cm^2), which was performed by dual energy X-ray absorptiometry (DXA, Lunar DPX-L densitometer) as described previously.[13] The Rotterdam Study has been approved by the

Medical Ethics Committee of Erasmus University Medical School and participants provided written informed consent.

Follow-up

Follow-up started either on 1st of January 1991 or, when later, at the time of inclusion into the study. For this analysis follow-up ended either at death or on March 31, 1997. The general practitioners of the participants provided data on morbidity including non-vertebral fractures and on mortality. For approximately 80 percent of the study population, medical events were reported through computerized general practitioner diagnosis registers. For the other participants this information was obtained by regularly checking the general practitioners' medical records. Two independent research physicians verified all reported fractures. In case of discrepancy, they had to achieve consensus. A third investigator finally approved the classification.

Statistics

All analyses were performed for the total group and for men and women separately. Incidence rates of fractures were calculated according to fracture site and subdivided in three main categories: upper extremity, lower extremity and other. The incidence rates were expressed as numbers of fractures per 1,000 person-years and 95 % confidence intervals (95% CI) were calculated using the exact Poisson formula. The incidence of fractures was also studied in 5-year age groups and the most frequent fractures were further analyzed and compared to age-related incidence rates of recent published population-based studies.[14,15] To determine which fractures were associated with low BMD, we estimated the relative risk for a first fracture of each type per standard deviation (SD) decrease in BMD using Cox' proportional hazards model. These analyses were based on first fracture in individuals because multiple fractures do not contribute to independent observations. To account for confounding by age we included age as a continuous variable in all models. To examine whether the association of fractures with BMD differed between men and women, we tested for significance of the interaction between BMD and gender. Fractures that were most strongly related to low BMD were additionally studied by age-adjusted quartiles of BMD.

RESULTS

Since 55 individuals died before 1st of January 1991, the starting point of the follow-up period, we excluded them from this study. Therefore, complete follow-up was achieved for 7,928 individuals (3,086 men (38.9%)) with a mean follow-up time of 4.6 years (range 0.01-6.25 years). Table 1 shows the baseline characteristics of the study population. Women were older and had lower mean BMD compared to men.

Table 1 Baseline characteristics by gender; the Rotterdam Study 1991-1997

Characteristics	Men	Women	Total
Number	3086 (38.9)	4842 (61.1)	7928 (100)
Age (years)	68.9 (8.6)	71.6 (10.2)	70.5 (9.7)
Age-groups			
50-59	508 (16.5)	708 (14)	1216 (15.3)
60-64	647 (21.0)	834 (18)	1481 (18.7)
65-69	642 (20.8)	760 (17)	1402 (17.7)
70-74	526 (17.0)	766 (17)	1292 (16.3)
75-79	389 (12.6)	641 (14)	1030 (13.0)
80-84	225 (7.3)	508 (10)	733 (9.8)
85+	149 (4.8)	625 (11)	774 (9.8)
Number with BMD measurement	2446 (79.3)	3376 (69.7)	5822 (73.4)
Femoral neck BMD (g/cm^2)	0.88 (0.14)	0.81 (0.13)	0.84 (0.14)

Values are expressed as numbers (percent) or means (SD)

Incidence of non-vertebral fractures

During the follow-up period a total of 676 non-vertebral fractures were reported within 585 (7.4 %) participants. Of those participants, 61 suffered two fractures, 11 had three fractures, one had four fractures and one participant five fractures. In total there were 36,450 person-years of follow-up (13.955 person-years in men).

Table 2 shows the fracture incidence rates by site and gender. Overall, the incidence of fractures was 8.8 (95% CI 7.4-10.5) per 1.000 person-years in men and 24.6 (22.6-26.7) in women. At each fracture site women had a higher incidence and, overall, the age-adjusted

Table 2. Gender- and site-specific incidence rates of non-vertebral fractures per 1000 person-years; the Rotterdam Study 1991-1997

	Men			Women			Total		
Type of fracture	**n**	**Incidence**	**95% CI**	**n**	**Incidence**	**95% CI**	**n**	**Incidence**	**95% CI**
Upper extremities									
Upper humerus	12	0.9	0.5 - 1.5	49	2.2	1.7 - 2.9	61	1.7	1.3 - 2.2
Wrist	9	0.6	0.3 -1.2	124	5.5	4.6 - 6.6	133	3.7	3.1 - 4.3
Hand	10	0.7	0.4 - 1.3	29	1.3	0.9 - 1.9	39	1.1	0.8 - 1.5
Other forearm[1]	5	0.4	0.2 - 0.9	35	1.6	1.1 - 2.2	40	1.1	0.8 - 1.5
Other upperarm[2]	7	0.5	0.2 - 1.1	24	1.1	0.7 - 1.6	31	0.9	0.6 - 1.2
Lower extremities									
Pelvis	4	0.3	0.1 - 0.8	20	0.9	0.6 - 1.4	24	0.7	0.4 - 1.0
Hip	39	2.8	2.0 - 3.8	132	5.9	5.0 - 7.0	171	4.7	4.0 - 5.4
Ankle	3	0.2	0.1 - 0.7	25	1.1	0.6 - 1.6	28	0.8	0.5 - 1.1
Foot	10	0.7	0.4 - 1.3	39	1.7	1.3 - 2.4	49	1.3	1.0 - 1.8
Other lower leg[3]	1	0.1	0.0 - 0.5	26	1.2	0.8 - 1.7	27	0.7	0.5 - 1.1
Other upper leg[4]	1	0.1	0.0 - 0.5	4	0.2	0.1 - 0.5	5	0.1	0.1 - 0.3
Other[5]	22	1.6	1.0 - 2.4	46	2.0	1.5 - 2.7	68	1.9	1.5 - 2.4
All non-vertebral fractures	123	8.8	7.4 -10.5	553	24.6	22.6 - 26.7	676	18.6	17.2 - 20.0

[1] Other forearm fractures include; fractures of the other parts of the radius and ulna
[2] Other upperarm fractures include; fractures of the scapula, clavicle and humerus shaft
[3] Other lower leg fractures include; fractures of the other parts of the tibia and fibula
[4] Other upper leg fractures include; fractures of the other parts of the femur and patella
[5] Other fractures include; fractures of the skull,face, ribs and sternum

incidence was 2.1 times (1.7-3.6) higher. In both men and women the predominant fracture sites were the hip, wrist and upper humerus.

Figure 1a and 1b show the age-related incidence of all non-vertebral, hip, wrist and upper humerus fractures in men and women separately. In both genders the incidence of hip fracture increased exponentially with age. For upper humerus fractures, a moderate increase with age was observed in women but no relation with age was found in men. In women, the incidence of wrist fracture increased markedly after the age of 55 with a peak incidence between the ages of 65 and 75. Thereafter the incidence remained stable. In men, the wrist fracture incidence did not change with age. Of all other types, only fractures of the pelvis and 'other' fractures showed an increasing incidence in elderly women (table 3). The age-related incidence rates of the most frequent types of fractures approximate those published by other population-based studies (fig 1a-b).[14,15]

Table 3 Fracture site-specific Incidence rates per 1000 person-years according to age and gender; the Rotterdam Study 1991-1997

		Age groups						
	Gender	**55-59**	**60-64**	**65-69**	**70-74**	**75-79**	**80-85**	**85+**
Hand	**M**	0.85	0.73	0.96	1.09	0.48	0.78	1.27
	W	1.82	1.60	1.54	1.52	0.86	1.10	.66
Other upper arm	**M**	0.00	0.36	0.00	0.36	0.96	0.00	3.82
	W	0.00	0.53	0.77	0.51	1.14	1.47	2.96
Other forearm	**M**	0.00	0.36	0.64	0.36	0.00	0.00	1.27
	W	0.00	1.07	2.05	1.78	1.43	1.84	1.97
Pelvis	**M**	0.00	0.00	0.00	0.00	0.96	0.78	1.27
	W	0.00	0.00	0.77	0.25	0.29	1.47	3.62
Ankle	**M**	0.00	0.36	0.00	0.00	0.48	0.78	0.00
	W	0.61	0.80	2.31	1.52	0.86	0.37	0.66
Foot	**M**	0.00	0.73	1.27	0.73	0.48	0.78	0.00
	W	1.82	2.14	2.05	1.52	2.00	1.10	1.31
Other upper leg	**M**	0.00	0.00	0.00	0.00	0.00	0.78	0.00
	W	0.00	0.00	0.00	0.25	0.29	0.37	0.33
Other low leg	**M**	0.00	0.00	0.00	0.36	0.00	0.00	0.00
	W	0.61	1.07	0.77	1.52	1.14	1.84	0.99
Other	**M**	1.70	0.36	2.23	1.45	1.44	1.57	3.82
	W	0.00	0.53	1.54	2.29	2.85	2.94	3.62

W = women M = men

Figure 1a Age-related Incidence of site specific fractures per 1000 person-years according to study in men

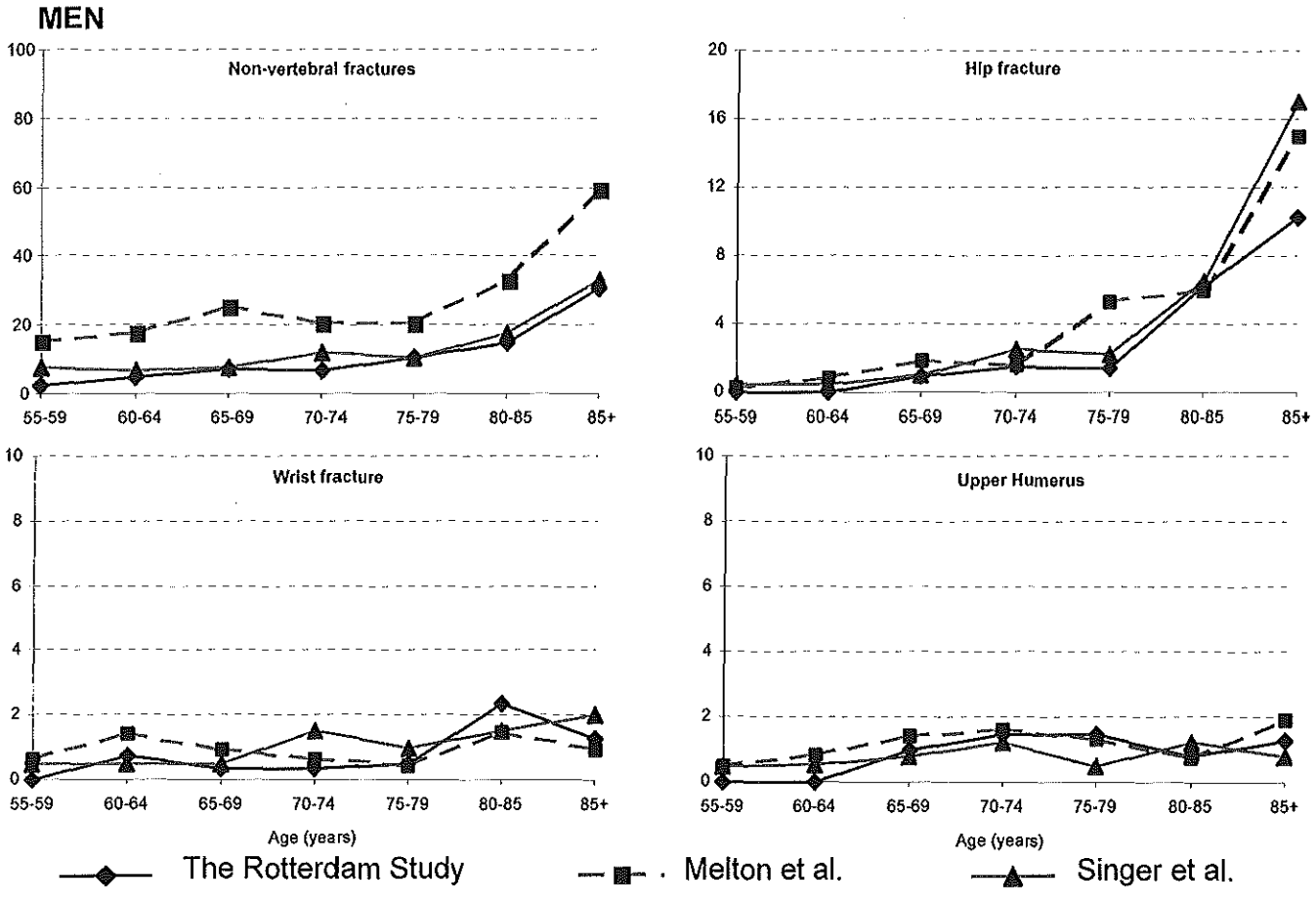
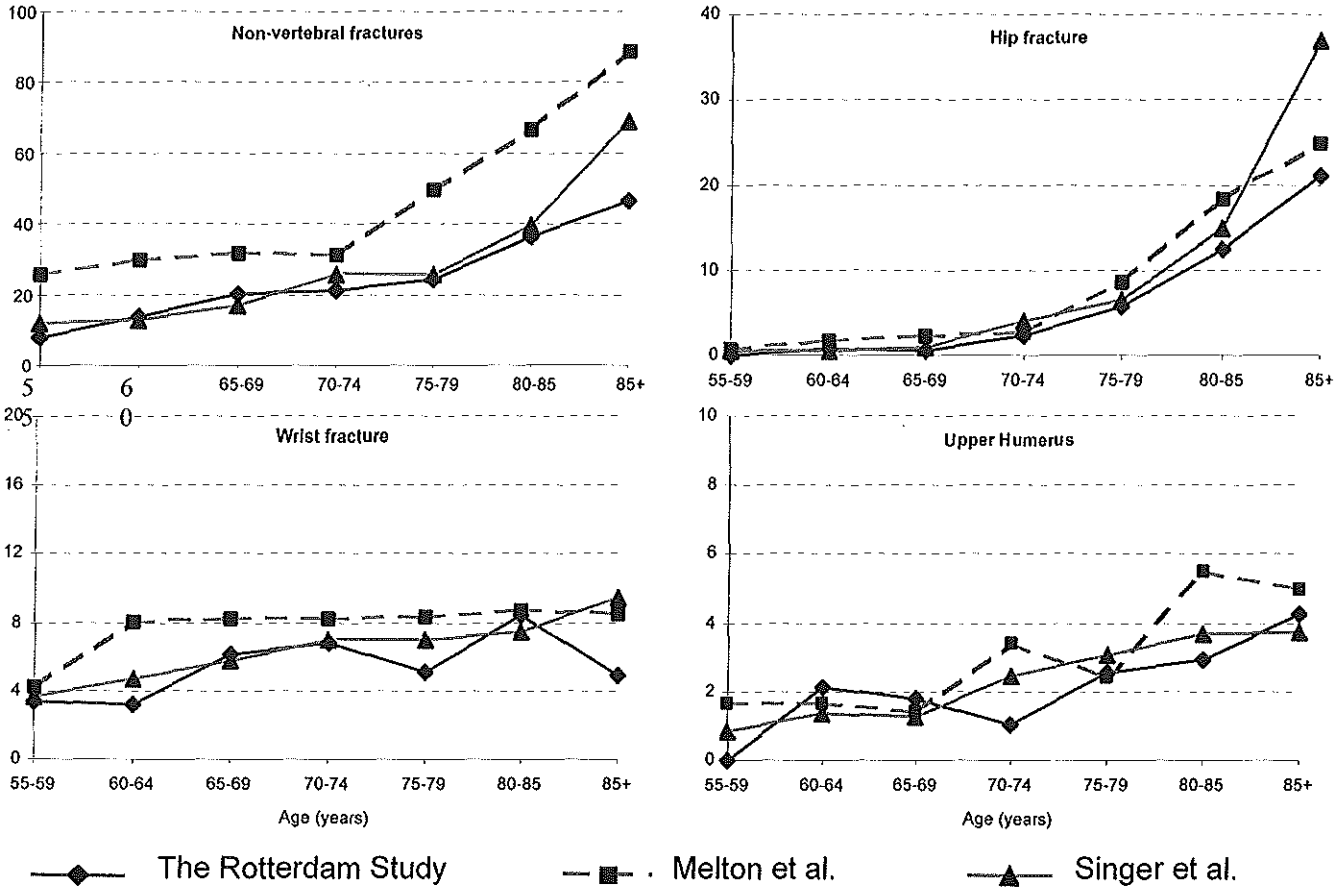


Figure 1b Age-related Incidence of site specific fractures per 1000 person-years according to study in women

WOMEN



Relation of BMD with non-vertebral fractures

BMD measurement was performed in 5,822 individuals who suffered 401 fractures during follow-up. In these younger (mean age was 68.0 vs. 77.6 years for the subgroup and individuals without BMD measurement respectively) independently living individuals, the incidence of first non-vertebral fracture was significant lower compared to individuals without BMD measurement (14.3 vs. 21.7 per 1,000 person-years, respectively). For hip fractures these were 3.2 vs. 8.8 per 1,000 person-years, respectively.

Table 4. Age-adjusted Hazard ratios for the predictive value of femoral neck BMD (g/cm^2) of non-vertebral fractures (95% CI)§; the Rotterdam Study, 1991-1997

Type of fracture	Men	Women	Total ‡	p-value †
Upper extremities				
Upper humerus	2.3 (1.0 - 5.2)	1.6 (1.1 - 2.3)	1.8 (1.2 - 2.5)	0.4
Colles	2.4 (1.1 - 5.0)	1.5 (1.2 - 1.9)	1.6 (1.3 - 2.1)	0.3
Hand	1.3 (0.6 - 2.7)	1.3 (0.9 - 2.0)	1.4 (0.9 - 2.0)	0.9
Other forearm	2.1 (0.7 - 6.1)	1.2 (0.8 - 1.9)	1.3 (0.9 - 2.1)	0.3
Other Upper arm	1.3 (0.5 - 3.1)	1.6 (0.8 - 2.9)	1.5 (0.9 - 2.5)	0.7
Lower extremities				
Pelvis	1.4 (0.4 - 5.2)	1.1 (0.4 - 2.7)	1.2 (0.5 - 2.5)	0.8
Hip	3.4 (2.1 - 5.3)	2.3 (1.8 - 3.0)	2.7 (2.1 - 3.4)	0.3
Ankle	0.6 (0.2 - 1.6)	0.9 (0.6 - 1.5)	0.9 (0.6 - 1.3)	0.5
Foot	1.6 (0.8 - 3.2)	1.3 (0.9 - 1.9)	1.3 (1.0 - 2.0)	0.8
Other upper leg	-	2.2 (0.5 - 9.6)	2.3 (0.5 - 11.4)	-
Other lower leg	14 (0.4 – 52.7)	1.9 (1.2 - 3.2)	2.2 (1.3 - 3.7)	0.3
Other	1.1 (0.7 - 2.1)	1.0 (0.7 - 1.5)	1.1 (0.8 - 1.5)	0.7
All non-vertebral	**1.7 (1.3 - 2.1)**	**1.4 (1.3 - 1.6)**	**1.5 (1.3 - 2.7)**	**0.4**

§ Site- and gender-specific hazard ratios (95% CI) per SD decrease of bone mineral density (BMD; 1 SD Total = 0.14 g/cm^2, Men = 0.14 g/cm^2, Women = 0.13 g/cm^2)
‡ Hazard ratios adjusted for age and gender
† p-value for age adjusted gender difference in the hazard function (interaction term)

The relative risks for site specific fractures per SD decrease in femoral neck BMD are shown in table 4. For all non-vertebral fractures combined, we observed a relative risk of 1.7

(1.3-2.1) per SD decrease in BMD in men and 1.4 (1.3-1.6) in women. Fractures of the hip, wrist, and upper humerus were most strongly related to low BMD, in both genders. Although for most fracture sites the overall relation with BMD was stronger in men than it was in women, this was not statistically significant.

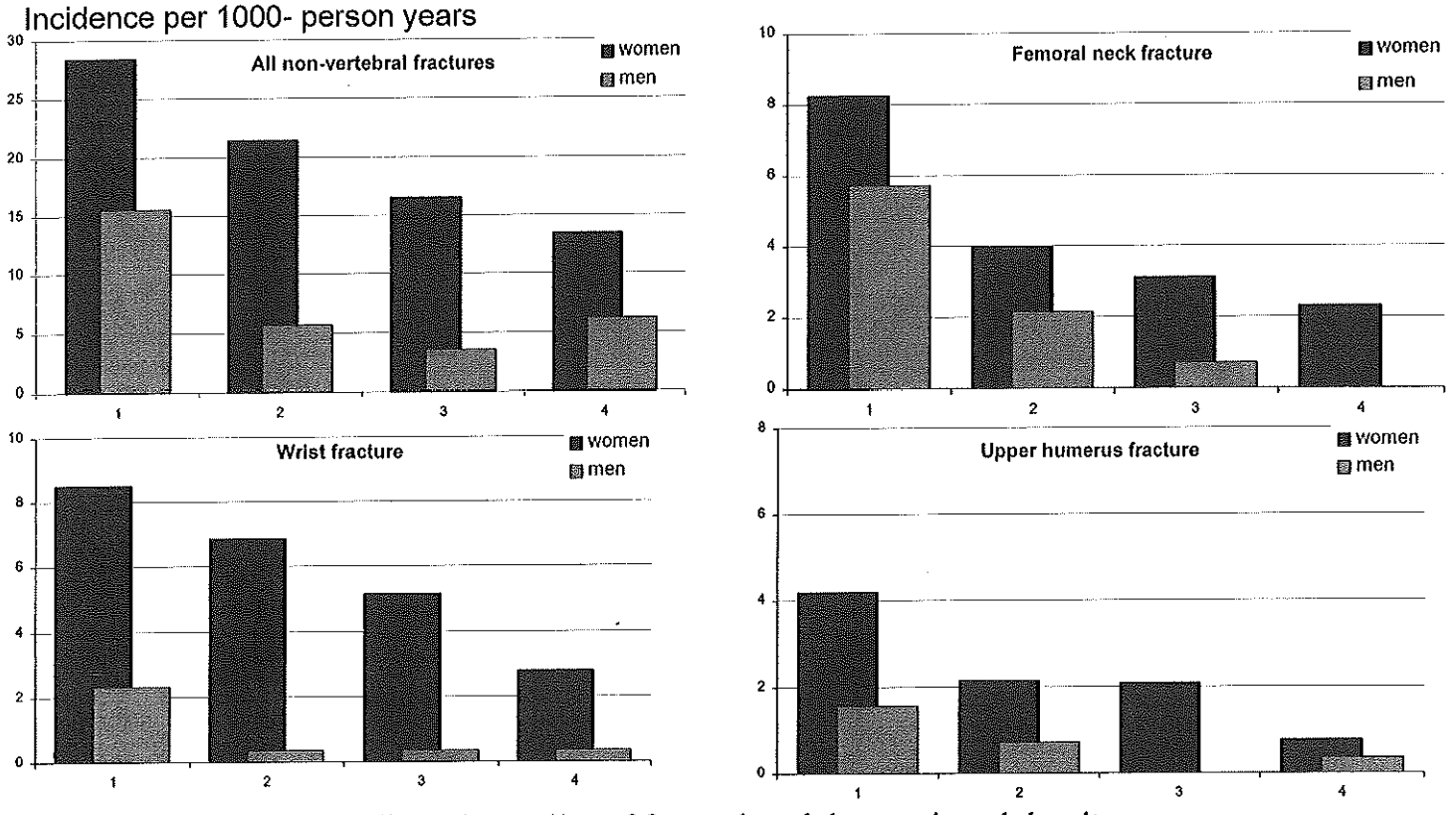
Figure 2 shows the incidence rates by age-adjusted quartiles of BMD for all non-vertebral fractures combined and for the three most common types. We observed a similar pattern of decreasing fracture incidence with age-adjusted quartiles of BMD in both men and women.

DISCUSSION

In this population-based prospective study of fractures, the most frequent non-vertebral fractures are those of the hip, wrist and upper humerus in both men and women. The risk of all non-vertebral fractures, but particularly for those of the hip, wrist and upper humerus increased with decreasing BMD. Although there appeared to be a tendency towards higher relative risk estimates in men, this was not statistically significant.

This study is, to the best of our knowledge, the first prospective study that describes the incidence of all non-vertebral fracture types and its relation to BMD in men and women from the same population-based cohort. We did not exclude fractures that resulted from high-energy trauma, thereby providing a valid estimate of the incidence fractures. We believe case ascertainment to be good, since in the Netherlands the GP is the gatekeeper of the healthcare system. Therefore, the GP record is the central repository of the medical information about a patient. Selective non-response of subjects with impaired mobility, or otherwise at an increased risk for fractures, may have occurred at baseline. Such non-response bias could decrease overall incidence rates. This is also suggested by the fact that the number of hip fractures we observed was 17 % lower than the number expected on the basis of nation-wide registers in the Netherlands.[16] This selective non-response might become more severe with age, resulting in an underestimation of the relation of fracture incidence with age. It is unlikely, however, that this would affect the age-adjusted relation between BMD and fractures.

Figure 2. Incidence of first fracture by age-adjusted quartiles of femoral neck bone mineral density in men and women



Only few previous population-based studies have assessed incidence rates of fractures at all skeletal sites, in both genders.[14,15] With regard to the overall incidence of fractures we confirm the estimates observed in the other Western-European study whereas we observed a lower incidence compared to the North American study.[14,15]. Although comparison of absolute incidence rates of fracture is complicated due to differences in age, gender, general health and fracture ascertainment,[17-20] the observed age-related incidence rates of the most common type of fractures, i.e. hip, upper humerus and wrist fractures, approximated those observed in both studies mentioned. Discrepancies between the present study and the North American study were marked for fractures of the hand, ankle, foot, rib and pelvis.[14] The present study represented only Caucasians whereas the North American study represented also non-white residents. It is unlikely however that the small proportion of non-whites explains the discrepancy in incidence rates.

The finding that the incidence of all non-vertebral fractures combined is inversely related to BMD is concordant with previous studies.[9-11,21-25] For women we show that fractures of the upper humerus, wrist and hip, as separate groups, have the strongest relation with low BMD, again in agreement with data reported previously.[9] We observed the highest estimates for hip fractures and this is probably due to the site (femoral neck) where we have measured BMD. It has been shown that the prediction of hip fractures is more accurate at the site where BMD is measured.[10] As was suggested by data from the Dubbo study,[9] we conclude that also in men the risk of hip, upper humerus and wrist fractures increases with decreasing BMD. In men, we found a slightly stronger relation between femoral neck BMD at most fracture sites compared to women, which was not statistically significant. However, if there were indeed a stronger relation in men this could not be explained by difference in bone volume since a higher bone volume is protective and therefore adjustment for bone volume should increase the risk difference between men and women. Nevertheless, the apparent increased risk in men might be explained by residual confounding: by using BMD we indirectly include other risk confounding factors for fractures such as alcohol, smoking and co-morbidity.[26] Those factors might have a higher prevalence in men than in women, and should therefore influences the risk of fractures in men more than in women.

We conclude that among non-vertebral fractures those of the hip, wrist and upper humerus occur most frequently and are strongly related to low BMD, in both elderly men and women. We further conclude that BMD is an equally important risk factor in men and women.

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2.2

Bone Resorption Rate as an Independent Risk Factor of Fractures in Postmenopausal Women; the Rotterdam Study 1991-1996

Abstract

Increased bone resorption rate predicts the risk of hip fractures, independent of bone mass. Whether this observation holds for other non-vertebral fractures is unknown. To determine the relationship between urinary free deoxypyridinoline crosslinks (fDPD) and non-vertebral fractures we conducted a case-control study within the Rotterdam Study.

The Rotterdam Study is a population-based cohort of 7983 individuals, aged 55 years and over, living in Rotterdam, the Netherlands, who were followed up for a mean period of 4.0 year (SD 0.8 year), from 1991 through 1996. Cases were 207 independently living women having a non-vertebral fracture during follow-up. Age-matched controls (n=220) were selected randomly from all independently living women without incident fractures. Overnight fastening urinary fDPD concentration corrected for creatinine was assessed by automated immunoassays.

Baseline fDPD concentrations above 4.5 nmol/mmol (equals the premenopausal mean) increased the risk of non-vertebral fractures, especially the hip and upper humerus fracture, 1.2 (95% confidence interval [CI] 1.0-2.2), 5.2 (95% CI 1.8-15.1) and 4.0 (95% CI 1.1-13.7) times, respectively. Adjustment for age, bone mass, previous fractures and disability, did not essentially change the estimates. Having both risk factors low bone mass (T-score $\leq$ -2.5) and high bone resorption rate (above premenopausal mean), increased the hip and upper humerus fracture risk 5.6 (95% CI 2.5-10.0) and 1.8 (95% CI 0.7-5.3) times, respectively.

We conclude that an increased bone resorption rate was independently associated with an increased risk of particularly hip and upper humerus fractures. Combining bone mass and bone resorption rate provides an even higher risk estimate.

Acknowledgement

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INTRODUCTION

Osteoporotic fractures are an important threat to general health because they induce a decreased mobility and increased mortality in the elderly,[1, 2] ultimately leading to increased cost for public health in Western Countries.[3, 4] The underlying pathogenic mechanism causing osteoporosis might be explained by an imbalance between bone formation and resorption, favoring bone resorption. In 1993, Akesson et al. showed that high urine concentrations of bone resorption markers were associated with an increased risk of hip fractures.[5] However, markers were measured after a fracture had occurred, which might reflect fracture-induced changes. Prospective studies were published by van Daele et al. and Garnero et al., who demonstrated an association between high levels of bone resorption and an increased risk of hip fractures, independent of bone mineral density (BMD).[6, 7] However, sofar all studies focused on hip fractures only. Prospective data on the association between bone resorption rate and other types of non-vertebral fractures are lacking and therefore it is not clear whether the association between bone turnover and fractures is a more general phenomenon.

To assess whether urinary fDPD, a specific marker of bone resorption,[8, 9] is associated with the risk of non-vertebral fractures, we conducted a nested case-control study within a large population-based cohort.

METHODS

Subjects

The Rotterdam Study, which was the source population, is a prospective cohort study including individuals aged 55 years or over. Its objective is to investigate the incidence of, and risk factors for, chronic disabling diseases. Rationale and study design have been described previously.[10] The focus of the Rotterdam Study is on neurologic, cardiovascular, ophthalmologic and locomotor diseases. All 10,275 inhabitants of a district in Rotterdam, the Netherlands, were invited for baseline examination between August 1990 and June 1993. Of

those, 7,983 participated, bringing the overall response rate to 78 percent. The Rotterdam Study has been approved by the Medical Ethics Committee of Erasmus University Medical School.

Study Population; Non-vertebral Fracture Cases and Controls

Follow-up started at the time of inclusion into the study from 1st of January 1991 onwards. For this analysis follow-up ended either at February 29, 1996, or, when earlier, at the time a specific fracture type occurred. The general practitioners of the participants provided data on morbidity, including non-vertebral fractures. For approximately 80 % of the study population, medical events were reported through computerized general practitioner diagnosis registers. For the other participants this information was obtained by regularly checking the general practitioners' medical records of the study participants.

All reported fractures were verified by reviewing the medical records of the participant. Two independent investigators classified all fractures and achieved consensus, which was approved by a third independent investigator. During the follow-up period, 467 non-vertebral fractures were reported. Fractures classified by the investigators as 'possible' (n= 42, 9.0 %) as well as pathologic fractures (n=6, 1.3%) were excluded leaving 419 definite non-vertebral fractures within 376 participants. For this study however, we included women only (n=317, 84.0 %). Urine samples were available for 207 (65.3 %) independently living women having an incident non-vertebral fracture.

Selection of controls was performed by matching each non-vertebral fracture case with one independently living women without a fracture during follow-up from the same cohort according to age (within the same 5-years age stratum as the case).

Baseline Measurement

Bone resorption was evaluated by the measurement of free DPD in overnight fasting urine. Urine samples were collected at baseline and stored until measurement at -20° C. Urinary free DPD was determined by an automated chemiluminescence immunoassay (ACS: 180 DPD, Chiron Diagnostics, USA).[8, 9] To correct for dilution, results were normalized against urinary creatinine and expressed as nmol DPD per mmol urinary creatinin (nmol/mmol).

Inter- and Intra assay coefficients of variation for the entire method were 6.4 and 3.1% respectively.

At baseline, measurement of femoral neck BMD (expressed in g/cm^2) was performed by dual energy X-ray absorptiometry (DXA, Lunar DPX-L densitometer) as described previously.[11] Disability was assessed by means of the Disability Index of the Stanford Health Assessment Questionnaire as described previously.[12, 13] The lower limb disability index was composed of the mean score (0 indicating no impairment, 3 indicating unable to perform) for six components questions on arising, walking, bending and getting in and out of a car. Previous fragility fractures were defined as a history of non-vertebral fractures within the previous 5-year before baseline examination.

Statistical Analysis

To increase power we used in case of analysis of a specific type of non-vertebral fracture all controls, for example in case of all 39 hip fracture cases we used all 220 controls in the analysis.

To test for significant differences in baseline characteristics and urinary fDPD concentrations between cases and controls, student-t test and Chi-square tests were used. Linear regression models were constructed to examine the association between fDPD, age and femoral neck BMD. Logistic regression was performed to estimate the association (expressed as odds ratio (OR)) between urinary fDPD and the risk of the first type-specific fracture occurred. A concentration of fDPD was entered into the model as a continuous or categorical variable. To adjust for potential confounders like age, BMD, lower limb disability and previous fractures we performed additional regression models. In order to explore the possible association between fDPD levels and fracture risk during follow-up, we used the Cox proportional hazard function in a sub-analysis. Furthermore, we compared the fDPD levels of the postmenopausal women of the study population with the mean value of 28 premenopausal women in which overnight fastening urine samples were captured and fDPD levels measured by the same protocol. In addition, we calculated the predictive value of having two risk factors, namely low bone mineral density, defined as 2.5 SD below the premenopausal mean

of Dutch women[14] and high bone resorption (above the premenopausal mean) against, one or none of these risk factors in the prediction of fractures.

RESULTS

During a mean follow-up time of 4.0 (SD 0.8) year, 207 women had a total of 223 non-vertebral fractures. Of those, 11 suffered from two fracture types, one from three and another one had four different types of fracture. Table 1 shows type and number of fractures reported. Most common were fractures of the wrist (n=75, 33.6%), hip (n=39, 17.5%) and upper humerus (n=23, 10,3%).

Table 1. Types and Numbers of Fractures

Types of fracture	Number (%)
Upper extremities	
Upper humerus	23 (10.3)
Wrist	75 (33.6)
Hand	13 (5.8)
Other[1]	8 (3.6)
Lower extremities	
Pelvis	6 (2.7)
Hip	39 (17.5)
Ankle	14 (6.3)
Foot	20 (9.0)
Other[2]	16 (7.2)
Other[3]	9 (4.0)
All fractures	223 (100)

[1]Other upper extremity fractures include; fractures of the scapula, clavicle and humerus shaft
[2]Other lower extremity fractures include; fractures of the other parts of the femur, patella, tibia and fibula
[3]Other fractures include; fractures of the skull, face, ribs and sternum

Baseline Characteristics

Women with a non-vertebral fracture did not significantly differ from the controls in age (due to matching), age at menopause, weight, height, lower limb disability and history of fractures (Table 2). However, when the case group was subdivided by site of fracture, a difference in age, history of fractures and lower limb disability was observed for certain types of fracture. Women with a hip fracture were significant older, had more previous fractures and showed a higher lower limb disability, whereas subjects with wrist fractures were younger and the disability index was even better compared to controls. Furthermore, as expected, bone mineral density measured at the femoral neck was significantly lower in the fracture cases, especially for women with hip and upper humerus fractures.

Bone Resorption Rate

Mean levels of urinary fDPD for premenopausal women, controls and fracture cases (according to type of fracture) are shown in Table 3. Postmenopausal women (mean of cases and controls combined) had a significant ($p < 0.05$) higher urinary concentration of fDPD (5.7 ± 2.9 nmol/mmol) compared to premenopausal women (4.4 ± 1.3 nmol/mmol). Women who suffered a hip or upper humerus fracture tend to have a higher mean level of urinary fDPD excretion compared to controls, whereas women suffering a wrist fracture had lower urinary concentration of the bone resorption marker, although not statistically significant. For other and all types of non-vertebral fractures combined there was no difference in urinary fDPD concentration compared to controls.

Table 2. Baseline characteristics of the study population by cases (all and according to type of fracture) and controls

	Controls	Cases	Hip	Wrist	Upper humerus	Other types
Number	220	207	39	75	23	75
Age (year)	71.3 ± 8.4	70.8 ± 8.4	78.5 ± 7.2*	69.2 ± 6.9*	71.8 ± 9.3	68.1 ± 7.7
Age at Menopause (year)	49.2 ± 4.2	49.5 ± 4.3	49.9 ± 4.6	49.3 ± 3.9	49.7 ± 4.7	49.3 ± 4.7
Height (cm)	160.9 ± 7.8	162.0 ± 7.1	160.7 ± 9.1	162.2 ± 6.8	160.3 ± 6.1	162.9 ± 6.7
Weight (kg)	69.2 ± 10.7	69.1 ± 11.2	65.9 ± 10.6	67.3 ± 10.8	68.7 ± 10.0	72.4 ± 11.5*
Previous fractures	17 (7.7)	26 (12.6)	7 (17.9)*	9 (12.0)	4 (17.4)	6 (8.0)
Lower limb disability	0.44 ± 05.7	0.45 ± 0.57	0.81 ± 0.67*	0.32 ± 0.49	0.40 ± 0.62	0.39 ± 0.49
BMD measurement available	200	185	34	68	22	66
FN-BMD (gr/cm^2)	0.80 ± 0.13	0.76 ± 0.14*	0.66 ± 0.14*	0.77 ± 0.13	0.74 ± 0.10*	0.81 ± 0.13

Values are mean ± SD or number (%)
*Case vs. control P-value < 0.05

Table 3 Urinary concentrations of f DPD / Creatinine (nmol/mmol) for premenopausal women, cases (according to fracture site) and controls

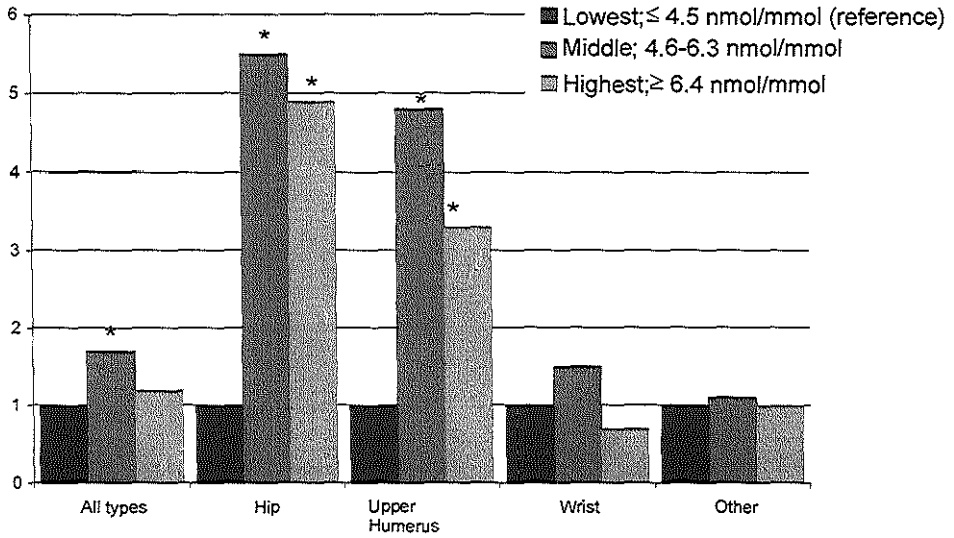
	n	FDPD / Creatinine
Premenopausal women	28	4.4 ± 1.3
Controls	220	5.7 ± 3.1
Fracture cases		
Hip	39	6.3 ± 2.0
Wrist	75	5.3 ± 2.6
Upper humerus	23	6.2 ± 1.8
All other types	75	5.7 ± 2.8
All non-vertebral	207	5.8 ± 2.5

Values are mean ± SD

Fracture Risk

When urinary fDPD levels were used as a continuous variable in the analysis to further explore the relationship between fDPD and different types of fractures, no significant association was found for any type of fracture. However, when the data were categorized by tertiles of urinary fDPD (lowest tertile ≤ 4.5 nmol/mmol, middle tertile 4.6-6.3 nmol/mmol and highest tertile ≥ 6.4 nmol/mmol), a significant association with specific types of fractures was observed (fig 1). Women with urinary levels of fDPD in the middle and highest tertiles were at increased risk for hip fractures compared to having levels in the lowest tertile with OR of 5.5 (95% CI 1.8-17.2) and 4.9 (95% CI 1.6-15.1), respectively. For upper humerus fracture cases this OR was 4.8 (95% CI 1.3-17.8) for the middle tertile and 3.3 (0.9-12.6) for the highest tertile, respectively. It should be emphasized that the cut-off value of the lowest tertile (≤ 4.5 nmol/mmol) is equivalent to the premenopausal mean. Therefore, women having a urinary concentration of fDPD above the premenopausal mean were at increased risk for hip and upper humerus fractures, expressed in OR this was 5.2 (95% CI 1.8-15.1) and 4.0 (95% CI 1.1-13.7), respectively. For any other type of fracture we did not observe a relationship with urinary concentration of fDPD. However, when all types of non-vertebral fractures were combined, still a limited significant association with fDPD was observed, meaning women having urinary fDPD values above 4.5 nmol/mmol had a 1.2 (95% CI 1.0-2.2) higher risk of any type of non-vertebral fracture compared to women below this premenopausal mean.

Figure 1. Unadjusted Fracture Risk by Tertiles of fDPD / creatinin (nmol/mmol)



*P-value < 0.05 vs. lowest tertile

Multivariate Analysis

To determine the influence of age and femoral neck on the association between fDPD and fracture risk, we first estimated the influence of both factors on bone resorption independently. As illustrated in figure 2, there was a positive relationship between age ($\beta = 0.04$, $p = 0.01$) and urinary fDPD, while an inverse relation was observed with bone mineral density ($\beta = -3.1$, $p < 0.01$), which consisted even after adjustment for age ($\beta = -2.4$, $p < 0.05$). Table 4 shows the additive analysis with separate models based on adjustment for age, BMD, lower limb disability and previous fractures. The OR for hip fractures was somewhat decreased after adjusting for femoral neck bone mineral density while lower limb disability and previous fractures did not change this OR. For wrist and upper humerus fractures adjusting for either of these variables did not influence the risk estimate.

Table 4 Odds ratio for fractures according to baseline levels of fDPD / creatinin (nmol/mmol) above vs. below the premenopausal mean

	Multivariate models*			
Fracture type	**Model 1**	**Model 2**	**Model 3**	**Model 4**
Hip	4.9 (1.6-14.5)	3.6 (1.2-11.2)	3.8 (1.2-11.9)	3.7 (1.2-11.7)
Upper humerus	3.9 (1.1-13.7)	3.7 (1.0-13.0)	3.6 (1.0-12.9)	3.6 (1.0-12.8)
Wrist	1.1 (0.6-1.8)	1.3 (0.7-2.3)	1.3 (0.7-2.3)	1.3 (0.7-2.3)
All other types	1.1 (0.6-1.9)	1.2 (0.7-2.2)	1.2 (0.7-2.2)	1.2 (0.7-2.2)
All non vertebral	1.5 (1.0-2.2)	1.6 (1.0-2.5)	1.6 (1.0-2.5)	1.6 (1.0-2.5)

* Multivariate logistic regression models;
Model 1; adjusted for age
Model 2; adjusted for age and bone mineral density
Model 3; adjusted for age, bone mineral density and lower limb disability
Model 4; adjusted for age, bone mineral density, lower limb disability and previous fractures

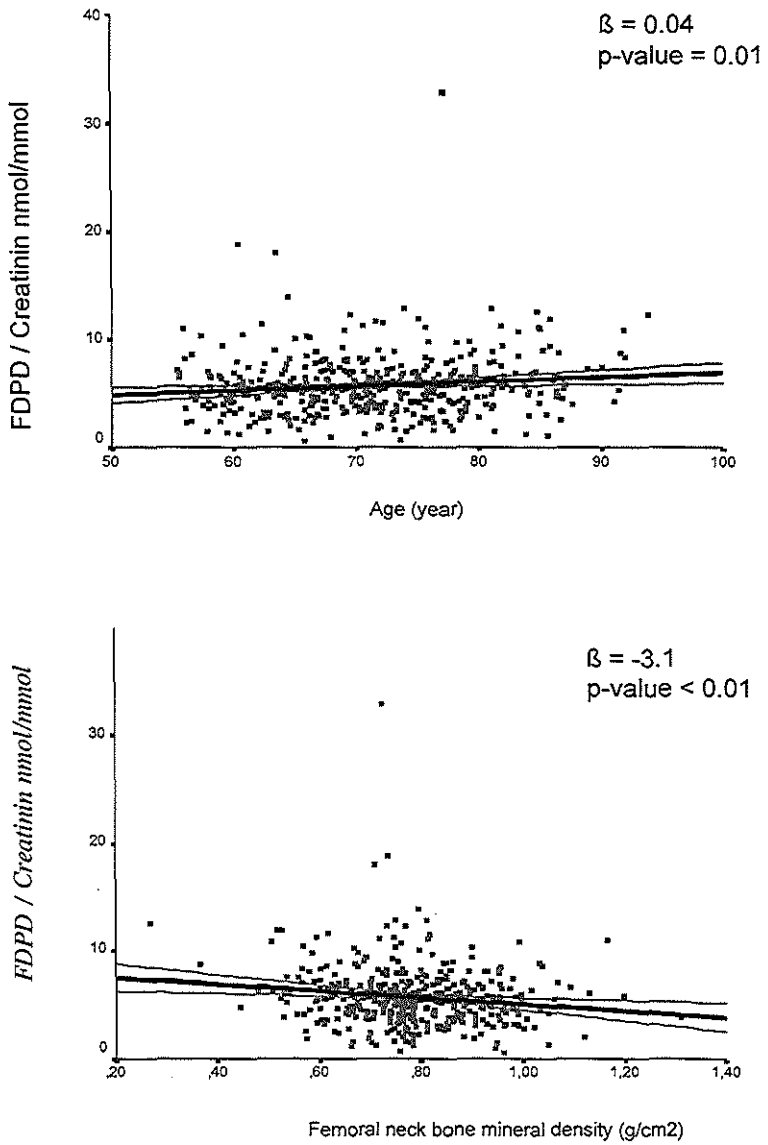
Influence of Time of Follow-up

Figure 3 visualizes the risk of a hip and upper humerus fracture during follow-up for women having a fDPD level above vs. below the threshold of 4.5 nmol/mmol creatinine. Already from the second year onward, the cumulative proportion of both hip and upper humerus fractures within the study population was increased for the women having urinary fDPD values above premenopausal level.

Fracture Risk for Two Risk factors

Women having one or two risk factors; i.e. low bone mineral density and/or high bone resorption had significantly more hip- and upper humerus fractures compared to women having no risk factors at baseline.(Figure 4). Women having both risk factors had 5.6 (95% CI 2.5-10.0) and 1.8 (95% CI 0.7-5.3) times more hip and upper humerus fractures, respectively, compared to women having only one of these risk factors.

Figure 2. Association between FDPD / Creatinin and
Age (upper panel) and bone mineral density (lower panel)



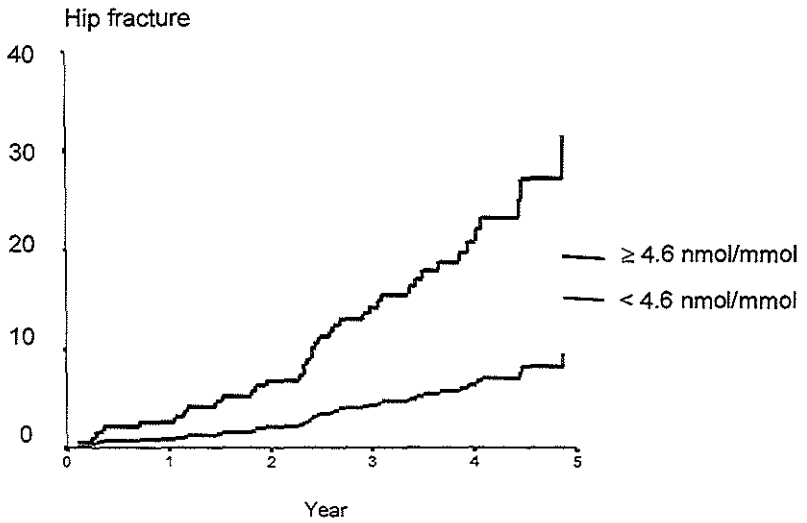
DISCUSSION

In this study of Dutch postmenopausal women we found that urinary concentrations of the bone resorption marker fDPD is associated with increased risk of non-vertebral fractures, especially those of the hip and upper humerus. This association was independent of age, bone mineral density, lower limb disability and history of fractures in the proceeding five years. Moreover, combining the assessment of bone mineral density and urinary fDPD improves the prediction of hip and upper humerus fracture.

This is the first prospective nested case-control study, with a relatively long follow-up, providing more detailed information on the relationship between bone resorption rate and the risk of different non-vertebral fractures. The observed association with hip fracture is in agreement with previously reported data from our group and the EPIDOS study.[6, 7] With respect to the latter study, mean urinary fDPD levels of hip fracture cases and controls are similar to the levels found in the present study. However, in the EPIDOS study a much higher cut-off level (6.8 nmol/mmol creatinine = 2 SD above the premenopausal mean) was observed as predictive value of the resorption marker compared to our study (4.6 nmol/mmol creatinine). This higher cut-off level in the EPIDOS study was probably found because follow-up was restricted to 22 months. In other words, only those with a very high bone resorption rate will have an increased risk to fracture their hip in the subsequent two years. The present study indicates that also those women with a lower (< 4.5 nmol/mmol) level of urinary D-Pyr are at risk. However, this risk became only apparent after 2 years of follow-up (Fig. 3). Furthermore, it is clear that the increased fracture risk associated with low urinary fDPD concentration is not only restricted to hip fracture, but also applies to the upper humerus.

In contrast to this present study, Akesson et al. did not observe an association between the bone resorption marker and all non-vertebral fractures combined.[15] By evaluating the non-vertebral fractures by site, it became evident that the overall association between urinary fDPD and non-vertebral fractures is mainly driven by increased risk of hip and upper humerus fractures. The relative low number of hip and upper humerus fractures in the Scandinavian study might explain the difference with this study.

Figure 3 Cumulative proportion of fracture cases within the study population during follow-up; the Rotterdam Study 1991-1996



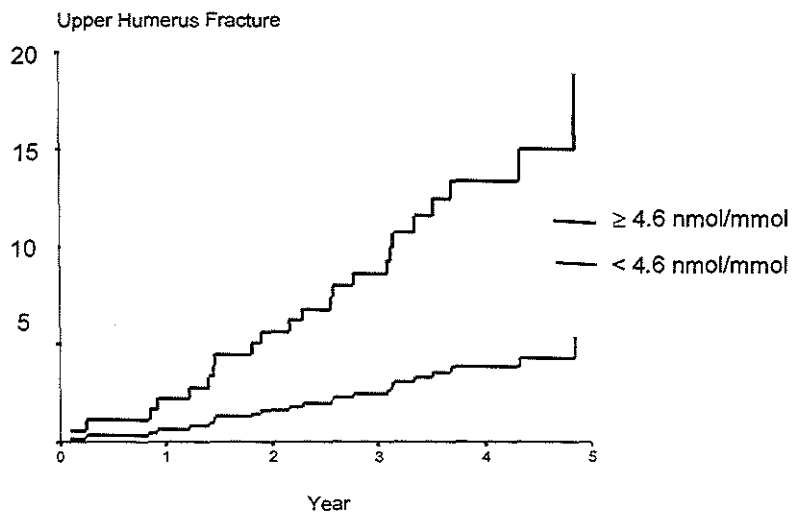
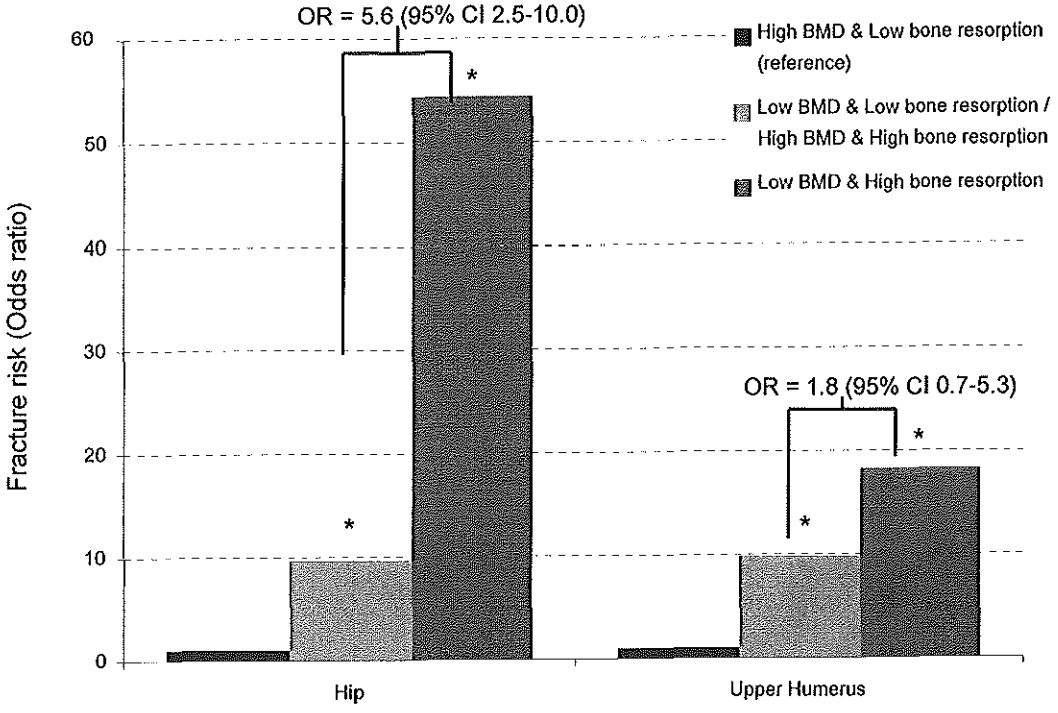


Figure 4. Fracture risk according to number of risk factors; BMD above vs. below T-score –2.5 and Bone resorption above vs. below premenopausal mean



* P-value < 0.02 vs. reference group

The results of the present study are supported by the evidence from previous retrospective studies.[5, 16] However, in contrast to those studies, the prospective design eliminates the potential interference of fracture-induced changes in the resorption marker.

Our results also indicate the absence of an association between bone resorption rate and other types of non-vertebral fractures. A potential explanation might be the low number of other types of non-vertebral fractures captured in this study, although this does not hold for the wrist fracture. It is known that wrist fractures occur predominantly after the onset of menopause, while the hip and upper humerus fracture are more frequent in the elderly. Therefore, we hypothesize that especially long term exposure to higher rates of bone resorption might be related to increased fracture risk. Furthermore, the ratio between cortical and trabecular bone at a specific skeletal site might play a role. The distal forearm is rich in trabecular bone, while the hip and upper humerus contain a substantial amount of cortical bone. More studies are needed to examine whether the urinary fDPD concentration reflects differences in the ratio of trabecular/cortical bone

Bone resorption rates, as measured by urinary fDPD levels, were related to age and, independent of age, to bone mineral density. This implicates that the association between bone resorption rate and fracture risk may be confounded by those factors, especially as hip fracture cases were significantly older and had significantly lower BMD than controls. However, adjustment for age, BMD and other potential confounders like lower limb disability and history of fractures did not change the association without adjustment. Moreover, we found that those women having two risk factors, i.e. a urinary D-Pyr level above the cut-off level of 4.5 mmol/nmol and a femoral neck BMD of 2.5 SD or more below the average mean of premenopausal Dutch women, were at higher risk than those with only one of those two risk factors. Therefore, our results indicate that a bone resorption rate above the premenopausal mean reflects bone fragility independent of bone mass.

The idea that not only decreased bone quantity (bone mass) but also loss in bone quality (number of affected remodeling units and perforations) ultimately leads to fractures, is supported by the observation that the relationship between bone resorption rate and fracture risk is not continuous, but exists above a certain threshold. Only above this threshold sufficient quality of bone is lost to increase fragility ultimately leading to fractures.

Strengths of this study include its prospective design and the fact that cases and controls were selected from the same cohort, thereby avoiding a selection bias. Furthermore, we used a marker for bone resorption rate that has been used in several studies.[6, 7] In contrast to those studies, we measured fDPD by a rapid and automated chemiluminescence immunoassay which is characterized by a substantially lower precision error than the manual method.[8, 9]

Of course there are also a number of limitations. We have measured only one marker of bone resorption and did not assess any marker of bone formation. With respect to the latter, previous results did not indicate an association between two markers of bone formation, namely osteocalcin and bone specific alkaline phosphatase, and the subsequent risk of hip fractures.[6] However, more recently, the same group reported results form a younger cohort,[17] where an increased risk of all types of fractures, including vertebral fractures, was observed in subjects with high levels of bone specific alkaline phosphate. In this relatively small study, a relationship with other markers of bone formation was absent. Another limitation of the present study is the fact that we did not include vertebral fractures. However, the number of incident symptomatic vertebral fractures in our cohort is very small and, therefore, does not allow a separate analysis. Furthermore, our findings only reflect independently living individuals, whereas it is known that a substantial number of fractures occur in individuals who do not live independently. Nevertheless, a lower incidence of fractures in independently living individuals should not affect the association between bone resorption and the occurrence of fractures as such.

We conclude that in a large population based cohort of postmenopausal women aged 55 years and over, increased bone resorption is associated with an increased risk of non-vertebral fractures, especially hip and upper humerus fractures. This association was found to be independent of age, BMD, lower limb disability and previous fractures. For both types of fractures, combined screening of BMD and of bone resorption rate provides more pronounced prediction estimates of fracture risk than measuring one of those factors alone.

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3

Genetic risk factors of osteoporosis

3.1.1

Estrogen Receptor αPolymorphism Predicts the Onset of Natural and Surgical Menopause

ABSTRACT

Age at menopause and risk of hysterectomy have strong genetic components but the genes involved remain ill defined. We investigated whether genetic variation at the estrogen receptor (ER) gene contributes to the variability in onset of menopause in 900 postmenopausal women aged 55-80 years of the Rotterdam Study, a population-based cohort study in the Netherlands. Gynecologic information was obtained and if women reported surgical menopause, validation of type and indication of surgery was accomplished by checking medical records. The ER genotypes (*PP*, *Pp* and *pp*) were assessed by polymerase chain reaction using the *Pvu*II endonuclease.

Compared with women carrying the *pp* genotype, homozygous *PP* women had a 1.1 year ($p < 0.02$) earlier onset of menopause. Furthermore, an allele-dose effect was observed, corresponding to a 0.5 year ($p < 0.02$) earlier onset of menopause per copy of the *P* allele. The risk of surgical menopause was 2.4 (95% CI 1.5-3.8) times higher for women carrying the *PP* genotype compared to the *pp* group, with the most prominent effect in women who underwent hysterectomy due to fibroids or menorrhagia.

We conclude that genetic variations of the ER gene are related to the onset of natural menopause and the risk of surgical menopause, especially hysterectomy.

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INTRODUCTION

Premature exposure to low estrogen levels, as occurs during early onset of menopause, has major implications for health of postmenopausal women. An early onset of menopause is associated with a higher risk of cardiovascular diseases, osteoporosis and ovarian cancer, and moreover, it increases the risk of mortality.[1-4] Therefore, from a clinical point of view, it is important to identify factors that influence the age at menopause. Although several environmental factors have been proposed as risk factors for early onset of menopause,[5-9] recently also genetic factors have been proposed to be determinants of age at menopause.[10,11] This notion is strongly supported by a recent twin study that showed that onset of menopause is genetically determined, yielding heritability for age at menopause of 63%.[12] In addition, undergoing a hysterectomy before reaching natural menopause, with menorrhagia or fibroids as main indications, showed considerable heritability (59%) in the same study.

Several approaches can be followed to identify genes that might contribute to the variation in onset of menopause, including the analysis of candidate genes. In the estrogen endocrine system the estrogen receptor (ER) is an important candidate, in this respect. This member of the family of steroid transcription factors functions as a regulator of expression of many genes and proteins,[13,14] and furthermore, the ER is an important regulator of growth and differentiation in many tissues, including the endometrium.[15-18]

The aim of the present study was to identify a genetic determinant of the onset of menopause. We investigated the association between an anonymous intronic *Pvu*II restriction fragment length polymorphism (RFLP) of the ER gene and both the natural and surgical onset of menopause in a population-based sample of postmenopausal women.

MATERIALS AND METHODS

All postmenopausal women included in this study were part of a population-based cohort study (n = 7983, 61.1% women) of persons aged 55 years and over, living in a district of Rotterdam, the Netherlands. The objective of the study is to investigate the occurrence of chronic disabling diseases in relation to several potential determinants. Rationale and design

have been described previously.[19] A total of 10,275 persons, of whom 9161 (89%) were living independently, were invited to participate in the study in 1990. Among those living independently, the overall response rate was 77% for the home interview and 71% for examination at the research center, where anthropometric characteristics and blood samples were taken. The Rotterdam Study was approved by the medical ethics committee of the Erasmus University Medical School, and written informed consent was obtained from each subject.

For the present study we included independently living subjects who were initially part of a large epidemiological study on osteoporosis in which subjects according to the following criteria: aged 80 years and over, use of thyroid hormone, use of cytostatics, use of diuretics and known diabetes mellitus type II, were excluded. Among the 4478 remaining independently living subjects, an age stratified sample of 1000 women was drawn with balanced numbers (n=200) in 5-years age-categories of age. DNA samples and menopause data of 900 postmenopausal women were available for the analysis.

During the home interview each woman provided information on her reproductive and gynecologic history, including ever use of sex steroids. Natural menopause was defined as menopausal after 12 continuous months of amenorrhoea, without gynecological surgery, or other procedures that would have stopped their menses. In case a gynecological surgical procedure prior to natural menopause was reported, we validated the date and indication of surgery by checking the medical records of the general practitioner (98% of cases). The health care system in the Netherlands well permits this validation as every individual has its own GP. The GP is the only access to specialist and hospital care and preserves all physician and hospital notes. Age at menopause was defined as age at natural menopause or age at surgical menopause (defined as the age at the date of operation). Surgical procedures were defined as hysterectomy (women with only hysterectomy and women with hysterectomy plus uni- or bilateral oophorectomy), oophorectomy (women with only uni- or bilateral oophorectomy) and unknown type of gynecologic surgery. Smoking habits (smoking defined as ever vs. never smoked) and socio economic status (SES defined as highest education level attained; class I-II primary school with/without lower secondary school vs. class III-IV secondary school with/without higher vocational school or university) were assessed by questionnaire. Height

and weight were measured at the clinical examination, with the subject in standing position without shoes.

Genotyping

The anonymous Pvu II restriction fragment length polymorphism (RFLP) is located in intron 1, 0.4 kb upstream of exon 2 of the ER gene and was assessed by a polymerase chain reaction (PCR) procedure.[20] Briefly, genomic DNA (100 ng) was extracted from peripheral leukocytes and used for PCR amplification in a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 150 ng of each primer and 0.2 unit of Super *Taq* polymerase (HT Biotechnology, Cambridge, United Kingdom). The reactions were performed in a DNA thermocycler (mode 480, Perkin-Elmer, Foster City, California, USA.) with a cycling protocol of 94°C, 60°C and 72°C for one minute each, for 25 cycles. Ten microliters (µl) of PCR products were digested with a *Pvu II* restriction enzyme (Life Technologies, Breda, the Netherlands) and 2.5 µl of a buffer (containing 150 mM Tris-HCl, pH 7.5, 250 mM NaCl and 35 mm $MgCl_2$), by incubation for 30 minutes at 37 °C. The digestion products were analyzed by 1.4% agarose gel electrophoresis in 0.5X TBE (1X TBE = 89 mM Tris, 89mM boric acid, 2mM Na_2EDTA) for 250 volt hours (Vh). Separation patterns were documented by Polaroid photography under UV-illumination (302 nm). Genotypes were defined as PP, Pp or pp. Capital letters denote absence and lowercase letters the presence of the site for the restriction enzyme *Pvu*II (P/p). To confirm the accuracy of the genotyping, repeated analysis was performed on 100 randomly selected samples. No discrepancies were found.

Statistical analysis

One-way analysis of covariance (ANOVA) and Chi-square analysis were used to compare anthropometric and environmental variables between the three genotype groups. To account for potential confounders like age of menarche, mean number of offspring, smoking, body mass index, socio economic status, hormone replacement therapy and use of oral contraceptives, we used multivariate regression models. Subsequently, we calculated the odds ratio (with 95% CI) as a measure of the relative risk for occurrence and indication of surgical menopause associated with ER genotype by using logistic regression models where women without premenopausal gynecologic surgery were the reference group. To visualize the genetic influences on

the lifetime risk of hysterectomy, we constructed a cumulative hazard function by using the Cox proportional hazard regression model where women without surgery were the reference group.

Table 4. Characteristics of 900 women according to their ER *Pvu*II genotype

	ER *Pvu*II genotype			P-value	
	PP	**Pp**	**pp**	**ANOVA**	**PP vs. pp**
Number (%)	205 (23)	435 (48)	260 (29)		
Age (year)	67.9 ± 7.0	67.5 ± 6.9	67.1 ± 7.1	0.4	0.2
Ever use HRT, (%)	32 (15.6)	62 (14.2)	37 (14.2)	0.9	0.7
Ever use oral contraceptive (%)	52 (25.4)	106 (24.4)	61 (23.5)	0.9	0.8
Height (cm)	162.5 ± 5.8	161.5 ± 6.3	161.7 ± 7.3	0.2	0.2
Weight (kg)	68.6 ± 10.9	68.4 ± 10.0	68.2 ± 10.6	0.9	0.5
BMI (kg/m^2)	26.0 ± 3.8	26.2 ± 3.5	26.1 ± 4.1	0.7	0.8
Ever smoked (%)	104 (50.7)	228 (52.5)	135 (52.1)	0.9	0.8
SES (education level I-II)	112 (54.6)	258 (59.4)	164 (63.3)	0.2	0.1
Number of offspring	2.2 ± 1.6	2.1± 1.6	2.1± 1.8	0.6	0.4
Offspring None (%)	34 (16.6)	85 (19.5)	55 (21.2)		
1 or 2 (%)	93 (45.4)	198 (45.5)	116 (44.6)	0.8	0.4
≥ 2 (%)	78 (38.0)	152 (34.9)	89 (34.2)		
Age at menopause (year)	48.1± 5.0	48.7 ± 5.0	49.2 ± 4.6	0.06	0.02
Median	49	49	50		
Age at natural menopause (year)*	48.7 ± 4.8	49.4 ± 4.3	49.8 ± 4.2	0.09	0.03
Age at surgical menopause (year)‡	46.1 ± 5.5	44.9 ± 6.3	46.4 ± 4.7	0.4	0.8
Age at menarche (year)	13.8 ± 1.7	13.7 ± 1.6	13.6 ± 1.7	0.4	0.3
Median	14	14	13		

*Natural menopause defined as onset of menopause without hysterectomy, oophorectomy or any other procedure that stopped menses.
‡Age at surgical menopause defined as age at date of hysterectomy and/or oophorectomy

RESULTS

The allele frequencies did not deviate from the Hardy-Weinberg equilibrium, which indicates that no selection has occurred among genotypes. Table 1 shows the general characteristics of the postmenopausal women according to their *Pvu* II genotype. The three genotypes did not differ significantly in age at menarche, use of sex steroids (hormone replacement therapy and oral contraceptives), smoking, body mass index and socio economic status. Furthermore, the mean number of offspring and the percentage of women having children did not differ between the three genotypes. Unfortunately we did not have data on the total number of pregnancies, which gives a better insight on fertility.

However, there was a significant difference in the mean age at onset of menopause between the genotypes. The mean and median age at menopause were 1.1 ($p < 0.02$) year earlier in women with the PP genotype compared to the pp genotype group. Furthermore, an allele-dose effect was observed, corresponding to a 0.5 ($p < 0.02$) year earlier onset of menopause per copy of the P allele. After adjustment for the potential confounders (age at menarche, mean number of offspring, use of sex steroids (HRT and oral contraceptives), smoking, BMI and SES) similar findings were observed.

After excluding women with artificial menopause the result remained essentially the same with an earlier onset of natural menopause for the women with the PP genotype. However, the frequency of women with surgical menopause differed between the three genotypes (Table 2). Therefore, we compared the risk of surgical menopause for women by their ER genotype. The prevalence of women with surgical menopause was highest in women with the PP genotype, lower in the heterozygous Pp women and lowest in women with the pp genotype (Table 2). This overrepresentation was most notable for the procedure hysterectomy. As shown in Table 2, the overrepresentation of women with surgical menopause among those with the PP genotype corresponded to a significant 2.4 (95% CI 1.5-3.8) times higher risk compared to women in the reference group with the genotype pp. When we repeated the analysis by type of surgical procedures, we observed the ER genotype dependent increased risk of surgical menopause to be due to hysterectomy and not oophorectomy. The odds ratios were 1.7 (95% CI 1.3-2.2) and 0.7 (95% CI 0.4-1.2) per copy of P allele for hysterectomy and oophorectomy,

respectively. Figure 1 shows the lifetime risk of hysterectomy according to the estrogen receptor genotypes as calculated by the Cox proportional hazard function. The PP group had a significant higher risk of premenopausal hysterectomy, which confirmed the results from the logistic regression analysis.

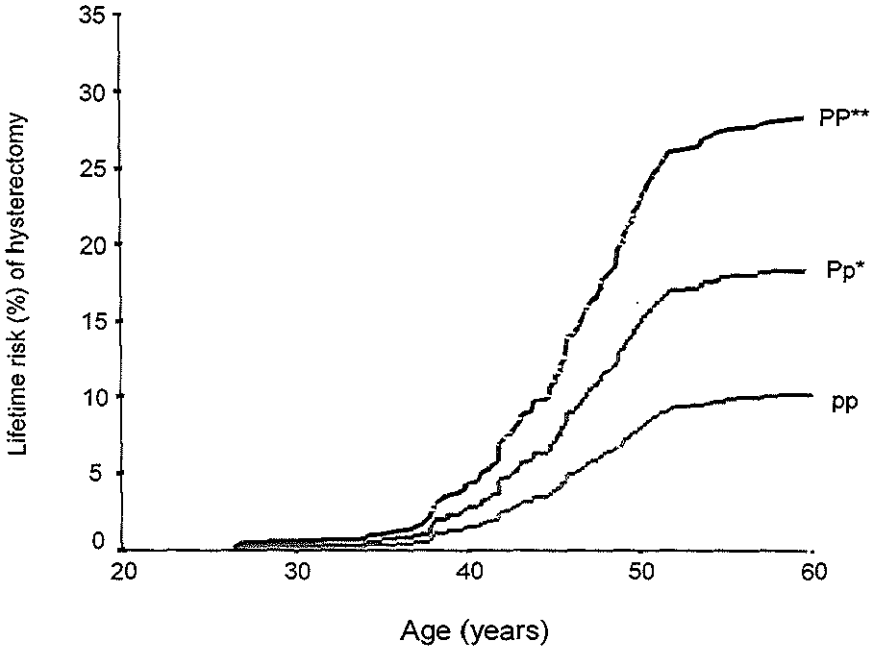
Table 5. Frequencies and ODDS ratios (95% CI) for premenopausal gynecologic surgical procedures according to ER *Pvu*II genotype.

	ER *Pvu*II-Genotype			
	PP	***Pp***	***pp***	**Total**
Surgery (%)	55 (26.8)	84 (19.3)	35(13.5)	174 (19.3)
Hysterectomy (%) *	49 (23.9)	70 (16.1)	24 (9.2)	143 (15.9)
Ovariectomy (%)	3 (1.5)	12 (2.8)	10 (3.8)	25 (2.8)
Unknown type (%)	3 (1.5)	2 (0.5)	1(0.4)	6 (0.7)
Odds ratio (95% CI) ‡	***PP***	***Pp***	***pp***	**allele-dose**
Surgery	2.4 (1.5-3.8)	1.5 (1.0-2.4)	1	1.5 (1.2-1.9)
adjusted*	2.7 (1.6-4.3)	1.7 (1.1-2.7)	1	1.6 (1.3-2.1)
Hysterectomy	3.1 (1.8-5.2)	1.9 (1.1-3.1)	1	1.7 (1.3-2.2)
Ovariectomy	0.4 (0.1-1.7)	0.8 (0.3-1.8)	1	0.7 (0.4-1.2)

* Adjusted for age, age at menopause, age at menarche, number of offspring, use of sex steroids, smoking habits, SES and BMI.
‡ Reference group is women without any premenopausal gynecologic surgery.

In Table 3 we stratified the analysis by indication for surgery (validated data not available for 91 of 174 (52.3%) women with surgery). The predominant indications were menometrorraghia (23%) and uterus myomatosis (fibroids) (43%). Surgical procedures performed for these indications were hysterectomy or hysterectomy plus oophorectomy. There was no oophorectomy solely performed for these indications. We observed that the ER genotype dependent risk of hysterectomy to be largely due to the higher frequencies of the indications menometrorraghia and uterus myomatosis (fibroids) in the PP genotype group (Table 3). The odds ratio for the allele dose effect was 2.6 (95% CI 1.3-5.2) and 1.8 (95% CI 1.1-2.9) per copy of the P allele for menometrorrhagia and uterus myomatosis, respectively.

Figure 1. Lifetime risk (%) of premenopausal hysterectomy according to the ER *Pvu*II genotypes.



** P-value < 0.001 (PP genotype vs. pp genotype), * P-value = 0.01 (Pp genotype vs. pp genotype) according to the Cox proportional hazard model

DISCUSSION

In this population-based study we show for the first time that the common allelic variants of the ER gene are associated with both natural and surgical menopause. Moreover, we provide evidence that women with the PP genotype will undergo hysterectomy due to menorrhagia and fibroids more frequently.

Table 3. Frequency and Odds Ratio for the indication of surgery according to ER *Pvu*II genotypes.

	ER *Pvu*II Genotype			
	PP	***Pp***	***pp***	**Total**
Surgical procedures	55	84	35	174
Indication available (%)	29 (52.7)	43 (51.1)	11 (31.4)	83 (47.7)
Subjects used for analysis	179	394	236	809
Menometrorrhagia (%)	9 (5.0)	8 (2.0)	2 (0.8)	19 (2.3)
Uterus myomatosis (%)	12 (6.7)	19 (4.8)	5 (2.1)	36 (4.4)
Other (%)*	8 (4.5)	16 (4.1)	4 (1.7)	28 (3.5)
Odds ratio (95% CI) ‡	***PP***	***Pp***	***pp***	**allele-dose**
Menometrorrhagia	6.7 (1.4-31.5)	2.6 (0.5-12.2)	1	2.6 (1.3-5.2)
Uterus myomatosis	3.6 (1.2-10.4)	2.4 (0.9-6.6)	1	1.8 (1.1-2.9)
Other*	3.0 (0.9-10.1)	2.6 (0.8-7.8)	1	1.6 (0.9-2.8)

*Other includes; prolapsed uteri, malignancy, ovarian cyst or other uncommon diagnosis.
‡ Reference group is women without any premenopausal gynecologic surgery.

There may have been biases that have lead to incorrect effect estimates. First, the self-report of the age of menopause was determined retrospectively, which has been shown to be unreliable in previous studies.[21] Nevertheless, it seems unlikely that this recall bias differs between the ER genotypes. Second, although regional differences in the frequency of hysterectomy have been reported,[22-23] it is unlikely that this would have influenced the relation between the ER genotype alleles and the risk of surgical menopause in this study. In addition, the allele frequencies of the ER *Pvu*II polymorphism did not differ from frequencies reported before by others and therefore argue strongly against selection bias in our population.[24] Third, although the frequencies of missing data on indication differed between the three genotypes, it is unlikely that the reason for missing these data differed between the ER genotypes. However, in view of the low frequency (48 %) of validated data on indication

for surgery, confirmation of our observations in another population with more extensive clinical and pathological data is needed to provide more robust risk estimates regarding indication. Finally, the ER genotype dependent effect on endpoints of menopause may have biased the results because women included in the study were probably healthier due to the in- and exclusion criteria applied. However, as far as we know, no studies have been published about the association of the ER gene and any of these criteria. Moreover, a separate analysis for the total cohort of the Rotterdam study (n= 4878 women) did not show a relation between the in- and exclusion criteria and age at menopause (data not shown).

Evidence that genetic factors are related to age at menopause has been observed previously in family and twin studies.[10,11,12] However, until now, only a limited number of genes have been studied in the association with the onset of menopause. One study showed that in a single family four woman had an interstitial deletion of the long arm of X-chromosome, which was associated with premature ovarian failure and premature menopause.[25] Furthermore, a link between menopause and galactose-1-phosphate uridyl transferase gene was reported.[26] This study, however, had limited generalizability due to population admixture and the small number of subjects examined.

In the present study we observed that common variations of the ER gene are associated with disorders of the uterus. This supports the hypothesis that a common factor is involved in the pathogenesis of abnormal bleeding and uterine fibroids.[27] The present observation seems logical due to the fact that the ER has been identified in endometrium, myometrium and fibroids.[15] In addition, estrogens have a direct effect on the uterus, which is emphasized by the fact that ER knockout mice demonstrated lack of uterine response on estrogen treatment compared to wild-type animals.[28]

Although it is unclear how the common allelic variations of the ER gene influence the action of the ER for its ligand oestradiol, the present study showed an earlier onset of natural menopause and a higher prevalence of hysterectomy due to fibroids and menorrhagia in women carrying the P allele. This is suggestive for a higher responsiveness of this ER gene allele to estrogen, which might affect the biological response and ultimately leads to irregularities in the differentiation and proliferation of endo - and myometrical cells. However, it must be emphasized that it is presently unclear what the molecular mechanism is of the

association we here report. Because the PvuII RFLP is an anonymous polymorphism it seems likely that the P allele is in linkage disequilibrium with a truly causative sequence variation elsewhere in the ER gene, or even in another nearby gene. To elucidate the precise molecular mechanism, extensive sequence analysis and functional studies of the ER gene variants are needed.

In conclusion, we have obtained evidence that a common allelic variation in the ER gene is associated with age at menopause as well as hysterectomy. This raises the possibility that genotyping at the polymorphic *Pvu*II ER gene may provide information on susceptibility to uterine disorders leading to early onset of menopause, which eventually might lead to other clinical entities like osteoporosis, cardiovascular diseases and ovarian cancer.

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3.1.2

The Effect of Estrogen Receptor α Polymorphism on Bone Mineral Density and Fractures in Men and Women

ABSTRACT

The estrogen endocrine system is an important regulator of a wide variety of biological processes. Changes in this system have been implicated in many clinical disorders including osteoporosis. We have investigated the influence of genetic variation of the estrogen receptor α (ERα) gene locus (6q25) on bone mineral density (BMD) and the risk of fractures in a large population.

For 2,257 men and women, information on BMD, incident non-vertebral fractures and ERα genotype was available. For 1,206 subjects baseline and follow-up photographs of the spine were available to analyze the occurrence of vertebral fractures. In total, 299 incident non-vertebral fractures were captured during 5 years of follow-up and 165 individuals had a vertebral fracture. ERα genotype was determined by analyzing a *C* to *T* substitution in intron 1 and subjects were categorized by their genotype as *CC*, *CT* and *TT*.

Compared to the *CC* group, BMD was lower in women carrying the *TT* genotype. This difference was 3.9 % at the lumbar spine ($p < 0.01$) and 2.5% at the femoral neck ($p= 0.02$), While in men these differences were less obvious. Compared to the *CC* genotype, the risk of vertebral fractures was higher for the *TT* genotype (OR = 2.3 (95%CI: 1.4-3.7) especially in women (OR = 2.9 (1.4-6.0). This increased risk was independent of BMD and not seen for non-vertebral fractures.

We conclude that the *T* allele of the ERα polymorphism is associated with decreased BMD and an increased risk of vertebral fractures. The molecular mechanism underlying this association needs further study.

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INTRODUCTION

Exposure to low estrogen levels may induce osteoporosis, which is characterised by low BMD and an increased risk of fractures.[1] In line with this, estrogen replacement treatment in early postmenopausal women decreases the risk of osteoporotic fractures.[2]This indicates the important regulating role of the estrogen endocrine system in bone metabolism. Estrogens act primarily via the estrogen receptor α (ERα), which is expressed in many cell types including bone cells like osteoblasts, osteocytes and pre-osteoclasts.[3] Interestingly, an ERα knockout mouse model showed that both female and male mice had decreased bone mass. Consistent with this, Smith et al. reported a man with a loss-of-function mutation of the ERα gene resulting in a phenotype with low BMD.[4]

Osteoporosis is considered to be multifactorial (environmental and genetic conditions interact) and multigenetic (genetic variations in a large number of genes play a role). Twin studies have suggested that up to 75% of the variance in BMD is genetically determined.[5,6] A potential candidate gene that may influence genetic differences in bone metabolism is the ERα gene. Several genetic variations in the ERα gene (6q25) have been described including a *C* to *T* substitution in intron 1, which is detected as a *Pvu*II RFLP.[7] Studies with this polymorphism in relation to BMD have been reported but results have been inconsistent. In part this is due to the limited sample size of most studies where lack of power can lead to spurious results. In addition, differences between populations can play a role, such as ethnicity, age, environment, and genetic make-up. Most studies focused on women and have not analyzed fractures, the clinically most relevant endpoint of osteoporosis. We therefore investigated, in a large and homogeneous population-based sample of Caucasian elderly men and women, the influence of the ERα polymorphism on BMD and fractures.

MATERIAL AND METHODS

Study population

Subjects were participants of the Rotterdam Study, a prospective population based cohort study of individuals aged 55 years and over, which has the objective to investigate the incidence of, and determinants of, chronic disabling diseases. Rationale and design have been described previously.[8] All 10,275 inhabitants aged 55 years and over, of a district in Rotterdam, the Netherlands, were invited for baseline examination between August 1990 and June 1993. Of those, 7,983 participated (61.1% women), bringing the overall response rate to 78 percent. For 2,257 participants, information on BMD, incident non-vertebral fractures and ERα genotype was available. For 1,051 participants no baseline and/or follow-up photograph were available. Therefore, the analyses on prevalent and/or incident vertebral fractures are performed in 1,206 subjects. The Rotterdam Study has been approved by the Medical Ethics Committee of Erasmus University Medical School.

Clinical examination

At baseline, BMD (expressed in g/cm^2) was measured at the femoral neck and lumbar spine by dual energy X-ray absorptiometry (DXA, DPX-L densitometer) as described previously.[9] Height and weight were measured in standing position in indoor clothing without shoes. Body mass index (BMI) was computed as weight in kilograms divided by height in meters squared (kg/m^2). Menopause status was assessed and validated as described previously.[10] The period of estrogen exposure was calculated by subtracting age at menarche from the age at menopause.

Vertebral fracture assessment

Both at baseline, between 1990 and 1993 and at a follow-up visit, between 1997-1999 radiographs of the spine were taken. All follow-up radiographs were scored for the presence of

vertebral fracture by the McCloskey/Kanis method.[11] If a vertebral fracture was present, the baseline photo was scored as well, to ascertain whether a fracture was incident or prevalent.

Non-vertebral fracture assessment

Follow-up started either at 1st of January 1991 or, when later, at the time of inclusion into the study. For this analysis follow-up ended either at March 31, 1997, or, when earlier, at the participants death. The general practitioners of the participants provided data on morbidity including non-vertebral fractures and mortality. For approximately 80 percent of the study population, medical events were reported through computerized general practitioner diagnosis registers. For the remaining 20%, research physicians collected data from the general practitioners' medical records of the study participants. All collected fractures were verified by reviewing discharge reports and letters from medical specialists. Fracture events were coded independently by two research physicians according to the International Classification of Diseases, 10th revision (ICD-10).[12] In case of discrepancy, consensus was attained in a separate session. A medical expert in the field reviewed all coded events for final classification. Data on all non-vertebral fractures were at the time of analysis available till 31st march 1997.

Genotyping

The e2-397 *C* to *T* substitution in intron 1, upstream of exon 2 of the ERα gene, was detected as a *Pvu*II restriction fragment length polymorphism (RFLP) as described previously.[10] Presence of the restriction enzyme recognition site is designated with '*p*' while '*P*' denotes absence. In this test the *C* allele corresponds to '*P*' and the *T* allele to '*p*'. Subjects were categorized as ERα genotypes *CC*, *CT* or *TT*.

Statistical analysis

Subjects were grouped according to genotype. Chi-square analysis was used to test for difference in the distribution of alleles by gender and for deviation from Hardy-Weinberg equilibrium. One-way analysis of covariance (ANOVA) was applied for testing differences in anthropometric variables between the three genotypes. For exploring an association between

BMD and ERα genotypes we used multivariate linear regression models. Additionally, for clinical purposes we calculated the gender specific Z-scores (as measure of age-adjusted values) of BMD at the lumbar spine and femoral neck by five-year strata of age. Allele dose effects were analyzed by comparing

Table 1. Baseline characteristics of 1021 men and 1236 women according to ERα e2-397 C/T genotype

	ERα genotypes			*p-value*
Women	CC	CT	TT	*ANOVA*
Number (%)	274 (22.2%)	608 (49.2%)	354 (28.6%)	
Age (yrs)	69.8 ± 8.7	69.1 ± 8.6	69.2 ± 8.6	*0.5*
Height (cm)	162.3 ± 6.4	161.4 ± 6.4	161.4 ± 7.5	*0.2*
Weight (kg)	69.2 ± 11.2	68.4 ± 10.2	68.0 ± 10.5	*0.4*
BMI (kg/m^2)	26.2 ± 3.9	26.2 ± 3.6	26.2 ± 4.0	*0.9*
Age of menopause (yrs)	48.0 ± 5.0	49.0 ± 4.6	49.4 ± 4.3	*0.01*
Age at menarche (yrs)	13.8 ± 1.7	13.8 ± 1.6	13.6 ± 1.7	*0.3*
Estrogen exposure (yrs)	34.2 ± 5.1	35.2 ± 4.9	35.8 ± 4.5	*< 0.001*
Men	CC	CT	TT	*ANOVA*
Number	216 (21.2%)	507 (49.7%)	298 (29.2%)	
Age (yrs)	67.3 ± 7.3	67.9± 7.5	67.2 ± 7.1	*0.3*
Height (cm)	175.6 ± 6.4	174.5 ± 6.6	175.0 ± 7.6	*0.2*
Weight (kg)	78.0 ± 10.2	78.2 ± 11.2	78.6± 11.8	*0.8*
BMI (kg/m^2)	25.3 ± 2.9	25.6 ± 3.0	25.5 ± 2.9	*0.4*

Values are numbers (percentage) or mean ± Standard Deviation

individuals carrying one copy of the test allele (heterozygotes) and two copies of the test alleles (homozygotes) with the reference group of individuals carrying no test alleles. To estimate the risk of vertebral fractures, odds ratios with 95% confidence intervals (95 % CI) were calculated using logistic regression models. To estimate non-vertebral fracture risk we

used Cox proportional hazard models and thereby taking potential differences in follow-up time into account. In addition, we estimated odds ratio's for the most frequent types of non-vertebral fractures. P-values are two-sided and 0.05 or less was considered significant.

RESULTS

Baseline Characteristics

Compared to individuals without information on BMD, incident non-vertebral fractures and ERα genotype (n= 5726), the study population (n=2257) included relativly less women (55% vs. 64%, p<0.05) who were younger (68.5 vs. 71.4 year, p<0.05) and more independently living (97.2% vs. 85.5%, p<0.05). Table 1 shows the characteristics of the study population at baseline according to ERα genotypes stratified by gender. Allele frequencies were 46% for the *C* and 54% for the *T* allele. ERα genotypes were equally frequent in men and women (p = 0.8) and the distribution did not deviate from the Hardy-Weinberg equation (p = 0.5 and p = 0.3 for men and women, respectively). In both men and women, age, height, weight and BMI did not differ significantly between the genotypes. However, there was a significant difference in age of menopause with evidence for a gene dose effect. Per copy of the *T* allele the onset of menopause started 0.7 year later (p = 0.001). In line with this, years of estrogen exposure were longest in duration for women having the *TT* genotype. The period of estrogen exposure was lengthened with 0.8 years per copy of the *T* allele (p < 0.001).

ERα genotype and BMD

For both sites measured, femoral neck and lumbar spine, BMD was similar for *CC* and *CT* but was lower in *TT* homozygotes suggesting a recessive effect of the *T* allele (Table 2). In women, BMD at the lumbar spine was 3.9 % lower (p < 0.01) compared to the *CC* group, whereas at the femoral neck this difference was 2.5% (p= 0.02). In men this difference was less obvious with BMD being 2.3% lower at the femoral neck (p=0.07) and 0.9% (p=0.3) at the lumbar spine. In all analyses, additional adjustment for age and BMI did not essentially change the differences. However, additional adjustment for years of estrogen exposure increased the difference in BMD among the genotypes groups in women somewhat. BMD

increased for women carrying the *CC* genotype whereas BMD decreased for those carrying the *TT* genotype group. In Figure 1 gender-specific Z scores for the lumbar spine and femoral neck BMD according to ERα genotype are presented. In women, a significant allele dose effect was observed meaning that the Z score was significantly lower per copy of the *T* allele at the femoral neck ($p= 0.05$) and lumbar spine ($p< 0.01$). For men similar trends were observed but these did not reach significance.

Table 2 BMD measures (mean ± SEM) according to ERα e2-397 C/T genotype

BMD measures	**ERα genotype**			***p-value***	
Women	**CC**	**CT**	**TT**	***ANOVA***	***CC vs TT***
Femoral neck(g/cm^2)					
Crude	0.80 ± 013	0.80 ± 0.13	0.78 ± 0.13	*0.1*	*0.06*
Adjusted[1]	0.80 ± 0.12	0.80 ± 0.11	0.78 ± 0.12		*0.02*
Lumbar spine (L2-4)					
Crude	1.02 ± 0.17	1.02 ± 0.18	0.99 ± 0.17	*0.02*	*0.02*
Adjusted[1]	1.03 ± 0.17	1.02 ± 0.16	0.99 ± 0.16		*< 0.01*
Men	CC	CT	TT	*p-value*	
Femoral neck (g/cm^2)					
Crude	0.88 ± 0.14	0.87 ± 0.13	0.86 ± 0.14	*0.5*	*0.3*
Adjusted[2]	0.88 ± 0.13	0.87 ± 0.13	0.86 ± 0.13		*0.07*
Lumbar spine (L2-4)					
Crude	1.16 ± 0.19	1.16 ± 0.20	1.15 ± 0.19	*0.7*	*0.5*
Adjusted[2]	1.16 ± 0.19	1.16 ± 0.18	1.15 ± 0.18		*0.3*

[1]values are adjusted for age, BMI and age at menopause
[2]values are adjusted for age and BMI

ERα genotype and non-vertebral fractures

In the total population of 2,257 subjects, during 4.8 years (SD 1.1 year) of follow-up 265 subjects died (11.7%) and 299 (13.2 %) non-vertebral fractures were reported (Table 3). Most non-vertebral fractures (n=231 (10.2%)) were reported in women. The frequencies of any non-vertebral fracture were 14.3%, 13.5% and 12.1% for the *CC*, *CT*, and *TT* genotype groups, respectively and did not differ significantly by ERα genotype (p=0.5) . Separate analysis by gender showed for men a protective effect of the *T* allele, although this did not remain statistically significant after adjustment for potential confounders (i.e., age, BMI and femoral neck BMD). For the risk of hip and Colles fractures (the most frequent types of non-vertebral fractures in this study, n = 77 and n = 64 for hip and Colles fractures, respectively) we could not observe genotype-dependent effects. In women, the frequencies of hip fractures were 3.3%, 4.8% and 5.1% for the *CC*, *CT* and *TT* genotype, respectively. Expressed as OR (95% CI) this corresponds to 1.4 (0.7-3.0) and 1.4 (0.6-3.2) for the *CT* and *TT* genotype, respectively. For the Colles fracture the frequencies were 4.4%, 4.9% and 4.5% for the *CC*, *CT* and *TT* groups, respectively. In men, the frequencies for hip fracture were equally distributed among the genotype groups with 2.8%, 2.2% and 1.3% for the *CC*, *CT* and *TT* genotype. Due to a low number of Colles fractures in men, we were not able to do separate analyses for this type of fracture.

ERα genotype and vertebral fractures

In the subgroup of 1206 individuals with data on vertebral fractures, the genotypes were in Hardy-Weinberg equilibrium (p=0.4). Compared to individuals without radiographs of the spine (n= 1051) this subgroup was relatively younger (65.9 vs. 71.6year, $p< 0.05$) and had a higher femoral neck BMD (0.84 vs. 0.82 g/cm^2, $p < 0.05$) whereas the lumbar spine BMD did not differ significantly. In men and women combined, 165 vertebral fractures (incident plus prevalent) were scored of which the frequencies were 10.0%, 11.2% and 20.1% in the *CC, CT* and *TT* ERα genotype groups, respectively (Table 3). The risk of vertebral fractures was 2.3 (95%CI: 1.4-3.7) times increased in individuals having the *TT* genotype compared to the *CC* group, whereas this was 1.1 (0.7-1.9) for the *CT* genotype. When we analyzed this relation by gender we found that the risk was mainly observed in women where the risk was 1.7 (1.2-2.5)

Table 3; Number (%) and Odds ratios (95% CI) of fractures according to ERα e2-397 C/T genotype by gender

	Women[1]			**Men**[2]		
	ERα genotype			ERα genotype		
Fracture site	CC	CT	TT	CC	CT	TT
Non-vertebral	50/274 (18.2)	118/608 (19.4)	63/354 (17.8)	20/216 (9.3)	32/507 (6.3)	16/298 (5.4)
OR crude	1	1.1 (0.8-1.5)	1.0 (0.7-1.4)	1	0.6 (0.4-1.1)	0.5 (0.3-1.0)
OR adjusted	1	1.1 (0.8-1.7)	1.0 (0.6-1.5)	1	0.7 (0.4-1.3)	0.6 (0.3-1.1)
Vertebral	11/139 (7.9)	41/310 (13.2)	46/196 (23.5)	14/112 (12.5)	24/271 (8.9)	29/178 (16.3)
OR crude	1	1.8 (0.9-3.6)	3.6 (1.8-7.2)	1	0.7 (0.3-1.4)	1.4 (0.7-2.7)
OR adjusted	1	1.6 (0.8-3.3)	2.9 (1.4-6.0)	1	0.6 (0.3-1.3)	1.2 (0.6-2.4)

[1]values are adjusted for age, years of estrogen exposure and BMD (non-vertebral type of fractures for femoral neck BMD, vertebral fractures for lumbar spine BMD)
[2]values are adjusted for age and BMD (non-vertebral type of fractures for femoral neck BMD, vertebral fractures for lumbar spine BMD)

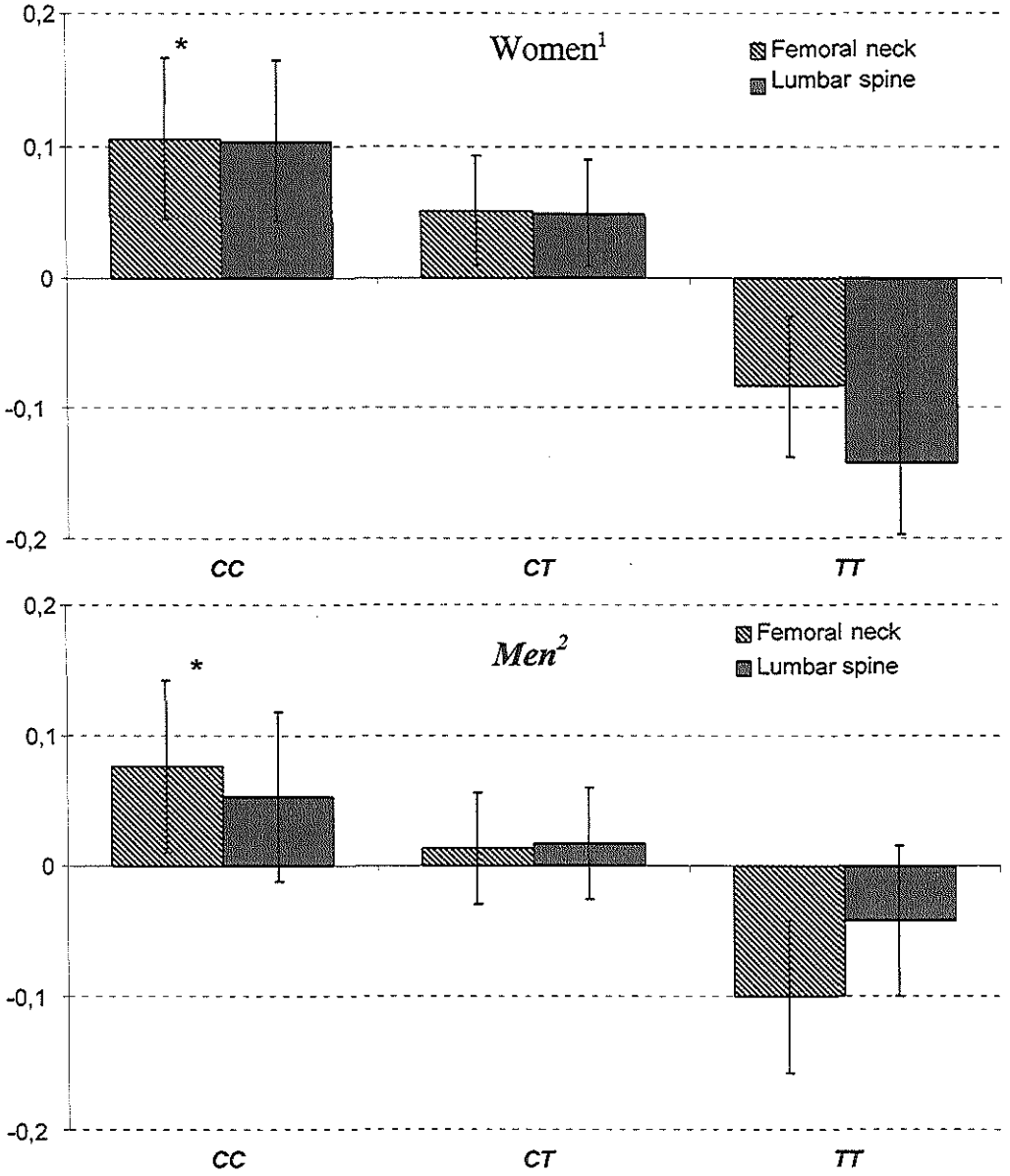
times increased per copy of the *T* allele. In view of differences in BMD and years of estrogen exposure we did additional analyses but the risk estimates did not essentially change after adjustment for age, lumbar spine BMD and years of estrogen exposure. In addition, to adjust for potential survival bias we analyzed the genetic effect of the ERα polymorphism for incident and prevalent fractures separately (Figure 2). During a mean follow-up time of 6.5 years (SD 0.5) 82 incident vertebral fractures were captured, of which 16 had already a prevalent vertebral fracture at baseline. The number of prevalent fractures determined from the follow-up radiographs was 99. The observed association described above was equally strong for both prevalent and incident vertebral fractures with allele-dose effects of 1.6 (1.0-2.5) and 1.7 (1.1-2.7) for prevalent and incident fractures, respectively.

DISCUSSION

In this large population-based sample, there are two main findings. First, the T allele of the ERα e2-397 C/T polymorphism is associated with a lower BMD at both the lumbar spine and the femoral neck. Second, the risk of vertebral fractures was increased in individuals carrying the T allele. Both findings were more prominent in women.

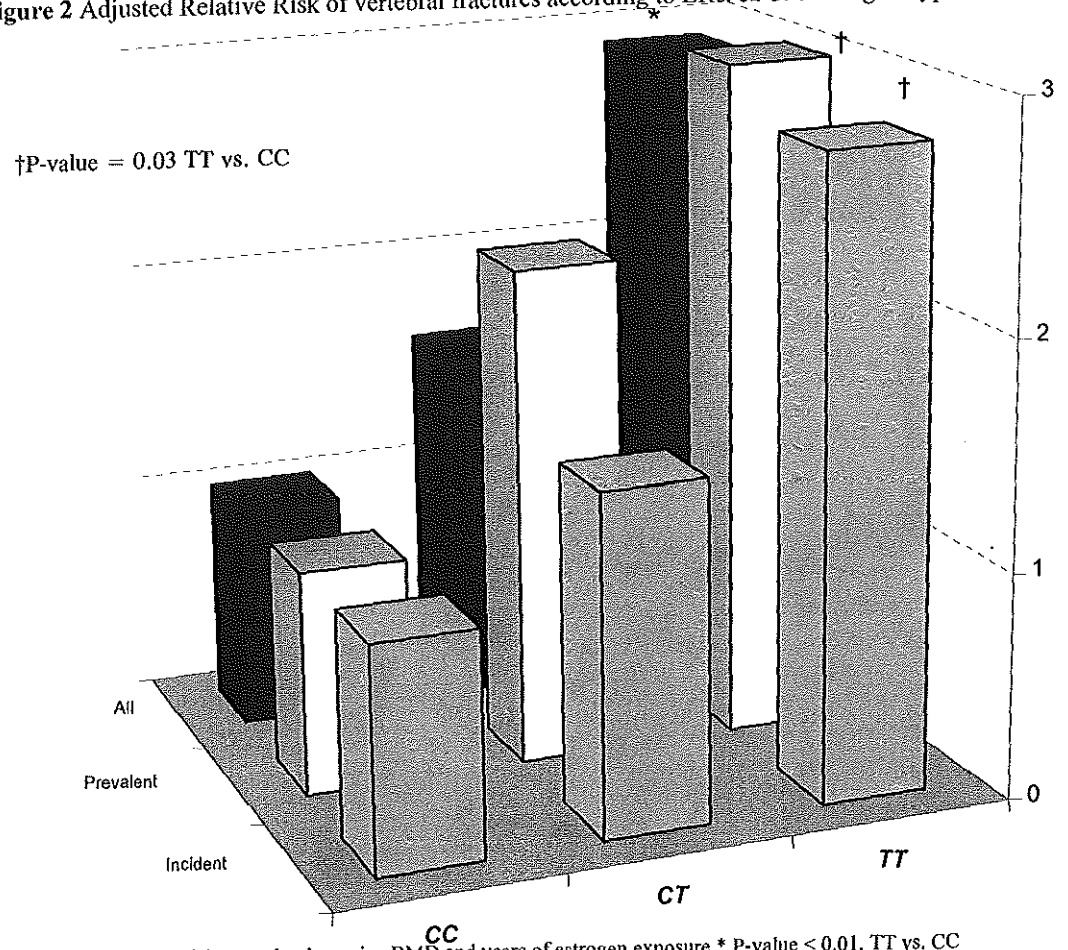
Our study has some limitations. When compared to the total source population, our study population contained fewer women who were younger and lived more independently. Although this might result in a healthy responder bias we do not think this to be genotype dependent and therefore do not expect this to influence our results. Furthermore, we observed similar risk estimates for prevalent and incident vertebral fractures, which argues against a potential survival bias. In addition we tried to avoid potential selection bias since cases and non-cases were derived from the same source population. Genetic association studies can be influenced by population heterogeneity. In this cohort study, all subjects were Dutch Caucasians; to our knowledge no systematic differences were present with respect to the part of the Netherlands in which this study was performed. Therefore our study population might be considered as an ethnically homogeneous and representative sample of the Dutch elderly. The distribution of the ERα genotype was in Hardy-Weinberg equilibrium, which indicates absence of genetic drift and selection bias. Moreover, the allele frequencies observed in this study are in close agreement to the ones observed in other Caucasian populations.[13-17] In the

Figure 1; BMD measures according to ERα e2-397 C/T genotype expressed in Z-score (SD)



[1] values are adjusted for BMI and years of estrogen exposure
[2] values are adjusted for BMI
† P-value < 0.01
* P-value < 0.05

Figure 2 Adjusted Relative Risk of vertebral fractures according to ERα e2-397 C/T genotype in women



Adjusted values are adjusted for age, lumbar spine BMD and years of estrogen exposure * P-value < 0.01, TT vs. CC

analysis on vertebral fractures, controls (non-fractures cases) could have suffered a non-vertebral fracture and vice versa. Nevertheless, this misclassification of disease is unlikely to be dependent on ERα gene status and if occurred, is likely to underestimate the true risk estimate.

Several studies in women have reported inconsistent associations between polymorphism of the ERα gene and BMD.[13-21] The existence of ethnic differences between the populations, the case-control designs and a health-based selection bias in several studies could explain the discordant findings. The present study confirms data of some previous studies,[14,16,21,22] including one conducted in men,[23] which report the *CC* genotype to have an increased BMD. In contrast, in Japanese women,[18] the *CC* genotype had a lower BMD compared to the *TT* group whereas others showed no association.[13,15,17,19,20] Our findings together with those of others suggest that there could be allelic heterogeneity at the ERα locus among different populations. However this should be accompanied with differences in genotype distributions. Arguing against this is the fact that the allelic frequencies were similar to those reported in studies of Caucasian subjects although no data were available for men.[13,15,17] Together with statistical reasons (such as lack of power) these phenomena could contribute to the controversial results published so far.

Our findings on the association with vertebral fractures are in line with those of Becharini et al and Langdahl et al.[15,22] These studies reported results using another polymorphic marker, i.e., a TA-repeat in the promotor region. Yet, there is strong linkage disequilibrium (LD) between this marker and the *C* to *T* substitution we analyzed.[15,22,24] The *T* allele we found associated with decreased BMD and increased vertebral fracture risk is in fact linked with a low number of TA-repeats as was demonstrated in other studies of Caucasians.[15,22] These studies reported a low number of TA-repeats to be associated with decreased BMD and increased fracture risk. Interestingly, the more than two-fold increase in fracture risk we found in the present study was not explained, as expected, by the 0.2 SD difference in BMD between the genotypes. Moreover, we observed the ERα genotype association with vertebral fractures to be independent of BMD and of age at menopause. Together with our previous observation

of the genetic effect of ERα polymorphism on menopausal status this illustrates the pleiotropic nature of the ERα protein.[10] The estrogen endocrine system can be simultaneously involved in several different metabolic pathways, such as the reproductive system, bone and cardiovascular function. Whereas the determination of BMD is to some extent ERα dependent, and as such indirectly influences the risk of fractures, the BMD-independent relation of ERα genotype with fractures suggests underlying biological mechanisms other than those reflected in BMD to explain the increased fracture risk. This same phenomenon was also shown for the relation between the Collagen type Iα1 Sp1 polymorphism, BMD and fracture risk,[25] and for VDR polymorphisms.[26] This suggests that BMD might not be the most suitable endpoint in genetic association studies, at least for these candidate genes.

Until now, no functional effects have been reported for this ERα polymorphism. Sofar the e2-397 *C* to *T* substitution is thought to be anonymous and therefore can not by itself explain the observed association. It is supposed that a truly functional sequence variation elsewhere in the gene is in LD with this polymorphism. Further delineating the area of LD is therefor crucial to be able to find such a functional polymorphism. Previous studies have suggested that variations in the promotor region might play a role in this respect. Such variation could include the TA-repeat near exon 1, which is 20 kb downstream and was shown to be in strong LD with the e2-397 *C* to *T* substitution. However future studies are necessary to clarify this issue.

In this study we showed that the associations observed were most prominent in women. This might be simply explained by lack of statistical power due to the lower number of fractures observed in men. However, it might also be a true effect and in this respect, higher estrogen levels in elderly men compared to postmenopausal women might mask the differences between genotypes. Furthermore, the associations were strongest at the spine (i.e. with lumbar spine BMD and with vertebral fractures). This is in line with previous data showing a higher response to estrogen replacement therapy at the lumbar spine in contrast to the femoral neck.[27-31] Probably, the effect of the ERα is more pronounced in the vertebral body, which is rich of trabecular bone, due to a higher rate of bone turnover. Trabecular bone has a higher rate of bone turnover than in cortical bone because trabecular bone presents relatively more surfaces per unit of bone volume.

We conclude that the *T* allele of the ERα PvuII RFLP is associated with low BMD and an increased risk of vertebral fractures, independently of BMD and menopause, and more pronounced in women than in men. Further studies are needed to elucidate the exact molecular mechanism underlying this association.

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3.2.1

Vitamin D receptor 3' polymorphism is associated with rate of bone turnover in women with low bone mineral density.

Abstract

A decreased bone mineral density is a strong risk factor of osteoporotic fractures, and is thought to result from changes in rate of bone turnover. Bone turnover rate has been shown to be under genetic control, but the genes involved remain ill defined. Active vitamin D ($1,25\text{-}(OH)_2D_3$) plays, via the vitamin D receptor (VDR), a central role in bone turnover rate. Therefore, we investigated whether genetic variations of the VDR could explain differences in bone turnover rate among 88 postmenopausal women with either a low (n = 41) or high (n = 47) BMD. Furthermore, we examined whether VDR genotype was associated with a divergent in biochemical response to a 7 days treatment with 2μg of $1,25\text{-}(OH)_2D_3$ orally. Indices of bone turnover were measured within overnight fastening urine and blood samples and VDR genotype was determined as haplotype of three clustered restriction fragment length polymorphism (*BsmI*, *ApaI* and *TaqI*) at the 3' end of the gene.

At baseline, the osteocalcin serum levels (p=0.09) and urinary Ntx/creatinine (p=0.02) ratio were increased in women having low BMD values compared to the high BMD group. Interestingly, in the low BMD group these biochemical markers were highest in women homozygous for haplotype 2 ('BAt') alleles, intermediate for the heterozygotes and lowest for the reference group without haplotype 2. The response of any of the biochemical markers to short-term substitution of 2ug $1,25(OH)_2D_3$ a day, did not differ significant among the BMD groups and responses were similar among the VDR genotypes.

In this pilot study, we observed higher osteocalcin and Ntx/creatinine levels in women having low BMD compared to women having high BMD. This increased bone turnover rate in women having low BMD was associated with VDR 3' haplotype polymorphism. This genotype-dependent bone turnover rate could not be explained by a divergent response to $1,25(OH)_2D_3$.

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INTRODUCTION

Fractures, the clinically significant endpoint of osteoporosis, are a major cause of increasing morbidity affecting the elderly population. One of the strongest predictors of fractures is a decline of bone mineral density (BMD) independent of age.[1] A decrease in BMD with age is associated with both an increased bone turnover rate, i.e. the ratio of bone formation and bone resorption, and an imbalance in bone turnover, favouring bone resorption. Recently, twin studies showed that the rate of bone turnover is under strong genetic influence, but the genes involved remain ill defined.[2,3]

One approach to investigate which genes are associated with bone turnover rate is the candidate gene approach. Such candidate genes can be selected on the basis of their involvement in a particular biochemical pathway in bone metabolism. An important candidate gene in this respect is the vitamin D receptor (VDR) gene. The hormonally active form of vitamin D ($1,25\text{-}(OH)_2D_3$) plays a central role in bone metabolism and exerts its effect via the VDR. $1,25\text{-}(OH)_2D_3$ regulates growth and differentiation of osteoblasts and osteoclast, which are cells of bone formation and bone resorption, respectively.[4,5] In line with this, substitution with vitamin D reduces the incidence of fractures in the elderly, although conflicting results have been reported.[6] A possible explanation for at least part of these contrasting results might be that genetic variability of the VDR can diverse the regulating effect of $1,25\text{-}(OH)_2D_3$ on bone turnover. This might result in VDR genotype dependent variances in BMD, which ultimately leads to differences in fracture rate.[7-11]

In light of the above we conducted a study to investigate whether differences in rates of bone turnover among women with either a low or high BMD could be explained by genetic variations of the VDR. Furthermore, we examined whether VDR gene polymorphism influenced the biochemical response to short-term substitution of $1,25\text{-}(OH)_2D_3$ in both BMD groups.

MATERIAL AND METHODS

Subjects

The postmenopausal women included in this analysis were part of the Rotterdam Study, a population-based cohort study of persons aged 55 years and over, living in a district of Rotterdam, the Netherlands. The objective of the Rotterdam Study is to investigate the occurrence of chronic disabling diseases in relation to several potential determinants. Rationale and design have been described previously.[12] All 10,275 inhabitants were invited for the study between August 1990 and June 1993. Of those, 7,983 participated bringing the overall response rate to 78 percent.

For the present study we included a sample of independently living women who were part of a large (n = 1782) genetic-epidemiological study on osteoporosis.[10] In that study subjects were excluded according to the following criteria: aged 80 years and over, use of thyroid hormone, use of cytostatics, use of diuretics and known diabetes mellitus type II. We re-invited, four years after the start of the initial study on osteoporosis, women (n=202) who had a femoral neck BMD at baseline in the lowest quintile ($\leq$ 0.75 g/cm^2) and those who had a femoral neck BMD in the highest quintile ($\geq$ 0.92 g/cm^2). Because of recent use of drugs known to influence the bone metabolism, 30 women could not be included and 84 women were not willing to participate. This brought the total number of participating women on 88 (44%) with n=47 in the highest and n=41 in the lowest BMD group. This sub-study within the Rotterdam study was approved by the medical ethics committee of the Erasmus University Medical School, and renewed written informed consent was obtained from each woman.

Intervention

Each subject received two microgram (2-μg) 1,25-$(OH)_2D_3$ each morning during 7 consecutive days. Fastening blood and overnight urine samples were collected at three time points: baseline, day 4 and day 7. To assess compliance, capsules were counted at each visit.

Measurements

A. Bone mineral density

At baseline (start of the Rotterdam Study) measurement of femoral neck BMD (expressed in g/cm^2) was performed by dual energy X-ray absorptiometry (DXA, Lunar DPX-L densitometer) as described before.[13]

B. Biochemical markers

Fasting blood and overnight urine samples were stored frozen at –20° C until all samples were analyzed. Analysis was performed without prior knowledge of the bone mineral density and genotype status. We measured 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), 25-hydroxy vitamin D (25-OH-D), osteocalcin, parathyroid hormone (PTH) and cross-linked N-telopeptide of type I collagen (NTX). For the quantitative determination of serum 1,25-Dihydroxyvitamin D a ^{125}I-radioimmunoassay (RIA) was used (Nichols Institute Diagnostics, San Juan Capistrano, CA) and expressed as pmol/l. The 25-OH-D serum level was measured by using a ^{125}I-RIA kit (Incstar Corporation, Stillwater, Minnesota), and expressed as nmol/l. Serum osteocalcin was measured with a radioimmuno assay (Incstar Corp, Stillwater, Minnesota) and expressed as µg/l. Serum PTH was measured by using a radioisotopic-assay of the intact parathyroid hormone (Nichols Institute Diagnostics, San Juan Capistrano, CA) and expressed as ng/l. An enzyme-linked immunosorbent assay was used for the measurement of urinary Ntx and the Ntx/creatinine ratio was calculated and expressed as nmol/µmol. Urinary excretion of creatinine was measured by autoanalyzer techniques. The inter-assay and intra-assay coefficient of variation of all these assays varied between the 3-18 % and 2-19 %, respectively.

C. Genotyping

Three anonymous polymorphic restriction enzyme recognition sites at the 3' end of the VDR gene, i.e., for *BsmI*, *ApaI*, and *TaqI* were assessed in relation to each other by a direct molecular haplotyping PCR procedure which we developed.[10] The alleles were named similarly as previously described for alleles defined by individual RFLPs;[14,15] in genotypes such as "BAt-baT" capitals denote absence and lower case letters denote presence of the site

for the restriction enzymes *Bsm*I (B/b), *Apa*I (A/a), and *Taq*I (T/t) on each of the alleles. The haplotype alleles were coded 1-5 in order of decreasing frequency in the population; genotypes are presented as combinations of two alleles (1 = baT, 2 = BAt, and 3 = bAT). Genotype 14, 15, 25, 33, 34, 35, 44 and 55 were not present in this study population. Detailed information on haplotype alleles and genotype frequencies in a larger sample from the Rotterdam Study, including this population, can be found elsewhere.[10]

Statistical analysis

Data for each biochemical marker of bone turnover of each visit were first examined for deviation from normal distribution by using the Kolmogorov-Smirnov test. If deviation was statistically significant, log10 transformations were performed.

We performed all analyses separately for women with high and low BMD. The comparison of continuous variables among groups was done with a two-sample t-test or ANOVA test. The categorical characteristics were compared by Pearson's chi-square analysis. To adjust for potential confounders at baseline we used multiple regression analysis. Differences in response to 1,25-$(OH)_2D_3$ between groups were analyzed by using repeated measurement models taking each visit into account.

To analyze the relation between VDR genotype and biochemical markers of bone metabolism we stratified the subjects by carrier status of the three most frequent alleles, i.e., haplotype alleles 1, 2 and 3, and made groups of subjects carrying 0, 1 or 2 copies of the test allele. For example, for haplotype2, the reference group consisted of subjects with genotype 11 or 13, the heterozygote group consisted of subjects with genotype 12, 23 or 24 while the homozygote group consisted of subjects with genotype 22. Allele dose effects were analyzed by comparing individuals of the reference group to individuals being heterozygous and homozygous for the test allele.

RESULTS

BMD groups

General characteristics according to BMD groups are show in Table 1. Compared to women with high BMD values, BMI was lower ($p < 0.01$) in women having a low BMD. For

other characteristics like age, dietary calcium- and vitamin D intake there was no significant difference between the BMD groups.

Table 6 Characteristics according to BMD group

	Bone mineral density groups		p-value
	High	**Low**	
Number (%)	47	41	
Age (yrs)	65.3 (4.2)	66.5 (4.2)	0.2
BMI (kg/m^2)	25.9 (3.5)(	23.9 (2.9)	<0.01
Calcium intake (mg/day)	1201 (331)	1123 (370)	0.3
Vitamin D intake (mg/day)	1.81 (1.06)	1.98 (1.40)	0.5
Femoral neck BMD (g/cm^2)	1.01 (0.08)	0.66 (0.05)	< 0.001
Haplotype 1 alleles (%)	46 (48.9)	37 (45.1)	0.7
Haplotype 2 alleles (%)	40 (42.6)	38 (46.3)	0.7
Haplotype 3 alleles (%)	7 (7.4)	7 (8.5)	0.7
Biochemical markers o	**High**	**Low**	p-value
1,25(OH)$_2$D$_3$ (pmol/l)	99.3 (1.3)	95.5 (1.3)	0.6
25 (OH) Vitamin D (nmol/l)	74.1 (1.5)	67.6 (1.5)	0.3
Osteocalcin (µg/l)	4.5 (2.1)	5.3 (2.4)	0.09
Ntx / creatinine (nmol/µmol)	57.5 (1.9)	75.8 (1.7)	0.02
PTH (ng/l)	31.6 (1.5)	30.9 (1.6)	0.9

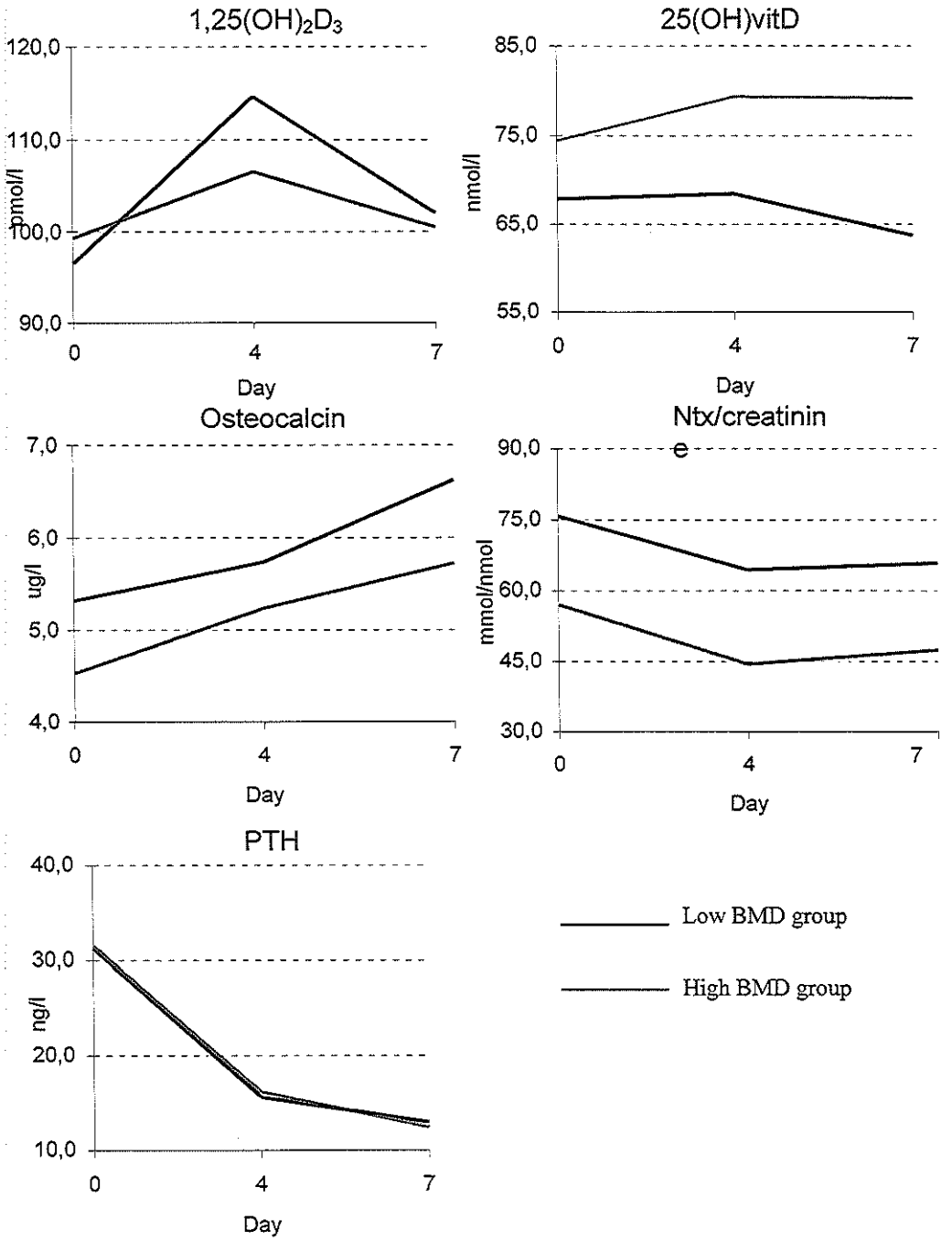
Values are number (%) or means (SD)

Biochemical markers

The distributions of values for biochemical markers of bone turnover were skewed at every visit, except for osteocalcin. Therefore, we log10 transformed those values to achieve normal distribution.

At baseline, increased osteocalcin serum levels (p=0.09) and urinary Ntx/creatinine (p=0.02) were observed in women having a low BMD compared to the high BMD group (Table 1). This observation did not essentially change after adjustment for age and BMI. All other levels of markers of bone metabolism were similar between women having either a low or high BMD.

Figure 1 Response in biochemical markers of bone turnover to $1,25(OH)_2D_3$ according to BMD group



Response to 1,25(OH)$_2$D$_3$

One woman stopped taking here medication at day two, bringing the compliance rate to 98% in the low BMD group whereas this was 100% in the high BMD group. We excluded this woman for further analysis on the response to 1,25(OH)$_2$D$_3$. Figure 1 shows the short-term response in biochemical markers of bone turnover on 1,25(OH)$_2$D$_3$ for women having either a low or high BMD. At day 4, both BMD groups showed a significant increase in 1,25(OH)$_2$D$_3$ levels ($p < 0.01$) and osteocalcin levels ($p < 0.01$), wheras the 25(OH)D level did not essentially change ($p=0.2$). A significant decrease was found for the Ntx/creatinine ratio ($p< 0.001$) and PTH levels ($p< 0.001$). At day 7, 1,25(OH)$_2$D$_3$ and 25(OH)D turned to baseline level, whereas osteocalcin increased further and Ntx/creatinine ratio and PTH remained low. The higher levels of biochemical markers of bone turnover at baseline observed in the low BMD group remained during follow-up. The absolute response to 1,25(OH)$_2$D$_3$ was similar between the two BMD groups. (Interaction terms for all biochemical markers $p > 0.5$).

VDR 3' haplotype

At baseline there were no significant differences among VDR 3' haplotype genotype groups concerning age, calcium- and vitamin D intake for both BMD groups measured (Table 2). A significant difference was observed in the high BMD group for femoral neck BMD when they were grouped according to VDR3'-haplotype 2. While BMI differed when they were grouped according to either VDR 3'haplotype 1 or haplotype 2 genotype. However, for both femoral neck BMD and BMI there was no evidence of a recessive, dominant or an allele-dose effect.

Biochemical markers

Table 3 shows the biochemical markers of bone metabolism at baseline according to VDR3' haplotype genotypes stratified by BMD group. Interestingly, in women with low BMD, the rate of bone turnover was observed to be genotype dependent in women carrying haplotype 2 with evidence of an allele dose effect. In the low BMD group, osteocalcin and Ntx/creatinine ratio were highest for the homozygote haplotype 2 group, intermediate for the heterozygote and lowest for the reference group. PTH, 1,25(OH)$_2$D$_3$ and 25(OH)D levels did not differ significantly among the subjects

Table 2 Characteristics according to carrier status for each VDR 3' Haplotype stratified by BMD group

	VDR 3' Haplotype							
	Haplotype 1			*Haplotype 2*			*Haplotype 3*	
High BMD	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**
Number (%)	16 (34)	16 (34)	15 (32)	18 (38)	18 (38)	11 (24)	40 (85)	7 (15)
Age (yrs)	66.8 (3.6)	64.3 (4.0)	64.7 (4.9)	64.9 (4.8)	64.3 (3.8)	67.5 (3.3)	65.4 (4.3)	64.9 (4.1)
BMI (kg/m^2)	25.2 (3.5)	28.1 (3.9)*	24.4 (1.7)	24.5 (2.0)	28.1 (3.9)*	24.7 (3.3)	25.9 (3.5)	26.0 (3.9)
Calcium intake (mg/day)	1248 (348)	1286 (324)	1032 (279)	1067 (263)	1301 (360)	1232 (332)	1196 (333)	1229 (346)
Vitamin D intake (mg/day)	1.50 (0.76)	1.96 (0.97)	1.97 (1.44)	2.08 (1.32)	1.90 (0.98)	1.25 (0.49)	1.72 (1.07)	2.33 (0.94)
Femoral neck BMD (g/cm^2)	1.05 (0.10)	0.98 (0.05)	1.01 (0.06)	1.01 (0.06)	0.99 (0.06)	1.07 (0.11)*	1.02 (0.08)	0.99 (0.09)
Low BMD	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**
Number (%)	13 (32)	19 (46)	9 (22)	10 (24)	24 (59)	7 (17)	34 (83)	7 (17)
Age (yrs)	64.7 (3.9)	67.1 (4.3)	67.9 (4.2)	67.6 (4.2)	67.0 (4.2)	63.3 (2.5)	66.6 (4.3)	66.0 (3.7)
BMI (kg/m^2)	23.7 (2.6)	24.2 (3.1)	23.6 (3.4)	23.4 (3.2)	24.3 (2.9)	23.3 (2.7)	23.9 (3.0)	23.8 (2.6)
Calcium intake (mg/day)	1064 (372)	1145 (325)	1171 (467)	1191 (444)	1165 (348)	901 (270)	1090 (367)	1270 (373)
Vitamin D intake (mg/day)	2.43 (1.96)	1.82 (1.13)	1.61 (0.57)	1.56 (0.56)	2.01 (1.15)	2.50 (2.55)	1.94 (1.46)	2.17 (1.16)
Femoral neck BMD (g/cm^2)	0.65 (0.05)	0.68 (0.05)	0.66 (0.03)	0.66 (0.03)	0.67 (0.06)	0.66 (0.04)	0.67 (0.04)	0.65 (0.06)

Values are means (SD) or numbers (%)
*p-value ANOVA <0.05

Table 3 Baseline levels of biochemical markers of bone turnover (mean (SD)) according to VDR 3' haplotype stratified by BMD group

	VDR 3' Haplotype							
	Haplotype 1			*Haplotype 2*			*Haplotype 3*	
High BMD	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**
Number (%)	16 (34)	16 (34)	15 (32)	18 (38)	18 (38)	11 (24)	40 (85)	7 (15)
$1,25(OH)_2D_3$ (ρmol/l)	93.3 (1.3)	100.0 (1.3)	104.7 (1.4)	104.7 (1.4)	100.0 (1.4)	89.1 (1.3)	97.7 (1.3)	104.7 (1.5)
25 (OH) Vitamin D (nmol/l)	81.3 (1.2)	63.1 (1.7)	79.4 (1.4)	77.6 (1.4)	70.8 (1.7)	74.1 (1.3)	72.4 (1.5)	85.1 (1.4)
Osteocalcin (μg/l)	4.56 (2.0)	4.51 (1.9)	4.51 (2.4)	4.44 (2.2)	4.74 (1.8)	4.31 (2.3)	4.52 (2.2)	4.60 (0.6)
Ntx / creatinine (nmol/μmol)	50.1 (2.2)	64.6 (1.5)	57.5 (2.1)	57.5 (2.0)	67.6 (1.4)	41.7 (2.4)	56.2 (2.0)	64.6 (1.6)
PTH (ng/l)	28.2 (1.5)	35.5 (1.6)	30.9 (1.5)	24.0 (1.5)	35.5 (1.6)	29.5 (1.6)	31.6 (1.5)	30.2 (1.4)
Low BMD	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**
Number (%)	13 (32)	19 (46)	9 (22)	10 (24)	24 (59)	7 (17)	34 (83)	7 (17)
$1,25(OH)_2D_3$ (ρmol/l)	102.3 (1.3)	93.3 (1.3)	93.3 (1.2)	95.5 (1.2)	91.2 (1.3)	117.5 (1.3)	97.7 (1.3)	91.2 (1.3)
25 (OH) Vitamin D (nmol/l)	75.9 (1.6)	66.1 (1.6)	61.7 (1.0)	64.6 (1.3)	63.1 (1.6)	93.3 (1.5)	69.2 (1.5)	61.6 (1.5)
Osteocalcin (μg/l)	5.43 (2.2)	5.45 (2.6)	4.89 (2.1)	4.40 (2.5)	5.34 (2.4)	6.57 (1.6)*	5.69 (2.2)	3.53 (2.6)
Ntx / creatinine (nmol/μmol)	89.1 (1.9)	70.8 (1.7)	74.1 (1.4)	69.2 (1.5)	72.4 (1.8)	109.6 (1.6)*	79.4 (1.6)	64.6 (2.0)
PTH (ng/l)	29.5 (1.5)	36.3 (1.6)	24.5 (1.6)	24.0 (1.5)	35.5 (1.5)	28.8 (1.7)	31.6 (1.6)	28.2 (1.4)

p-value allele dose effect =0.06

grouped by VDR3' haplotype 2. After adjustment for potential confounders like age and BMI, these differences did not essentially change. For haplotype 1 and haplotype 3 a less obvious but inverse relation was found. In women with high BMD we did not observe such a genotype-dependent association.

Response to 1,25(OH)$_2$D$_3$

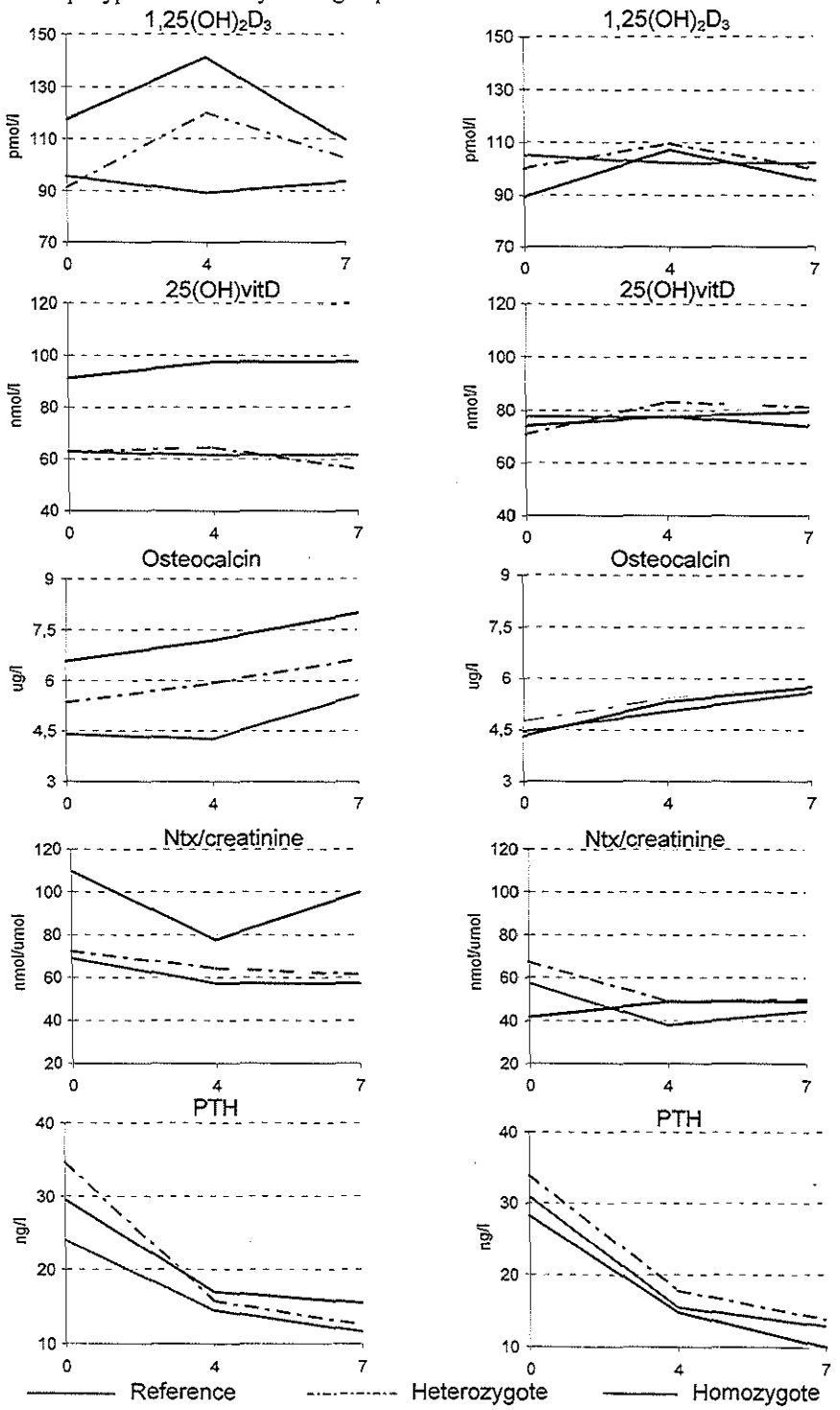
Figure 2 shows the response in biochemical markers of bone turnover to 1,25(OH)$_2$D$_3$ among VDR haplotype 2 genotype groups. The observed VDR genotype dependent effect on the biochemical markers of bone metabolism in women having low BMD at baseline remained consistent during the follow-up visits. The absolute response of none of the biochemical markers to 1,25(OH)$_2$D$_3$ differed significantly among the haplotypes. However, the homozygote haplotype 2 group showed maximum increase of 1,25(OH)$_2$D$_3$ levels at day 4 while the levels remained similar in the reference group. For haplotypes 1 and 3 no difference in response to 1,25(OH)$_2$D$_3$ was found.

DISCUSSION

In this study performed in postmenopausal women, we observed higher osteocalcin and Ntx/creatinine levels in women having low BMD compared to women having high BMD. Interestingly, this increased bone turnover rate in women having low BMD was associated with VDR polymorphism measured as haplotypes of 3 RLFP sites (*Bsm*I, *Apa*I and *Taq*I) at the 3' end of the VDR gene. Furthermore, this VDR3' genotype dependent bone turnover rate could not be explained by difference in sensitivity for 1,25(OH)$_2$D$_3$ of the different VDR genotypes.

Before interpreting the results some elements have to be discussed. With respect to the design of the study, the number of participants was small resulting in insufficient power to detect small differences. For example, the estimated power within this study group of 88 women was 40% for the observed differences in osteocalcin levels between the BMD groups. To get sufficient power of 80% at least 240 participants had to be studied. It is therefore important to look for consistency in the effects. Several studies have shown that the fastening morning samples are most accurate for

Figure 2 Response in biochemical markers of bone turnover to $1,25(OH)_2D_3$ according to VDR3' haplotype 2 stratified by BMD group



measuring bone turnover rate.[16] We used overnight fastening urine samples and therefore, circadian variances of bone markers is not likely to influence the results Furthermore, since the inter- and intra coefficients of variation of the assays of bone markers are high, the observed effect on the regulation of the calcium and bone metabolism after stimulation of $1,25(OH)_2D_3$ may have been imprecise. The study population has a relatively high calcium- and vitamin D intake compared to other populations, which might lead to an underestimation of the treatment effect.[17,18] In our study the biochemical markers of bone turnover were determined four years after BMD was measured. It is unlikely however, that low BMD leads to a higher rate of bone turnover while having low BMD as a results of a high bone turnover rate is more likely.[19] Furthermore, we do not expect that these shortcomings of the study are genotype dependent, therefore it should not have influenced the true association between genotypes and biochemical markers of bone turnover.

Regarding the short-term response to $1,25(OH)_2D_3$ in general, our data confirm observations made by others who also showed an increase in osteocalcin levels and a decrease in PTH.[20-24] Interestingly, those studies observed an increase in bone resorption in contrast to the decreased Ntx/creatinine ratio of the present study.[23,24] The decreased bone resorption rate can be explained by a decrease in PTH, which is the most prominent effect modifier of treatment with $1,25(OH)_2D_3$,[25-27] Furthermore, those studies used other bone resorption markers while the Ntx/creatinine ratio is the most sensitive and specific marker of bone resorption.[28]

In our study the association between VDR polymorphism measured as haplotypes of 3 RLFP sites (*Bsm*I, *Apa*I and *Taq*I) at the 3' end and bone turnover rate was only obvious in women having low BMD values. A possible explanation for this contradicted finding might be residual confounding like osteoarthritis for example that could be more frequent in the high BMD group. Individuals with osteoarthritis have increased growth hormone levels that subsequently leads to higher rates of bone turnover.[29-31] In contrast to this, individuals not carrying a VDR haplotype 2 allele are more at risk for osteoarthritis.[32] Therefore theoretically, the observed lower bone turnover rate in the reference group (individuals not carrying haplotype 2 alleles) compared to individuals carrying a haplotype 2 allele, could have been neutralised by the

growth hormone induced increased bone turnover rate in the reference group. Subsequently this would have diluted the difference among the VDR haplotype genotypes.

Several studies have analyzed the relation between VDR 3' polymorphism and bone turnover.[7,9,14,33,34] Our data are in line with observations by others who used only the *Bsm*I RFLP and showed that the baseline level of markers of bone turnover (in particular osteocalcin) is higher in the *BB* genotype.[9,14,33] The *BB* genotype group corresponds to the homozygous haplotype 2 carriers (see methods). Thus, putting all the data together we conclude that the haplotype 2 allele is associated with an increased rate of bone turnover favoring bone formation, which implies that other haplotypes, i.e. haplotype 1 and/or 3, are associated with lower bone formation levels. In view of the close relation between bone turnover and BMD this in turn might be reflected in lower BMD values in subjects carrying those haplotypes and/or in increased fracture risk. Indeed, in a much larger sample of this study population we observed haplotype 3 to be associated with low BMD,[10] while we have recently observed haplotype 1 to be associated with increased fracture risk[11]

Concerning the short-term response to $1,25(OH)_2D_3$ we did not see a significant genotype effect as observed by Howard et al. in Australian women.[33] A possible explanation might be that we studied postmenopausal women whereas they only included premenopausal women (including two pair of dyzygotic twins). Premenopausal women are known to have substantial variance in bone turnover rates during this stage in life. [35] Therefore, the observed difference between the genotypes in the Australian study could have been confounded by age differences between the genotypes. Nevertheless, in the present study we showed a continuous increase in bone formation rate during short-term administration of active vitamin D whereas the rate of bone resorption remained stable after 4 days. This suggests that during long term administration of vitamin D, the relatively higher bone turnover rate in individuals carrying haplotype 2 alleles will be superimposed by a higher rate of bone formation that might lead to different BMD values on the long-term. This notion is supported by the observation reported by Graafmans et al. who showed a significant increase in BMD in postmenopausal women homozygous for the '*B*' allele of the *Bsm*I RLFP (corresponding to haplotype 2) after a 2 year administration of vitamin D.[36]

The precise molecular mechanism causing the observed effect of the VDR 3' gene polymorphism in relation to bone turnover remains to be elucidated. The polymorphisms we analyzed are anonymous and, therefore, are not likely by them selves to explain the associations observed. Supposedly they are in linkage disequilibrium with a truly functional polymorphism elsewhere in the VDR gene. Recent studies from our laboratory indicate that the VDR 3' haplotypes accurately indicate a linkage group of several polymorphisms across at least 20 kb in this part of the gene. (Fang et al., manuscript in preparation) They can therefore be considered to be helpful markers to find the functional sequence variants.

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3.2.2

Consequences of vitamin D receptor gene polymorphisms for growth inhibition of cultured human peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D_3

Abstract

In the vitamin D receptor (VDR) gene a *BsmI* restriction fragment length polymorphism (RFLP) in intron 8 and a translational start-site polymorphism, identified as a *FokI* RFLP, have been described. Crucial for a proper interpretation of these polymorphisms in association studies is the knowledge whether they have direct consequences for 1,25-$(OH)_2D_3$ action at cellular level. The present study was designed to assess functional significance of the *FokI* and *BsmI* VDR gene polymorphisms in peripheral blood mononuclear cells (PBMC) with a natural occurring VDR genotype for cell growth inhibition by for 1,25-$(OH)_2D_3$.

PBMC of women were isolated, VDR genotyped and *in vitro* inhibition by 1,25-$(OH)_2D_3$ of Phytohemagglutinin (PHA)-stimulated growth of PBMC was examined in relation to VDR genotype.

PHA-stimulated growth and maximal growth inhibition were independent of VDR genotype. However, the FF genotype had a significant lower ED_{50} than the Ff genotype corresponding to an allele dose effect of 0.32 nM per f allele copy ($p=0.0036$). For *BsmI* genotypes no differences in ED_{50} were observed.

The present study demonstrates for the first time in cells with a natural VDR genotype a direct functional consequence of the VDR gene translational start-site polymorphism for the action of 1,25-$(OH)_2D_3$. Especially under conditions of vitamin D insufficiency these findings might have clinical implications.

INTRODUCTION

Over the last years the genetic basis of osteoporosis has been intensively studied. In relation to this, several polymorphisms in the vitamin D receptor (VDR) gene have been identified. Firstly, the *BsmI* restriction fragment length polymorphism (RFLP) is located in intron 8 at the 3' end of the VDR gene. Several studies have shown an association between this VDR polymorphism and bone mineral density,[1-4] but other studies have not found such relationship.[5-7] Putting all these data together, there seems to be a weak association with bone mass.[8] Also associations with other phenotypes, like primary hyperparathyroidism, prostate cancer, and radiographic osteoarthritis have been described.[9-12]

Recently, a polymorphism of the translational start-site has been identified in the VDR gene.[13-16] This polymorphism is characterised by the presence of either two ATG start codons separated by 6 nucleotides or due to a T to C substitution in the 5'-ATG site only the presence of the most 3'-ATG codon. The presence of the 5'-ATG-site results in recognition sequence for the *FokI* restriction enzyme.[17] For this VDR polymorphism also both the presence and the lack of association with bone mineral density has been described.[16-19] In contrast to the *BsmI* polymorphism, the *FokI* polymorphism has distinct structural consequences for the VDR. The absence of the *FokI* restriction site, indicated as F, predicts a VDR protein of 424 amino acids, whereas the presence of the *FokI* site results in a VDR of 427 amino acids.

Crucial for proper interpretation of genetic association studies is the demonstration of functional consequences of these polymorphisms. For the *BsmI* polymorphism some *in vivo* studies have been performed showing potential association with parameters related to bone turnover and bone mineral density.[20-24] However, these *in vivo* studies are complex and do not provide direct insights into the consequences for $1,25\text{-}(OH)_2D_3$ action at cellular level. A few *in vitro* studies have been performed to address functionality of VDR gene polymorphisms. In these studies cells were transfected with either one of the VDR genotypes.[1,16] A potential disadvantage of transfecting cells with VDR genotypes is the absence of control over subtle differences in expression of the gene of interest. Especially, these differences may be very important for the differential phenotypic effect of natural occurring gene polymorphisms. The present study was designed to investigate the functional consequences of the *BsmI* and *FokI*

RFLP of the VDR gene for the action of 1,25-$(OH)_2D_3$ in cells, peripheral blood mononuclear cells (PBMC) expressing the natural VDR genotypes. PBMC form a readily accessible target of vitamin D which is used as a model for studying genotype-dependent 1,25-$(OH)_2D_3$ effects.

MATERIALS AND METHODS

Subjects

Fasting blood samples from 72 healthy postmenopausal women, aged 59 to 75 years (mean age ± SD = 65.7 ± 4.3 years) were taken. Women were randomly selected from independently living subjects of the Rotterdam Study[25] whereby those using hormonal replacement therapy, cytostatics, vitamin D, thyroid hormone or known to have diabetes were excluded. Four women were excluded from analysis as no growth dose-response curves were present because no genotype data were available. Written informed consent was obtained from each participant. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam.

Cell culture

PBMC were prepared from heparinized blood by Ficoll-Hypaque (Pharmacia, Sweden) density gradient centrifugation.[26] The cells were suspended in phenol red-free RPMI 1640 medium (Sigma Chemical Co, St. Louis, MO) supplemented with 10% charcoal-treated FCS, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Breda, The Netherlands) and 24 mM sodium bicarbonate. Immediately after isolation the cells were used for the proliferation analysis. For this purpose cells ($4,4 \times 10^6$ cells per ml) were cultured for 96 hrs in 24-wells plates (300 µl/well) in the presence of 3 µg/ml phytohemagglutinin (PHA) and 10^{-10} M to 10^{-7} M 1,25-$(OH)_2D_3$ (kindly donated by Dr. L. Binderup of LEO Pharmaceutical Products, Ballerup, Denmark). At the end of the incubation, DNA content of adherent and non-adherent cells was measured according to the ethidium bromide method of Karsten and Wollenberger.[27] For each individual two PBMC cultures were performed each consisting of two DNA measurements.

Due to ethical reasons it was not possible to perform both PBMC growth analyses and VDR measurements in one and the same individual. For the PBMC growth study 3-4 tubes of blood were needed. In addition to this 3-4 other tubes had to be taken for clinical analyses. This would mean that over 10 tubes of blood from volunteers were needed in order to have at least the possibility to measure VDR. For measurement of VDR in monocytes only we have calculated that even 21 tubes would be necessary.

Table 1: Growth stimulation by PHA and maximal growth inhibition by $1,25\text{-}(OH)_2D_3$ of PBMC according to VDR genotype.

Genotype	n (%)*	PHA-stimulated growth§ (fold-stimulation)	Maximal $1,25\text{-}(OH)_2D_3$ effect (% inhibition)
All#		2.25 ± 0.07	72.8 ± 1.46
FF	34 (50.0)	2.18 ± 0.10	71.0 ± 2.15
Ff	25 (36.8)	2.28 ± 0.12	71.6 ± 2.18
ff	9 (13.2)	2.31 ± 0.19	79.5 ± 3.73
BB	10 (15.2)	2.31 ± 0.14	72.4 ± 4.55
Bb	35 (53.0)	2.21 ± 0.11	73.7 ± 5.91
bb	21 (34.9)	2.30 ± 0.13	71.5 ± 2.33

* n = the number of subjects.
§ PHA growth is expressed as fold stimulation over cell growth in the absence of PHA.
# All = Effect independent of genotypes. Data are presented as means SEM.

Genotyping procedure

The *BsmI* RFLP at the 3' end of the VDR gene was assessed by a direct haplotyping PCR procedure as previously described.[4] The *FokI* RFLP was analysed by PCR with the primers described previously.[17] The amplification protocol consisted of 28 cycles of 94, 60 and 72°C for 1 minute each. Ten microliters of the PCR products were digested with 10 U *FokI* and 1.2 µl of a 10x buffer (containing 150 mM Tris-HCl, pH 7.5, 250 mM NaCl and 35 mM $MgCl_2$) by incubating for 30 minutes at 37°C. Digestion products were analyzed on a 3% NuSieve agarose

gel run in 0.5X TBE. Capital letters denote absence and lowercase letters the presence of the site for the restriction enzyme *BsmI* (*B/b*) or *FokI* (*F/f*).

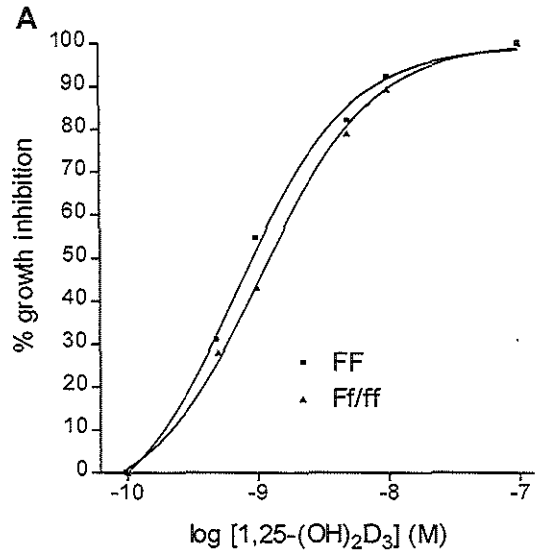
Statistical analysis

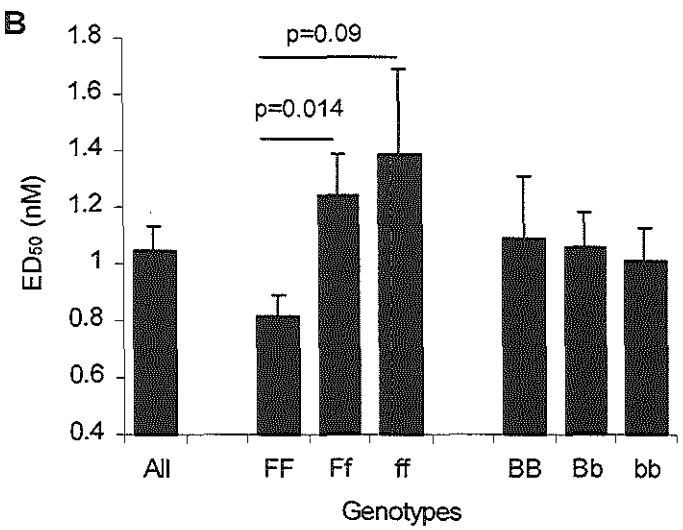
For each individual a best fitted growth curve and maximal inhibition and ED_{50} values were calculated and analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, USA). Next, per genotype the mean maximal inhibition and ED_{50} (± SE) were calculated. Analysis of variance (ANOVA) was performed and for pairwise comparison student's *t*-test was used. The relation between allele dose and ED_{50} was quantified by linear regression analysis.

RESULTS

As shown in Table 1 incubation of PBMC with PHA resulted in an almost 2.3 times stimulation of growth compared to control cells. PHA-stimulated cell growth was strongly dose-dependently inhibited by 1,25-$(OH)_2D_3$ (Figure 1A) with a maximal inhibition of about 75% at 10^{-8} - 10^{-7} M (Table 1). The half-maximal inhibition was achieved at 1.05 nM 1,25-$(OH)_2D_3$ (Figure 1B). Neither PHA-stimulated cell growth nor maximal inhibition by 1,25-$(OH)_2D_3$ appeared to be VDR genotype dependent. However, the ED_{50} showed differences between *FokI* genotypes in a gene-dose dependent manner (Figures 1A and B). The PMBCs with the *FF*-genotype had the lowest ED_{50} value (0.82 ± 0.072) *Ff* heterozygotes had an intermediate ED_{50} value (1.24 ± 0.15), while *ff* homozygotes had the highest ED_{50} value (1.39 ± 0.3). Linear regression analysis demonstrated an allele dose effect of 0.32 nM per *f* allele copy (P=0.0036). No significant differences were observed when the 3'-end of the VDR was genotyped by the direct haplotyping procedure,[4] which may be due to too low numbers per genotype group to allow analysis (data not shown). Also when the analysis was restricted to the single *BsmI* polymorphism no with differences in ED_{50} (Figure 1B) and maximal inhibition were observed (Table 1). The *ApaI* and *TaqI* polymorphisms were also not associated with differences in maximal inhibition by 1,25-$(OH)_2D_3$ and ED_{50} (data not shown).

Figure 1





Dose-dependent inhibition of PHA-stimulated growth by 1,25-$(OH)_2D_3$ (A) and calculated ED_{50} of PMBC growth inhibition according to the *FokI* and *BsmI* VDR genotypes (B). For clarity reasons the dose-response curves of Ff and ff are combined. Data are transformed according to a maximal inhibition of 100 % (A). Data are presented as mean ± SEM (B). *FF*: number (n) = 34; *Ff*: n = 25; *ff*: n = 9; *BB*: n = 10; *Bb*: n = 35; *bb*: n = 21. All = ED_{50} independent of genotypes.

DISCUSSION

The current study demonstrates direct functional consequences of natural occurring VDR gene polymorphisms for the cellular action of 1,25-$(OH)_2D_3$ in PBMC of healthy postmenopausal women. In most previous studies the functional consequences of VDR genotypes at the cellular level were only studied in cells transfected with different VDR alleles.[1,16,28] Our study demonstrates that the half maximal concentration for 1,25-$(OH)_2D_3$ inhibition of PHA-stimulated growth is significantly different between PBMC characterized by different alleles of the VDR translational start-site while the maximal inhibition is similar for all genotypes. The ED_{50} for cells homozygous for the 424 amino acids long VDR, i.e. the *FF* genotype, is lower than the ED_{50} for cells containing the 427 amino acids long VDR, i.e. the *Ff* and *ff* genotypes. A significant allele dose effect was observed which means that PBMC containing both forms of the VDR, the heterozygotes, have an intermediate ED_{50}. Arai et al. have also shown a more potent effect by the short VDR using HeLa cells transfected with a VDR *FokI* genotype and a 24-hydroxylase VDRE-reporter construct. They only tested one concentration of 1,25-$(OH)_2D_3$ and it is not clear whether this reflects the maximal concentration or approaches the half-maximal effective dose for the response analyzed by Arai et al.[16] Also others showed a more active 424 amino acids VDR,[29,30] while one other study did not observe differences between VDR *FokI* genotypes.[28] Together, our data support the hypothesis that the 424 amino acids-long VDR is more efficient in exerting 1,25-$(OH)_2D_3$ effects than the 427 amino acids-long VDR.

Although we were not able to measure VDR levels in the same PMBC of the individuals used in the growth study (see Materials and Methods), it is unlikely that the differences in effect are due to increased VDR expression by the *FF* genotypes.[16] Arai et al.,[16] even reported that the *FF* genotype appears to have a somewhat (± 20 %) reduced VDR expression. Maybe the numbers of ff are too low to show significance, but the somewhat higher maximal inhibition by the ff genotypes may reflect an increased VDR level to compensate for the lower affinity.

In view of the localization of the polymorphism in the N-terminal A/*B* region of the VDR it is unlikely that it will have an effect on 1,25-$(OH)_2D_3$ binding to the ligand binding domain in

C-terminal E/*F* region. It is tempting to speculate that the difference in amino terminal sequence between the VDR genotypes directly affects binding of the VDR to its target genes. In this respect it is noteworthy that the VDR has a very short A/*B* region and, therefore, the sequence variation is located close to the DNA binding domain (C-region). Interesting in this respect are the observations by Jurutka et al.[29] that the 424 amino acids VDR interacts more efficiently with the transcription co-factor TFIIB and possesses elevated transcriptional activity compared to the 427 amino acids VDR.

The VDR *FokI* genotype effect on ED_{50} and not on maximal inhibition has potential clinical significance. In this way it is conceivable that dependent on the vitamin D status a VDR genotype effect is present or absent. So, especially under conditions of vitamin D insufficiency, for instance in elderly subjects, the biological consequences may become apparent, while at sufficient 1,25-$(OH)_2D_3$ levels the genotype consequences will be absent.

In contrast to the *FokI* RFLP, no relationship was observed between the VDR *BsmI* RFLP and 1,25-$(OH)_2D_3$ action which is in line with earlier findings.[31] An explanation for the dissociation with some *in vivo* data showing an association between *BsmI* VDR genotypes and response to 1,25-$(OH)_2D_3$ is unknown and yet purely speculative.[19-23] Considering the localization of the *BsmI* polymorphism it is unlikely that it will have a direct structural effect on the VDR. The *BsmI* RFLP can be in linkage with polymorphisms in the 3' UTR,[1,11] and in this way be related to stability of VDR mRNA and VDR expression.[32] Maybe the present experimental system is not sensitive sufficient to observe effects of potential differences in VDR mRNA stability between *BsmI* genotypes. Some data showed that the *BsmI* RFLP does not affect the abundance of the VDR mRNA,[33] while other studies indicated a difference in abundance of VDR mRNA between TaqI genotypes without an effect on VDR mRNA stability.[34] Unfortunately as mentioned above, it was not possible to relate our present observations directly to VDR levels in PBMCs of the individuals in this study. Although the 3' polymorphisms are not in linkage with the 5'-start site polymorphism analysis of combinations VDR genotypes may reveal further diversity in VDR activity.[30] Preliminary analysis of the various combinations of *FokI* and *BsmI* genotypes in the current study didn't provide additional information over the *FokI* analysis alone. It must, however, be noted that the number of individuals per genotype combination group is low and limits statistical analysis.

In conclusion, the current study provides evidence for direct functional consequences of the VDR translational start-site polymorphism for the cellular action of 1,25-$(OH)_2D_3$ and provides insights into the VDR genotype-phenotype association studies. The relationship with 1,25-$(OH)_2D_3$ concentrations is interesting and potentially important under conditions related to low 1,25-$(OH)_2D_3$ levels like vitamin D deficiency and renal impairment. More general, the present data demonstrate that on basis of natural occurring variants of a single gene differences in responsiveness between individuals may occur. Finally, it should be noted that in the present study one specific response of one specific target cell of 1,25-$(OH)_2D_3$ has been examined. For a full 1,25-$(OH)_2D_3$ response interaction of the VDR with co-factors (e.g. TFIIB, see above) is essential and these co-factors may vary between responses and cell types. Therefore, additional analyses of other responses and cell types are needed to further assess the generality of the concept that the 424 amino acids VDR is the more active form.

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3.3

Interaction between the vitamin D receptor gene and collagen type Iα1 gene in susceptibility for fracture

ABSTRACT

Osteoporosis is a common disease with a strong genetic component. Polymorphisms in the vitamin D receptor (VDR) gene have been implicated in osteoporosis but explain only a small part of the genetic effect on bone mineral density (BMD) while their effect on fractures is still uncertain. Recently, a G to T polymorphism in a Sp1 site in the collagen type Iα1 (COLIA1) gene was found to be associated with reduced BMD and with increased fracture risk. To analyse the combined influence of polymorphisms in the VDR gene and the COLIA1 gene in determining the susceptibility to osteoporotic fracture, we studied 1004 postmenopausal women. The "baT" VDR haplotype, constructed from three adjacent restriction fragment length polymorphisms, was found to be overrepresented among fracture cases (p=0.009). This corresponded to an Odds Ratio of 1.8 (95% confidence interval 1.0 - 3.3) for heterozygous carriers and 2.6 (95%CI 1.4 - 5.0) for homozygous carriers of the risk haplotype. The effect was similar for vertebral and non-vertebral fractures and, most importantly, independent of BMD. We observed significant interaction (p=0.03) between VDR and COLIA1 genotype effects. Fracture risk was not VDR genotype-dependent in the COLIA1 "reference" group (genotype GG) while in the COLIA1 "risk" group (genotypes GT and TT) the risk of fracture was 2.1 (95%CI 1.0 - 4.4) for heterozygous and 4.4 (95%CI 2.0 - 9.4) for homozygous carriers of the VDR risk haplotype. We conclude that both the VDR and COLIA1 polymorphisms are genetic markers for osteoporotic fracture in women, independent of BMD. Our data indicate that interlocus interaction is likely to be an important component of osteoporotic fracture risk.

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INTRODUCTION

Osteoporosis is a common disease characterized by reduced bone mineral density (BMD), deterioration of bone microarchitecture and increased fracture risk.[1] Low bone mineral density (BMD) is an important risk factor for fractures, the clinically most relevant feature of osteoporosis. Different aspects of osteoporosis have been shown to have genetic components. Twin- and family studies have suggested that BMD has a strong genetic component[2-6] and is under polygenic control.[7,8] In addition, biochemical markers of bone turnover,[9-11] and measurements of bone geometry and structure such as hip axis length and ultrasound measurements of the calcaneus have been shown to have strong genetic components.[12] Finally, maternal history of fracture is strong risk factor for hip fracture in older women, independent of BMD, suggesting heritability of hip fracture.[13]

Several candidate genes have been analysed in relation to BMD but the first and most widely studied gene in this respect, the vitamin D receptor (VDR) gene,[14] explains only a small part of the genetic effect on BMD.[15-17] Indeed, in our study population of elderly subjects we previously demonstrated a small effect of VDR genotype on BMD.[18]

Most genetic analyses have focussed on BMD as a determinant of fracture risk and not so much on fractures themselves as an endpoint in the analysis. While a recent study[19] and an early unpublished report[20] suggested VDR genotype to predict osteoporotic fracture, other studies have not found significant associations between VDR polymorphisms and fracture.[21,22] Recently, the T allele of a polymorphism in an Sp1 binding site in the first intron of the COLIA1 gene, encoding the most abundant bone matrix protein, was found to be associated with reduced BMD and, more importantly, also with increased risk of osteoporotic fracture.[23,24] Interestingly, the COLIA1 genotype-dependent fracture risk was largely independent of BMD.[24] This suggests a genetic effect on the pathogenesis of osteoporotic fracture by mechanisms that are, at least in part, independent of an effect on BMD.

Osteoporosis can be considered a complex genetic trait with variants of several genes underlying the genetic determination of the variability of the phenotype. Sofar no studies have addressed the possible interaction between osteoporosis candidate genes in relation to the

clinically most important endpoint, i.e. fractures. The COLIA1 gene and the VDR gene are interesting genes in this respect because the VDR is a transcription factor also regulating the expression of the COLIA1 gene.[25,26] There is now increasing evidence that a VDR allele carrying a particular haplotype of the three 3' RFLPs (baT; haplotype 1) is associated with aberrant expression levels of VDR mRNA, possibly through changes in mRNA stability.[14,27-29] Genetic variation in the VDR gene can therefore be expected to influence the association of COLIA1 gene variants with osteoporosis. Thus, we first analysed the relation between fractures and VDR genotype and, second, studied interaction between the VDR and the COLIA1 polymorphisms.

METHODS

Study subjects.

The Rotterdam Study is a population-based cohort study of 7983 subjects aged 55 or more years, residing in the Ommoord district of the city of Rotterdam in the Netherlands. The study was designed to document the occurrence of disease in the elderly in relation to several potential determinants.[30] A total of 10,275 persons, of whom 9161 (89 %) were living independently, were invited to participate in the study in 1991. In the independently living subjects, the overall response rate was 77 percent for home interview and 71 percent for examination in a research center, including measurement of anthropometric characteristics, bone mineral density and blood sampling. The Rotterdam Study was approved by the Medical Ethics Committee of the Erasmus University Medical School and written informed consent was obtained from each subject. The analysis of the association between VDR genotype, COLIA1 genotype and osteoporotic fracture was performed in a subgroup of women participating in the study. Baseline measurements of bone mineral density were available for 5931 independently living subjects from the study, but 1453 of these were excluded on the basis of age (>80 yrs), use of a walking aid, diabetes mellitus or use of diuretic, estrogen, thyroid hormone or cytostatic drug therapy. From the 4478 remaining subjects, we studied a random sample of 1500 women aged 55 to 80 years. Anthropometric data, DNA samples or genotype data for both loci were not

available for 481 women, and we excluded women with the rare VDR haplotypes 4 and 5 (n=15) resulting in a final study group of 1004 women.

Measurements.

Height and weight were measured at the initial examination. Bone mineral density (in g/cm^2) was determined by Dual Energy X-ray Absorptiometry (Lunar DPX-L densitometer; Lunar Corporation, Madison, WI, USA) at the femoral neck and lumbar spine (vertebrae L2 to L4) as described elsewhere.[31] Dietary intakes of calcium (mg/day) during the preceding year were assessed by food frequency questionnaire and adjusted for energy intake. Age at menopause and current cigarette smoking was assessed by questionnaire. For 732 women (73 percent), lateral radiographs of the spine from the fourth thoracic to the fifth lumbar vertebrae were obtained at baseline examination and analysed for the presence of prevalent vertebral fractures by morphometric analysis as previously described.[32] The occurrence of incident non-vertebral fractures, including hip, wrist and other fractures, were recorded, confirmed and classified by a physician over a mean follow-up period of 3.8 years, as described previously.[33] In brief, follow-up for fractures was achieved through a link with the computer systems of the general practitioners of the district and on hospital admission data, covering about 80% of the study population. For all participants not covered by this system, annual checks were performed on the complete medical records of their general practitioners. Reported fractures were verified by retrieval and review of the appropriate discharge reports from the patient record. In total, 49 prevalent vertebral fracture cases and 52 incident non-vertebral fracture cases were recorded (7 hip, 6 upper humerus, 22 wrist, 4 hand, 4 ankle, 3 foot, and 5 other fractures). Four subjects, in which both a vertebral and a non-vertebral fracture were present, were each counted as one fracture case, resulting in 97 cases with one or more fractures.

Determination of COLIA1 and VDR genotypes

Genomic DNA was extracted from peripheral venous blood samples according to standard procedures and the polymorphism in the COLIA1 gene was detected by PCR with a mismatched primer that introduces a di-allelic restriction site, as previously described.[23] The

test discriminates two alleles named S and s, corresponding to nucleotides G and T, respectively at the first base of the Sp1 binding site in the first intron of the gene for COLIA1. Three anonymous polymorphic restriction enzyme recognition sites at the 3' end of the VDR gene, i.e. for *BsmI*, *ApaI* and *TaqI* were assessed in relation to each other by a direct molecular haplotyping PCR procedure which we developed.[18] This allowed us to determine phase of the alleles at each of the RFLP loci and as a result three frequent haplotype alleles are discerned, encoded 1 (baT;frequency 48%), 2 (BAt;40%), and 3 (bAT;10%) combining to 6 genotypes encoded 11, 12, 13, 22, 23, and 33. We excluded the less frequent haplotypes 4 and 5 from the analysis. Women with genotypes containing these haplotypes (n=15) represent 1.5% of this population. Detailed information on haplotype alleles and genotype frequencies in the Rotterdam Study can be found elsewhere.[18]

Statistical Analysis.

Clinical variables were compared between the genotype groups by analysis of covariance to adjust for confounding factors. For the comparisons we made reference, heterozygote and homozygote groups for each of the COLIA1 and VDR alleles. For COLIA1 the groups comprised the GG genotype group for the reference group, GT for the heterozygote risk group and TT for the homozygote risk group. For VDR haplotype 1 the groups comprised genotypes 22, 23, and 33 for the reference group, genotypes 12, and 13 for the heterozygote risk group, and genotype 11 for the homozygote risk group. The Likelihood Ratio test statistic was used to test for genotype distribution in women with and without fractures. Odds ratios (with 95 percent confidence interval) were calculated by multivariate logistic regression analysis to estimate the relative risk of osteoporotic fracture by genotype. For regression analysis using combinations of VDR and COLIA1 genotype we defined the "reference" group to include women with the COLIA1 GG genotype in combination with the VDR 22, or 23 or 33 genotype. The regression analysis included an interaction term defined as VDR genotype multiplied with COLIA1 genotype. All p-values for statistical tests were two-sided.

Table 1 Number of postmenopausal women with fractures according to VDR Genotype

VDR Genotype	No. with fracture / total No. (%)
11	34 / 255 (13.3)
12	35 / 375 (9.3)
13	13 / 101 (12.9)
22	7 / 179 (3.9)
23	6 / 82 (7.3)
33	2 / 12 (16.7)
Chi2	13.3
P Value	0.04

RESULTS

When we analysed the distribution of fractures in women grouped according to VDR genotype, we observed an overrepresentation of fractures in women carrying the haplotype 1 (Table 1). Women were subsequently grouped according to carrier status for this VDR haplotype as heterozygous carriers (including the genotypes 12 and 13) and homozygous carriers (consisting of genotype 11) of the risk haplotype and compared to women not carrying the haplotype (including genotypes 22, 23, and 33). No significant differences in known risk factors for osteoporosis could be observed between women grouped according to VDR haplotype 1 (Table 2). Similar results were obtained when the women were grouped according to VDR haplotypes 2 or 3 (data not shown).

We went on to determine the distribution of fractures in women according to their carrier status for VDR haplotype 1 (Table 3a). Significantly more women heterozygous for VDR haplotype 1 had fractures than the women in the reference group and for women homozygous for the VDR haplotype 1 this difference further increased. When women were grouped according to VDR haplotype 2, we observed an under representation in fracture cases ($p=0.002$) while for VDR haplotype 3 no differences were observed ($p=0.65$;data not shown). Logistic regression analysis showed that women heterozygous for the VDR haplotype 1 had 1.8

times the risk for fractures compared to women in the reference group. This was further increased for women homozygous for the VDR haplotype 1 to 2.6 times the risk for fracture compared to women in the reference group (Table 3a).

Table 2. Characteristics of 1004 postmenopausal women according to their VDR haplotype 1 genotype

Characteristic*	**VDR genotype**			**P Value**
	Reference	**Heterozygotes**	**Homozygotes**	
Number	273	476	255	
Age (yr)	66.4 ± 7.0	67.4 ± 7.0	67.1 ± 6.7	0.19
Height (cM)	162.3 ± 6.3	162.1 ± 6.0	161.7 ± 7.5	0.66
Weight (Kg)	68.9 ± 9.7	68.6 ± 10.5	69.3 ± 10.5	0.70
Age at menopause (yr)	49 ± 5	49 ± 5	49 ± 5	0.35
Dietary calcium intake (mg/day)	1076 ± 335	1103 ± 329	1073 ± 287	0.42
Current smoker (%)	20	21	24	0.51
Fem. Neck BMD (g/cm^2)	0.82 ± 0.15	0.80 ± 0.12	0.81 ± 0.13	0.21

* Plus-minus values are means ± SD
+"Reference" includes VDR genotypes 22, 23, 33;
"Heterozygotes" includes 12, 13; "Homozygotes" includes 11

When we analysed by type of fracture we observed the VDR genotype effect to be similar for prevalent vertebral fracture cases and incident non-vertebral fracture cases. The age-adjusted Odds Ratio for vertebral fracture for women who are heterozygous or homozygous for VDR haplotype 1 is 1.7 (95% CI 0.7 – 3.9) and 2.7 (95% CI 1.1 – 6.6), respectively. The age-adjusted relative Odds Ratio for non-vertebral fracture for women who are heterozygous or homozygous for VDR haplotype 1 is 2.1 (95% CI 0.9 – 4.9) and 3.0 (95% CI 1.2 – 7.3), respectively. When we limited the analysis of fracture risk to the group of women for whom we had radiographic data (n = 732 with 88 fracture cases) the data remained essentially unchanged. In this group the age-adjusted Odds Ratio for any fracture for women who are heterozygous or

Table 3a-b. Number of postmenopausal women with fractures and Odds Ratios for fracture according to VDR haplotype 1 genotype and according to COLIA1 genotype

Genotype+	**Fracture cases/ total No. (%)**	**Odds Ratio (95% CI)**	
A. VDR allele 1 genotype		**Age-adjusted**	**Multivariate***
Reference	15 / 273 (5.5)	1.0	1.0
Heterozygotes	48 / 476 (10.1)	1.8 (1.0-3.3)	1.6 (0.8 - 3.1)
Homozygotes	34 / 255 (13.3)	2.6 (1.4-5.0)	2.4 (1.2 - 4.8)
Chi2	9.47	-	-
P Value	0.009		
B. COLIA1 genotype			
GG	53 / 679 (7.8)	1.0	1.0
GT	37 / 293 (12.6)	1.7 (1.1-2.7)	1.6 (1.0-2.6)
TT	7 / 32 (21.9)	3.7 (1.5-9.2)	3.3 (1.3-8.4)
Chi2	11.1	-	-
P Value	0.004		

+"Reference" includes VDR genotypes 22, 23, 33;
"Heterozygotes"includes 12, 13;
"Homozygotes" includes 11
*Multivariate Odds Ratios were adjusted for age, weight, and femoral neck BMD.

homozygous for VDR haplotype 1 is 1.7 (95% CI 0.9 – 3.2) and 2.6 (95% CI 1.3 – 5.0), respectively. Also when we analysed the risk for non-vertebral fractures in this group (43 fracture cases) we found that the age-adjusted Odds Ratio for women who are heterozygous or homozygous for VDR haplotype 1 was 1.7 (95% CI 0.7 – 4.2) and 2.7 (95% CI 1.1 – 6.6), respectively. Thus, for reasons of power we combined the prevalent vertebral and incident non-vertebral fracture cases in one group of "any fracture". The risk of fracture did not essentially change after adjustment for potential confounding factors such as age, weight, and bone density in the multivariate regression analysis (Table 3).

In this group of women we also determined the distribution of fractures according to COLIA1 genotype (Table 3b). In correspondence with what we previously found (24) we observed the COLIA1 T allele to be associated with increased fracture risk, independent of BMD. To assess whether there was interaction between the VDR haplotype effect and the COLIA1 genotype effect on fracture we determined the distribution of fractures according to VDR haplotype 1 in the different COLIA1 genotype groups (Table 4). The distribution of fracture cases according to VDR genotype did not differ in the group of women with the COLIA1 GG genotype. However, in the COLIA1 risk groups of women with the GT and TT genotypes the distribution of fracture cases was strongly VDR genotype dependent (Table 4a). Logistic regression analysis showed that the effect of VDR genotype on fracture risk is absent in women with the COLIA1 GG genotype while the VDR genotype effect is large in the COLIA1 heterozygous GT and homozygous TT risk group (Table 4b). When age, VDR genotype, COLIA1 genotype and fracture were considered together in a multivariate regression model we found that VDR genotype significantly modified the COLIA1 genotype effect ($p=0.03$ for the interaction term). The effect was found to be similar for incident non-vertebral fracture cases and prevalent vertebral fracture cases and when bone density was entered into the model the results did not change indicating the interaction effect to be independent of bone density.

DISCUSSION

Although VDR gene polymorphisms have been implicated in the genetic regulation of BMD,[14,15] meta-analyses showed that the effects on BMD are small[16, 17] as we also demonstrated earlier in our study population.[18] While two reports suggested VDR genotype to predict osteoporotic fracture,[19,20] no associations with osteoporotic fracture have been reported in two other studies.[21,22] The studies were small, however, and used only the BsmI RFLP in their analysis. As we argued previously,[18] analysing only the BsmI RFLP can compromise the outcome of such studies because heterogeneous groups are compared. For example, the "b" allele (58.9% in our population;[18]) is in fact a group of three alleles when defined as haplotypes, i.e., "baT" (48.2%), "bAT" (10.5%), and "bAt" (0.2%). Thus, there is

Table4. Number of postmenopausal women with fractures.*(Number with Fractures/total number (%))* and Odds Ratios for fractures according to combined VDR haplotype 1 and COLIA1 genotype

VDR genotype+	**COLIA1 genotype**			
	GG	**GT**	**TT**	**GT + TT**
Reference	13 / 194 (6.7)	2 / 70 (2.9)	0 / 9 (0)	2 / 79 (2.5)
Heterozygotes	27 / 315 (8.6)	18 / 149 (12.1)	3 / 12 (25.0)	21 / 161 (13.0)
Homozygotes	13 / 170 (7.6)	17 / 74 (23.0)	4 / 11 (36.4)	21 / 85 (24.7)
Chi2	0.59	13.3	3.94	17.3
P Value	0.74	0.001	0.14	0.0002

*b. Age-adjusted Odds Ratio (95% CI)**

Reference	1.0	0.4 (0.1 – 2.0)	-	0.4 (0.1 – 1.8)
Heterozygotes	1.3 (0.6 - 2.5)	1.9 (0.9 – 4.1)	4.8 (1.1 – 21)	2.1 (1.0 – 4.4)
Homozygotes	1.2 (0.5 - 2.7)	4.1 (1.9 – 8.5)	7.1 (1.8 – 29)	4.4 (2.0 – 9.4)

+"Reference" includes VDR genotypes 22, 23, 33; "Heterozygotes" includes 12, 13; "Homozygotes" includes 11

*Odds Ratios were calculated with the VDR allele 1 reference/GG genotype group as reference group.

Zero cases in the cell precluded the calculation of the Odds Ratio in the TT COLIA1 genotype group.

Based on the small numbers of the TT COLIA1 genotype group and the similar trends in the GT and the TT COLIA1 genotype groups, we calculated Odds Ratios for the combined GT+TT COLIA1 genotype group.

extensive linkage disequilibrium at the 3' end of the VDR gene[14,18,34,35] which can be accurately measured by the molecular haplotypes constructed from the three 3' RFLPs for BsmI, ApaI, and TaqI. Thus, these haplotypes, which by themselves are not functional polymorphisms, can be used as good markers for truly functional polymorphisms elsewhere in the 3' end of the VDR gene. This notion is underlined by the findings of some studies that a particular haplotype, "baT" (or haplotype 1), is associated with aberrant mRNA expression and/or stability levels.[14,27-29]

Our findings suggest the VDR to be involved in bone metabolic pathways other than those reflected in BMD but still leading to increased fracture risk. The VDR genotype dependent increased fracture risk is especially pronounced in interaction with COLIA1 genotype of which we already reported the COLIA1 Sp1 "T" allele to increase fracture risk in our study population.[24] The interaction between VDR and COLIA1 genotype we here describe raises the possibility of biological interaction of the gene products. The VDR is a member of the steroid transcription factors, known to be important regulators of gene expression. Vitamin D dependent regulation of expression of bone-specific genes, such as osteocalcin, has been well-documented[36] and also includes regulation of the expression of the collagen type Iα1 at the level of transcription.[25,26] Furthermore, in RT-PCR experiments the Sp1 polymorphism has been shown to lead to differential binding affinity of the Sp1 transcription factor[23] and also to genotype- dependent differences in COLIA1 mRNA- and protein expression levels and to differences in bone strength.[37] Therefore, the VDR regulated expression of the collagen type Iα1 gene may differ across COLIA1 alleles and VDR alleles and could be an important factor in the interaction we observe. However, while the exact molecular mechanism underlying the associations we here describe remains to be elucidated, our observations on the interaction should be considered as preliminary.

It is likely that interactions between genetic loci involved in a complex trait are a common phenomenon and several examples have already been demonstrated but mostly in model organisms. Our data represent the first example of interlocus interaction in relation to fractures between two well-known candidate genes in osteoporosis, a complex trait in humans. We show that the interaction leads to increases in the risk of fracture, the clinically most relevant feature of osteoporosis, and that this increase in risk is independent of BMD, the most widely used diagnostic criterium for osteoporosis. This has important consequences not only for the analysis of the genetic basis of osteoporosis but also for the identification of individuals at risk of the disease. However, larger studies will be required to assess the attributable risk of these genetic variations, including their interaction, also in relation to other known risk factors for fracture. We are currently collecting fracture data for the complete cohort in the Rotterdam Study in order to tighten the fracture risk estimates we here observed. Finally, our observation also raises the possibility of developing new therapeutic intervention strategies based on the known

involvement of the VDR and the COLIA1 gene in bone metabolism and the determination of fracture risk.

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4

General Discussion

GENERAL DISCUSSION

In this chapter we will briefly describe the most important findings and discuss the methodological issues of the conducted studies. Furthermore, the identified determinants of osteoporosis will be interpreted in relation to the pathogenesis of osteoporosis. This discussion will finish with suggestions for future research on the (genetic) etiology of osteoporosis.

MAIN FINDINGS

SKELETAL RISK FACTORS

Fracture incidence and BMD

We prospectively studied the frequency of non-vertebral fractures and the role of BMD as a risk factor for these fractures among participants from the Rotterdam study (Chapter 2.1) In both men and women, hip, wrist and upper humerus fractures were predominant and most strongly related to low BMD. We further conclude that bone mineral density is an equally strong risk factor in men and women. These data serve as reference for further studies on the etiology of osteoporotic fractures.

Bone resorption

We investigated the role of bone resorption rate in osteoporosis within a case-control study. Bone resorption rates have been shown to influence the risk of hip fractures,[1,2] however, for other types of fractures this was unknown so far. In our study, we showed an association of increased bone resorption rate with the risk of non-vertebral fractures. This risk was most pronounced for hip and upper humerus fractures and independent of low BMD (Chapter 2.2). Having a low BMD partly explains the risk of fractures while there probably is a concomitant effect of bone structure and quality resulting in substantially increased fracture risk.

GENETIC RISK FACTORS

Estrogen receptor α polymorphism

The estrogen receptor α (ERα) gene is considered a candidate gene since estrogen, which acts via the estrogen receptor, plays an important role in maintaining BMD. Furthermore, the age at menopause influences the risk of osteoporosis and appears to be under genetic control. In Chapter 3.1 we investigated whether genetic variation at the ERα gene contributes to the variability in onset of menopause and the risk of osteoporosis. Women having a *C* to *T* substitution in intron 1 on both alleles of the ERα gene had a later onset of menopause and moreover, were at lower risk of surgical menopause, i.e. in particular due to hysterectomy. Interestingly, those *TT* homozygous women had a lower BMD and, independent of BMD, had a higher risk of especially vertebral fractures.

Vitamin D receptor polymorphism

Recently, several studies have demonstrated that genetic variants of the gene locus encoding the vitamin D receptor (VDR) are associated with osteoporosis.[3,4] Until now, the functional consequence of the genetic variance of the VDR polymorphism, except for the *FokI* polymorphism, is unknown. It is likely, however, that genetic variability of the VDR can modify the effect of 1,25-$(OH)_2D_3$ on the cellular level (Introduction section). In light of the above we have investigated the genetic effect of the VDR on bone turnover rate and on the response of 1,25-$(OH)_2D_3$ *in vivo* and *in vitro* in postmenopausal women (Chapter 3.2) . As presented in Chapter 3.2.1 we observed at baseline a higher rate of bone turnover in women having low BMD compared to women having high BMD. Interestingly, this increased bone turnover rate was under influence of the VDR 3' gene polymorphisms. However, this VDR genotype dependent effect on bone turnover could not be explained by a difference in response to short-term administration of 1,25$(OH)_2D_3$. In Chapter 3.2.2 data from an *in vitro* study with peripheral blood mononuclear cells (PBMC) are presented. The half maximal concentration for 1,25-$(OH)_2D_3$ inhibition of PHA-stimulated growth is significantly different between

PBMC carrying different alleles of the VDR translational start-site while the maximal inhibition is similar for all genotypes. This supports the hypothesis that the 424 amino acids-long VDR is more efficient in exerting the effects of 1,25-$(OH)_2D_3$ than the 427 amino acids-long VDR.

Gene- gene interaction

So far few studies have addressed the possible interaction between osteoporosis candidate genes in relation to the clinically most important endpoint, i.e. fractures. Genetic variations in both the VDR gene as well as the COLIA1 gene have been implicated in osteoporotic fractures. We analyzed the combined influence of polymorphisms in the VDR gene and the COLIA1 gene in determining the susceptibility to osteoporotic fracture (Chapter 3.3). We showed that both the VDR and COLIA1 polymorphisms are genetic markers for osteoporotic fracture in women, independent of BMD. In addition, we found evidence for interaction of these effects. Interlocus interaction is therefore likely to be an important component of osteoporotic fracture risk.

METHODOLOGICAL CONSIDERATIONS

Osteoporosis; a complex trait disease

The term complex trait disease refers to a trait phenotype with a multifactorial origin that usually is not characterized by simple Mendelian inheritance, early onset and straightforward diagnostic criteria. In fact they involve interaction of genetic and environmental factors and involvement of multiple genes. We performed all studies within the Rotterdam study, a population-based cohort study with a large data set. The great benefit of a large data set is that besides various environmental factors can be studied, also enough power is present to unravel individual genetic effects, and, in addition, to study interaction.

Study design

In this thesis two types of studies are described, i.e. observational (cohort and case-control studies) and experimental studies, that all involve analytical study designs. In analytical studies the investigator assembles groups of individuals to determine whether the risk of a specific outcome parameter is different for individuals exposed or not exposed to a factor of interest. Furthermore, to explore genetic factors in the etiology of osteoporosis we conducted genetic association studies within a cohort design.

Longitudinal studies

In Chapter 2.1, 3.1 and 3.3 we performed longitudinal studies. In longitudinal studies at the time exposure status is defined, all potential subjects are free from disease status and are followed over a period of time to assess the occurrence of that outcome. In Chapter 2.1 the aim was to obtain precise estimates of incidence of fractures and therefore a substantial person time experience is required. The follow-up period of 5 years was appropriate to investigate the relation of BMD with fractures in women, although longer follow-up time is needed to get more stable estimates for every type of fracture, especially in men. In Chapter 3.1.1, ERα polymorphism and menopausal state were assessed simultaneously. However, the ERα polymorphism exposure is stable since conception of life, therefore the definition of a longitudinal study design is still valid. In Chapter 3.1.2, the aim was to assess the relation between ERα genotype and osteoporosis determined by BMD and fractures. For vertebral fracture we were able to do separate analysis for prevalent and incident cases and therefore avoid potential survival bias (disturbing factor). In Chapter 3.3, gene-gene interaction was studied in relation to fractures. Although the investigation will be repeated in a much larger study population, the substantial number of fracture cases allowed calculating fairly accurate risk estimates.

Case-control study

In chapter 2.2 we applied a case-control study. Case-control studies are particularly efficient in terms of both time and costs. Nevertheless due to its design case-control studies are susceptible to bias when cases and controls are selected from different populations. For

example, most studies have selected fracture cases from a hospital database whereas controls were selected from the general population. In the hospital-based cases probably co-morbidity is more frequent than in the 'healthy' controls, which subsequently leads to questionable results. To reduce this bias we conducted a nested case-control study within the population-based cohort study in which cases and controls are selected from the same source population.

Experimental studies

Chapters 3.2.1 and 3.2.2 are based on experimental studies. Experimental studies are often considered as reliable studies in research due to the unique strength of determining exposure status (investigator can influence the exposure). Epidemiologic data obtained *in vivo* generally do not result in unambiguous insights in the mechanism underlying the association observed. Therefore additional experimental studies like cell biological and molecular biological studies are necessary. In chapter 3.2.1 we performed an epidemiologic experimental study without using a placebo group. During the very short time of follow-up we did not expect that the subjects were influenced by external factors other than known biochemical effects of vitamin D. Moreover, if any placebo effect was applicable, it is unlikely that this would be genotype dependent.

Validity

Selection bias

Selection bias leads to incorrect estimates of true associations, especially in case-control studies since exposure and disease both occurred at the time the subjects are selected for the study. Since controls were chosen from the same source population from which cases were derived it is not likely that selection bias has occurred due to an ill defined sampling frame of controls.

In cohort studies selection bias can occur when the relation between the determinant and the outcome is different for those who participated and those who were eligible but did not participate. Such selective non-response may have occurred at baseline or at follow-up. All

longitudinal studies described in this thesis were conducted within the cohort of the Rotterdam study. The non-response rate in the Rotterdam study was low at baseline (22%). Furthermore, we achieved complete follow-up of all participating individuals concerning non-vertebral fractures.

Non-responders might be subjects that are older, with impaired mobility or otherwise at an increased risk for osteoporosis. Assuming that we have selected a relatively healthy cohort this would result in an underestimation of the incidence rates of fractures. However, for evaluating associations with exposures such as BMD, ERα, VDR 3' and Collal genotypes in relation to fractures, it is unlikely that non-response has accounted for biased estimates. For those who were not included in the study it is improbable that the cause of not participating is both related to exposure and, independent of the exposure, to the outcome variable (fractures). Still, if any difference in exposure effects between these groups exists, this only would concern generalizability of the study results.

Concerning genetic association studies selection bias by genotype might have occurred if a population is small or relatively isolated such as in Finland or Iceland. In the studies presented in this thesis we found no deviation from Hardy-Weinberg equilibrium, which indicates the absence of genetic selection, drift and/or mutations that have recently occurred in the study population during the last generations. Genetic factors are stable which means that they remain the same during life and are not influenced by external factors. Still there might have been a healthy responder bias although we do not think this to be genotype-dependent and therefore not to influence our genetic association results. Furthermore, we were able to do separate analyses on prevalent and incident vertebral fractures to adjust for a potential survival bias.

Information bias

Misclassification of outcome or exposure may lead to information bias. Non-differential misclassification, which is a random distribution of information bias over the compared groups, leads to underestimated effects. The primary requirement for the validity of a cohort and a nested case-control study is the ability to obtain complete and accurate information on all participants. In the studies described in this thesis, we believe case ascertainment to be good, since in the Netherlands the GP is the gatekeeper of the healthcare system. Therefore,

the GP record is the central repository of the medical information about fractures that have or have not occurred in a participant. Furthermore, we fixed diagnostic criteria a priori. This minimizes the possibility of introducing misclassification that, if any, would be non-differential. Determinants were measured using standardized and generally approved protocols, but non-differential misclassification of risk factors and confounders could not be totally excluded.

Confounding

An important disturbing factor that must be considered in discussing study results is bias due to confounding. Confounding involves the possibility that the observed association is due to the effects of differences between study groups other than the exposure status. Important confounders in studies on the relation between potential determinants and osteoporosis are age and gender. Old age and being female are strong risk factors for osteoporosis, and related to most determinants we studied. We controlled for the effect of age and gender by stratification or analysis of multivariate models.

Theoretically, some determinants we used may be also an intermediate in the association of, therefore over-adjustment might have occurred. For example, in the study on the association between bone resorption rate and the risk of fractures, BMD is likely to be both a confounder and intermediate. Therefore, additional adjustment for BMD in the analysis might have under estimated the true effect.

Population admixture is one of the problems introducing bias in genetic association studies since there are racial difference in BMD and in the frequency of fractures. Nevertheless, all studies mentioned in this thesis were performed within the Rotterdam study that almost exclusively consists of Dutch Caucasian individuals and therefore we did not adjust for racial and ethnic background.

GENERAL INTERPRETATION OF STUDY RESULTS

Definition of outcome

The identification of genetic and environmental determinants of osteoporosis is important for risk stratification and for understanding the pathogenesis of the disease. In this respect, the definition of osteoporosis forms a significant lead in unraveling the disease. Osteoporosis is defined as a systemic skeletal disease, characterized by low bone mass and microarchitectural detoriation of bone tissue with a consequent increase in bone fragility and susceptibility to fracture.[5] At first sight, the clinically relevant end-point of osteoporosis, i.e. fractures, may seem the diagnostic criteria of choice. However, which type of fracture constitutes an osteoporotic fracture is not clear. Until now, in epidemiologic terms a fracture is considered to be osteoporotic if the incidence increases with age and is related to BMD. [6] Nevertheless, the incidence of wrist fracture does not increase with age but is still considered to be osteoporotic.[6] In addition, different types of fractures will have different types of risk factors and most of them usually occur at more advanced age, and are therefore severely complicated by other co-morbidity.

Definition of determinants

BMD might also be used as a surrogate diagnostic criterion in etiologic studies on osteoporosis but again the definition is not a straightforward one. What is a low BMD? The most widely used clinical criterion of low BMD is the T-score, i.e., the number of SD different from the premenopausal mean. However, due to different bone densitometry machines (DEXA, dual energy X-ray absorptiometry) used worldwide we have to deal with large differences in BMD measured. This can be explained by variances in absolute values measured and by different reference populations used.[7]

Concerning the definition of bone turnover rate identical issues can be identified. Again, what defines a high bone turnover rate? The most applicable definition is also based on a T-score, however, in contrast to BMD that stays relatively constant until the menopause, bone turnover rate declines with age in premenopausal women.[8] This makes it impossible to compare study results of estimates based on different premenopausal reference groups. Moreover, several

different assays are used to measure markers of bone formation and/or resorption and moreover, each assay had a large precision error. In contrast to most studies, we measured a marker of bone resorption by a rapid and automated chemiluminescence immunoassay, which is characterized by a substantially lower precision error than the manual method.[9,10]

The use of anonymous polymorphisms as genetic markers in osteoporosis makes testing much more feasible since they are abundant in human genome. However, it is important to realize that an association between the anonymous candidate gene polymorphism and the outcome of interest is likely to be explained by linkage disequilibrium (LD) with a truly functional variation elsewhere in the gene. Such LD is not constant among different populations and can contribute to controversial results. This phenomenon will be discussed below in more detail for each genotype separately.

PATHOGENESIS

Skeletal factors

Unraveling the genetic and environmental basis of osteoporosis makes it possible to get new insights in the pathogenesis. The most common factors associated with osteoporosis are advanced age and female gender. The effect of age on osteoporosis is mainly via its effect on BMD and is stronger in women.[11] An important BMD independent component, falling, is clearly responsible for suffering a fracture and frequencies of falling increase with age and are higher in women.[12] Nevertheless, the increase in frequency of falling and the decrease of BMD with age do not fully explain the risk of fractures, therefore other factors can alter the risk of osteoporosis not only in women but also in men.

BMD is an indirect estimate of bone quantity while also bone quality is an important aspect of fracture risk. These aspects are in turn presumed to be related to the interplay of bone formation and bone resorption.[13] Recently, van Daele et al. showed a BMD-independent relation between bone resorption rate and the risk of hip fracture.[2] In Chapter 2.2 we report that increased urinary concentrations of the bone resorption marker free deoxypyridinoline crosslinks (fDPD) is associated with an increased risk of non-vertebral fractures, especially those of the hip and upper humerus. Not only decreased bone quantity but also losses in bone

quality (see Introduction) ultimately leads to fractures. This supports the observation that the relationship between bone resorption rate and fracture risk is not continuous, but exists above a certain threshold. Only above this threshold sufficient quality of bone is lost to increase bone fragility, ultimately leading to fractures.

Genetic factors

Recently, both quantitative and qualitative aspects of bone have found to be genetically determined but the genes involved are ill defined. Until now some candidate genes have been studied but findings are not always consistent and might be explained by interaction of the genetic factors with other risk factors like, environmental factors or other genes. Important candidate genes in studying the etiology of osteoporosis are the VDR gene, the ERα gene and the COLIA1 gene since they play a central role in bone metabolism and bone structure.

Estrogen receptor α polymorphism

The ERα genotype dependent variability of BMD only explains a small part of the vertebral fracture risk (Chapter 3.1.2). In relation to this, in Chapter 2.2 we showed a BMD-independent association with bone turnover and fracture risk. The influence of the ERα polymorphism was more pronounced at the lumbar spine where a relatively high bone turnover rate exists compared to the femoral neck. This raises the possibility that genetic differences in bone turnover rate are part of the explanation of the BMD-independent fracture risk. This hypothesis should be investigated in future studies.

We performed the association study with a supposedly anonymous polymorphism, and thus, it is assumed that this *C* to *T* substitution is in linkage disequilibrium (LD) with a truly functional sequence variation. Indeed, strong LD has been observed around this polymorphism which encompassed an area of about 20 kb. A TA-repeat in the promotor region has been shown to be in LD with the *C* to *T* polymorphism; a low number of TA repeats was predominantly found on the PX-allele, corresponding to T-A at position e2-397 and e2-347, respectively.[14,15] Interestingly, the variation in TA-repeats has been suggested to be associated with differences in expression levels of the ERα mRNA.[16] Therefore, although the truly functional sequence variant is currently unknown and could involve protein isoforms or different levels of mRNA, the latter possibility is a possible and attractive explanation.

Although the differences in mRNA levels are likely to be small, over a lifetime they can have important consequences

In Chapter 2 we reported the ERα gene to be associated with both the onset of menopause and different endpoints of osteoporosis. Interestingly, we reported that the ERα polymorphism responsible for a later onset of menopause was not associated with higher BMD and a lower risk of fractures. However, estradiol (E_2) is a regulator of cell growth, differentiation and function of numerous tissues such as tissues of the female as well as the male reproductive system, bone, and the cardiovascular and central nervous system. Thus, for a pleiotropic "master" gene such as the ERα one can expect to find associations of this gene with multiple traits and disease phenotypes. Indeed, the ERα gene has shown to be associated a number of different phenotypes like breast cancer, osteoporosis, hypertension, generalised osteoarthritis, and some autoimmune diseases such as rheumatoid arthritis has been reported.[17-23] In addition, the potential confounding effect that arises from this pleiotropy, can influence the associations observed. For example, it can be hypothesized that ERα gene variants can influence calcium metabolism through differential absorption in the intestine and, at the same time, influence bone turnover, while also the onset of menopause can be influenced. All together this will result in a net effect on BMD measured at a certain site, at a certain age and in a subject with a certain diet.

Vitamin D receptor polymorphism

In the analysis of the VDR polymorphism, the ligand1,25-$(OH)_2D_3$ is necessary for activation of the receptor. The natural ligand has potential therapeutic effect but the overall effect is not consistent in osteoporotic patients. A plausible explanation would be that variations in the function of the receptor exist as a result of genetic variability. This has been supported by the finding in Chapter 3.2.1 where the bone turnover rate was found to be VDR genotype dependent but also cellular growth inhibition by active vitamin D was associated with genetic variation of the VDR (Chapter 3.2.2). In line with this is the observation reported by Graafmans et al., who showed a VDR genetic dependent change in BMD after long-term administration of calcitriol.[24]

However, the 3' *Bsm-Apa-Taq* RFLPs itself are not functional and, thus, consequently, linkage disequilibrium (LD) between them and the truly functional allele might explain the findings. The alleles of the *Bsm*I, *Apa*I, and *Taq*I polymorphisms in intron 8 and exon 9 are closely linked and haplotypes can be constructed over this 2.2 kb region.[3,25] The LD of these RFLPs extends into the 3' untranslated region (UTR) which is a 3.2 kb sequence immediately adjacent to exon 9.[25-27] More than 10 different sequence variations in the 3'UTR have been described including a poly (A) repeat polymorphism. Morrison and colleagues provided evidence of differential luciferase activity for the two UTRs that are linked the two most frequent haplotypes 1 (baT) and 2 (BAt) (see introduction).[25] Durrin and colleagues have shown certain parts of the UTR, so-called destabilizing elements, to be involved in determining stability of the VDR-mRNA.[27] Yet, when 8 individuals, selected by their poly(A)- genotype, were sequenced no polymorphisms were found in the destabilizing elements of the 3'UTR. Furthermore, the UTRs linked to the two most common variants (the baT and BAt haplotype) were not found to differ with respect to mRNA stability.[27] However, only few individuals were sequenced so variations could have still been missed while also heterologous constructs (human VDR-UTR with a rabbit β-globin gene) and cell types (mouse NIH3T3 cells) were used to test for functionality. Especially since it is known that UTRs display cell-type specific effects on mRNA stability this could be important in demonstrating functionality of sequence variations in the UTR.

The finding presented in Chapter 3.2.2 where genetic variation of the *Fok*I RFLP site was associated with the variance in response to calcitriol whereas the VDR 3'genotype had no influence, is in line with the observation that the *Fok*I RFLP site was not in linkage disequilibrium with the 3' polymorphisms.[28,29] In view of the considerable distance between the two sites (±40 kb) and the different nature of the polymorphisms, they should be treated as a different marker. Taken together, these data indicate that multiple polymorphic variations exist in the VDR gene, which could each have different types of consequences. Thus, 5' promotor variations may affect mRNA expression patterns and levels while 3' UTR sequence variations will affect the mRNA stability. In combination these genotypic differences are likely to affect the VDR protein levels and/or function, depending on the cell type, developmental stage and activation status.

Vitamin D receptor polymorphism and Collagen type Iα1 gene polymorphism

Besides gene-environment interaction, gene-gene interaction might occur: genetic variants of a protein will interact with other protein variants in the same biological pathway. Such interaction implicates that polymorphism of one gene can influence the effect of another gene. This can result in additive, multiplicative or subtractive results. This gene-gene interaction mechanism might explain the finding described in chapter 3.3 where the VDR 3' polymorphism and the COLIA1 gene interact. The VDR is a member of the steroid transcription factors, known to be important regulators of gene expression. Vitamin D dependent regulation of expression of bone-specific genes, such as osteocalcin, has been well documented and also includes regulation of the expression of the collagen type Iα1 at transcription level.[30-32] In RT-PCR experiments the Sp1 polymorphism has been shown to lead to differential binding affinity of the Sp1 transcription factor and also to genotype dependent COLIA1 mRNA expression levels.[33-35] Therefore, while the exact molecular mechanism underlying the associations we described in chapter 3.3 remains to be elucidated, it can be speculated that VDR genotype dependent regulation of COLIA1 gene expression could be the basis of the synergistic interaction we observed and may differ across VDR alleles.

RECOMMENDATIONS FOR FUTURE RESEARCH

Studies conducted to identify risk factors for osteoporosis are hard to compare due to different definitions of endpoints as well as determinants of osteoporosis. Therefore, to establish guidelines for treatment and prevention, one of the first goals is to reach consensus over terminology and measures used.

In this thesis we showed incidence rates of all types of non-vertebral fractures not only in women but also in men. Since worldwide only few prospective cohort studies are available, data on gender and especially population differences in incidence rates are scarce. To address this issue, large prospective studies are needed throughout the world.

We showed an increased risk of most types of fractures with decreased BMD. Besides BMD also bone quality (bone resorption rate) influenced the risk of non-vertebral fractures, especially the hip and upper humerus fracture. Moreover, the VDR polymorphism, ERα polymorphism and the COLIα1 gene polymorphism were found to be associated with different endpoint of

osteoporosis. This raises the possibility that specific risk factors determine the risk of specific fractures. Therefore, it would be interesting to conduct prospective studies on all types of fractures including large numbers of (genetic and/or environmental) risk factors from which risk profiles could be made.

It has been a challenge to identify the genes that are responsible for this disease. Linkage analysis using multigenerational families with osteoporosis will be helpful but collecting DNA from several affected generations will be difficult if not impossible. Another possible manner to identify genes is sib pair analysis. Affected sib pairs (brothers and sisters with osteoporosis) who share the same allele with each other and with different sib pairs, give some insight in the locus associated with the disease. Nevertheless this approach can only be fruitful when it is conducted on an international level since large numbers are necessary. Taking these considerations and cost-effectiveness into account the most effective approach will be a combination of genome search and candidate gene association studies.

Our findings provide evidence for the importance of the anonymous VDR, ERα and ColIA1 polymorphism in the risk of osteoporosis. However, finding functional sequence variants that matter, establishing the phase of alleles across the entire candidate gene, and defining haplotype patterns is crucial to better understand the associations that are necessary for new therapeutic intervention strategies.

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5

Summary / samenvatting

Summary

Osteoporosis is one of the major diseases facing the ageing population. Fractures, the clinical endpoint of osteoporosis, contribute considerably to overall morbidity, mortality and financial healthcare costs. With an increasing number of elderly subjects in modern Western Countries, the coming 50 years a 4-fold increase in the worldwide incidence of osteoporotic fractures is expected. To limit this increase in fracture rate, studies on etiology may therefore be needed. In the last decade, there has been a considerable increase in knowledge of incidence rates of osteoporosis. Furthermore, the role of bone mineral density (BMD) as important quantitative measure of bone, has been demonstrated to be an important factor in the risk of osteoporotic fractures. However, most studies were performed in women and focused on hip fractures only. Moreover, only 70-80% of the bone fragility can be explained by a decrease in BMD whereas the other 20% could be contributed to qualitative aspects. Such qualitative aspects are the architecture of bone, aspects of bone matrix and the rate of bone turnover, i.e. the ratio between bone formation and bone resorption. Recently, both quantitative and qualitative aspects of bone have found to be genetically determined but genes involved remain defined.

The objective of the work presented in this thesis was to get insight into the magnitude of the problem and we aimed to study both skeletal and genetic determinants of osteoporosis not only in women but also in men. The studies described in this thesis were performed within the Rotterdam Study. The Rotterdam Study is a large population-based cohort study of subjects aged 55 years and over, which aim is to study specific diseases affecting the elderly and to determine their risk factors.

In **Chapter 2.1** we reported incidence rates of non-vertebral fractures and we determined whether BMD was a risk factor for fractures in both men and women. In both genders fractures of the hip, wrist and upper humerus were predominant. Those specific fractures were also most strongly related to BMD. We further conclude that BMD is an equally strong risk factor in men and women. In **Chapter 2.2** we studied aspects of bone quality and its involvement in the susceptibility for fractures. Bone quality was determined by a urinary biochemical marker for bone resorption, i.e. free deoxypyridinoline crosslinks (fDPD) and corrected for creatinine. We found that increased concentrations of fDPD are associated with increased risk of non-vertebral fractures, especially those of the hip and upper humerus. This supports the idea that not only decreased bone quantity but also loss in bone quality, ultimately leads to fractures. This is

supported by the observation that the relation between bone resorption rate and fracture risk is not continuous, but exists above a certain threshold. Only above this threshold, sufficient quality of bone is lost to increase fragility ultimately leading to fractures.

Recently, research has included genetic factors as potential determinants of both bone quality and bone quantity. Findings, however, are not always consistent and might be explained by interaction of the genetic factors with other risk factors like, environmental factors or other genes. Important candidate genes in studying the aetiology of osteoporosis are the vitamin D receptor (VDR) gene, the estrogen receptor α (ERα) gene and the collagen type Iα1 gene since they play a central role in bone metabolism and in bone structure. In **Chapter 3.1** we investigated whether genetic variation at the estrogen receptor α (ERα) gene contributes to the variability among women in onset of menopause, as determinant of osteoporosis. Furthermore, we investigated the association between this polymorphism and the risk of osteoporotic fractures. Women having a *C* to *T* substitution in intron 1 on both alleles of the ERα gene had a later onset of menopause and moreover, were at lower risk of surgical menopause, i.e. particularly hysterectomy (**Chapter 3.1.1**). Interestingly, those women with a *C* to *T* substitution had a low BMD and, independent of BMD also had a higher risk of fractures, especially vertebral fractures. For men this ERα genotype-dependent BMD and fracture risk was not obvious (**Chapter 3.1.2**).

Until now, the functional consequence of the polymorphism of the VDR gene is unknown. It is likely however that genetic variability of the VDR diverse the regulating effect of 1,25-$(OH)_2D_3$ on cellular level. In light of the above we have examined the genetic effect of the VDR on bone turnover rate. In addition, we observed in *in vivo* and *in vitro* studies whether there was a genotype-dependent difference in response to active vitamin D substitution in a small sample of postmenopausal women (**Chapter 3.2**). In **Chapter 3.2.1** we observed a higher rate of bone turnover in women having low BMD compared to women having high BMD. Interestingly, this increased bone turnover rate was under influence of the VDR gene polymorphism. However, this genotype-dependent difference in bone turnover rate could not be explained by divergent response to short-term administration of active vitamin D. In **Chapter 3.2.2** data of the *in vitro* study performed with peripheral blood cells with different VDR genotypes are shown. We used VDR polymorphisms that are known to code different VDR proteins, i.e., a 424 amino acids-long VDR and a 427 amino acids-long VDR. The half maximal concentration for 1,25-

$(OH)_2D_3$ inhibition of cellar growth is significantly different among the VDR genotypes while the maximal inhibition is similar for all genotypes. This support the hypothesis that the 424 amino acids-long VDR is more efficient in exerting the effects of 1,25-$(OH)_2D_3$ than the 427 amino acids-long VDR.

Genetic variants of a protein might interact with other protein variants resulting in additive or subtractive results. Such interaction implicates that polymorphism of one gene can influence the effect of another gene. This gene-gene interaction mechanism might explain the finding described in **Chapter 3.3**. We analysed the combined influence of polymorphism in the VDR gene and the COLIA1 gene in determining the susceptibility to osteoporotic fractures. We showed that both the VDR and COLIA1 polymorphism are genetic markers for osteoporotic fracture in women, independent of BMD. But moreover, inter-locus interaction is likely to be an important component of osteoporotic fracture risk.

Chapter 4 reflects our main findings and we discussed the general methodological issues of the studies described in this thesis. In addition, we discussed the possible underlying mechanism of osteoporosis and strategies for future research.

Samenvatting

Osteoporose, ook wel botontkalking genoemd, is een frequent voorkomende aandoening onder ouderen. Fracturen (botbreuken), het klinische kenmerk van osteoporose, zijn door hun aanzienlijke bijdrage aan morbiditeit (ziekte), mortaliteit (sterfte) en kosten, een belangrijk probleem voor de volksgezondheidszorg. Door de 'vergrijzing' van de moderne westerse samenleving wordt de komende 50-jaar wereldwijd een 4-voudige toename in het aantal osteoporotische fracturen verwacht. Om deze toename in fracturen te voorkomen, zijn studies naar oorzaak van osteoporose noodzakelijk. Het laatste decennium is de kennis toegenomen met betrekking tot het voorkomen van osteoporose. Ook is enig inzicht verkregen in de rol van botmineraal dichtheid (BMD), als belangrijkste maat voor kwantiteit van bot, bij het voorspellen van het risico op fracturen. Echter, de meeste studies zijn uitgevoerd bij vrouwen en beperken zich tot heupfracturen. Bovendien kan maximaal 70-80% van de degeneratie van bot verklaard worden door een afname in BMD terwijl de overige 20-30% toe is te schrijven aan kwalitatieve veranderingen van bot. Hieronder vallen de bot architectuur, bot matrix aspecten en de verhouding tussen botaanmaak en botafbraak ('botombouw'). Recent is tevens gebleken dat zowel bot kwantiteit als kwaliteit voor een groot deel genetisch (erfelijk) bepaald zijn. Echter de hiervoor verantwoordelijke genen (erfelijke factoren), zijn tot nu toe niet goed vastgesteld.

Het doel van de studies beschreven in dit proefschrift, was inzicht te verkrijgen in de omvang van het fractuur probleem voor de volksgezondheid in Nederland en het bestuderen van zowel skelet als genetische oorzaken van osteoporose, niet alleen bij vrouwen maar ook bij mannen. Alle studies zijn uitgevoerd binnen het Erasmus Rotterdam Gezondheid en Ouderen (ERGO-onderzoek (the Rotterdam Study)) onderzoek. Dit is een groot bevolkingsonderzoek onder deelnemers van 55 jaar en ouder waarbij een aantal ouderdomsziekten en hun mogelijke risico factoren worden onderzocht.

In **Hoofdstuk 2.1** rapporteren wij incidentie cijfers (aantal per 1000 persoonsjaren) van alle niet-wervel fracturen en bestudeerden we de invloed van BMD op het risico van fracturen bij zowel mannen als vrouwen. Bij beiden komen de fracturen van de heup, pols en bovenarm het meest voor. Deze specifieke fracturen zijn ook het sterkst geassocieerd met een afname in BMD. Verder concluderen wij dat BMD een risico factor is voor deze fracturen, die even sterk is voor mannen en vrouwen. In **Hoofdstuk 2.2** hebben wij gekeken naar de voorspellende waarde van bot kwaliteit voor het risico op fracturen. De kwaliteit van bot werd hierbij

uitgedrukt als een biochemische bepaling in de urine (free deoxypyridinoline crosslinks (fDPD)) als merker voor botafbraak. Uit onze studie blijkt dat een toegenomen concentratie fDPD (gecorrigeerd voor kreatinine) geassocieerd is met een toegenomen fractuurrisico van met name heup en bovenarm fracturen. Dus niet alleen een afname in kwantiteit van bot maar ook een afname in kwaliteit van bot, leidt uiteindelijk tot het ontstaan van fracturen. Deze gedachtegang wordt ondersteund door de bevinding beschreven in dit hoofdstuk, dat the relatie tussen botafbraak en fracturen niet continue is maar pas boven een bepaalde 'drempel waarde' bestaat. Alleen boven die drempelwaarde is er voldoende kwaliteits verlies wat leidt tot bot degeneratie en uiteindelijk tot een hoger risico op het ontstaan van fracturen.

Onlangs is gebleken dat genetische factoren risicofactoren zijn van kwaliteit en kwantiteits aspecten van bot. Echter de gepubliceerde resultaten zijn niet consistent. Dit kan onder andere verklaard worden door interactie van de genetische factoren met andere factoren zoals omgevingsfactoren of andere genen. Belangrijke kandidaat genen in studies naar de oorzaak van osteoporose zijn het vitamine D receptor (VDR) gen, het oestrogeen receptor α (ERα) gen en het collageen type Iα1 gen daar zij een belangrijke rol spelen in het botmetabolisme en de bepaling van de botstructuur. In **Hoofdstuk 3.1** hebben wij onderzocht of polymorfismen (genetische variaties die meer dan 1% voorkomen in de bevolking) van het ERα gen bijdragen aan de variatie tussen vrouwen onderling betreffende de leeftijd waarop de menopauze aanvangt. Een vroege start van de menopauze is een risicofactor van osteoporose. Tevens bestudeerden wij de relatie tussen dit gen en het risico op osteoporose. Vrouwen die een *C* naar *T* substitutie in intron 1 op beide allelen van het ERα gen hadden, kwamen later in de postmenopauzale fase en hadden bovendien een lager risico op een chirurgisch geïnduceerde menopauze, met name op het ondergaan van een hysterectomy (baarmoeder verwijdering) (**Hoofdstuk 3.1.1**). Opvallend is dat deze vrouwen een lagere BMD waarde hebben en, onafhankelijk van BMD een hoger fractuurrisico, met name van wervelfracturen.(**Hoofdstuk 3.1.2**). Voor mannen was deze ERα genotype-afhankelijke BMD waarde en fractuurrisico niet duidelijk aanwezig.

Tot nu toe is de functionele betekenis van polymorfismen van het VDR gen onbekend. Het is echter zeer waarschijnlijk dat de genetische variabiliteit van de VDR, het regulerende effect van actief vitamine D op cellulair niveau beïnvloedt. In dit kader hebben we de associatie tussen VDR polymorfismen en botombouw bestudeerd in postmenopausale vrouwen.

Aanvullend bestudeerden wij in zowel *in vivo* (klinisch) als *in vitro (*fundamenteel) studies VDR gen afhankelijke verschillen in biochemische response op vitamine D toediening (**Hoofdstuk 3.2**). In **Hoofdstuk 3.2.1** zagen wij een hoger niveau van botombouw bij vrouwen met een lage BMD vergeleken met vrouwen met een hoge BMD waarde. Bovendien blijkt dat deze toegenomen botombouw-ratio onder invloed te staan van VDR polymorfismen. Echter deze bevinding kon niet verklaard worden door een genotype afhankelijk verschil in response op kortdurende toediening van actief vitamine D. In **Hoofdstuk 3.2.2** worden gegevens getoond van de *in vitro* studie uitgevoerd in perifere bloedcellen met een verschillend VDR genotype. We hebben een bepaald VDR polymorfisme onderzocht waarvan bekend is dat de verschillende polymorfismen staan voor verschillende VDR uitend in een 424 amino zuur-lange VDR of een 427 amino zuur-lange VDR.

De half maximale concentratie van de door actief vitamine D gestuurde remming van celgroei verschilt tussen de VDR polymorfisme terwijl de maximale remming gelijk is voor alle genotypen. Dit bevestigt de hypothese dat de 424 amino zuur-lange VDR meer efficiënt is in het uitoefenen van het stimulerende effect van actief vitamine D dan de 427 amino zuur-lange VDR.

Genetische varianten van een eiwit kunnen interacteren met andere eiwitten wat resulteert in een additief effect of een afgenomen effect. Zo kan een gen-gen interactie impliceren dat het polymorfisme van een gen het effect van een ander gen kan beïnvloeden. Dit gen-gen interactie patroon kan mogelijk de bevinding beschreven in **Hoofdstuk 3.3** verklaren. We analyseerden het gecombineerde effect van polymorfismen in het VDR gen en het COLIA1 gen als voorspellende factor voor fracturen. We rapporteren dat zowel het VDR als het COLIA1 polymorfismen genetische merkers zijn voor fracturen bij vrouwen, onafhankelijk van verschillen in BMD. Bovendien blijkt inter-locus interactie een belangrijke factor te zijn voor het risico op fracturen.

Hoofdstuk 4 reflecteert onze hoofdbevindingen en we bediscussïeren methodologische aspecten met betrekking tot de uitgevoerde studies. Verder bespreken we mogelijk onderliggende mechanismen en geven strategieën voor toekomstig wetenschappelijk onderzoek.

6

Epilogue

AWARDS

Young Investigator Award; European Calcified Tissue Society (ECTS), 1999
Estrogen Receptor Polymorphism Predicts the Onset of Natural and Surgical Menopause

Young Investigator Award; American Society for Bone and Mineral Research (ASBMR) 1999
Which Fractures are Associated with High Bone Resorption: the Rotterdam Study

List of publications

Weel AEAM, Redekop WK, Weening RS. Increased risk of malignancy for patients with chronic granulomatous disease and its possible link to the pathogenesis of cancer. *Eur J Cancer 1996;32A(4):734-5.*

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Colin EM, Weel AEAM, Uitterlinden AG, et al. Consequences of vitamin D receptor gene polymorphisms for growth inhibibition of cultured peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D3. *Clin Endocrinology 2000;.52(2): 211-216*

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Uitterlinden AG, Weel AEAM, Yue F, et al. Interaction between the vitamin D receptor gene and collagen type Ial gene in determining susceptibility for osteoporotic fracture in postmenopausal women. *J Bone MIner Res 2001; 16(2); 379-385*

Weel AEAM, Burger H, de Laet CEDH , Hofman A, van Leeuwen JPTM, Pols HAP. Fractures in Elderly Men and Women:Incidence and Association with Bone Mineral Density. *Submitted.*

*Weel AEAM, M Seibel, Hofman A, van Leeuwen JPTM, Pols HAP.*B one resorption rate as an independent risk factor of fractures in postmenopausal women; the Rotterdam Study 1991-1996 *Submitted.*

Weel AEAM, Uitterlinden AG, et al. The effect of estrogen receptor gene polymorphism on BMD and Fractures in Men and Women *Submitted*

*Weel AEAM, Collin E, Uitterlinden AG, et al.*Vitamin D receptor 3' polymorphism associated with the rate of bone turnover in women with low bone mineral density *Submitted*

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Lieve Michael, wie heeft het? Wij maar vooral jij !

Curriculum Vitae Auctores

Angelique Weel werd op 1 oktober 1967 geboren te Berkhout, Nederland. Zij behaalde het HAVO diploma in 1985 aan de "Scholengemeenschap Werenfridus" te Hoorn. Na het behalen van het Atheneum B diploma werd in 1987 met de studie geneeskunde aangevangen aan de Vrije Universiteit te Amsterdam, Nederland. In 1994 werd het artsexamen afgelegd. Aansluitend was zij tot 1996 werkzaam als arts-onderzoeker bij stichting ROMERES te Rotterdam, waarbij de interesse werd gewekt voor de ziekte osteoporose. Vanaf juli 1996 werd aangevangen met het onderzoek naar de "skeletal and genetic determinants of osteoporosis" op de afdelingen Interne Geneeskunde (Prof.dr. H.A.P. Pols and Dr J.P.T.M. van Leeuwen) en Epidemiologie & Biostatistiek (Prof.dr. A. Hofman), waarvan de resultaten in dit proefschrift zijn beschreven. Gedurende deze periode behaalde zij de Master of Science in Clinical Epidemiology aan de Netherlands Institute for Health Sciences te Rotterdam. In mei 1999 ontving zij de "Young Investigator Award" van de European Calcified Tissue Society. In oktober 1999 ontving zij tevens de "Young Investigator Award" van de American Society of Bone and Mineral Research. Sinds september 1999 is zij werkzaam als arts-assistent op de afdeling Inwendige Geneeskunde van het Academisch Ziekenhuis "Dijkzigt" te Rotterdam, alwaar zij in opleiding is tot internist.