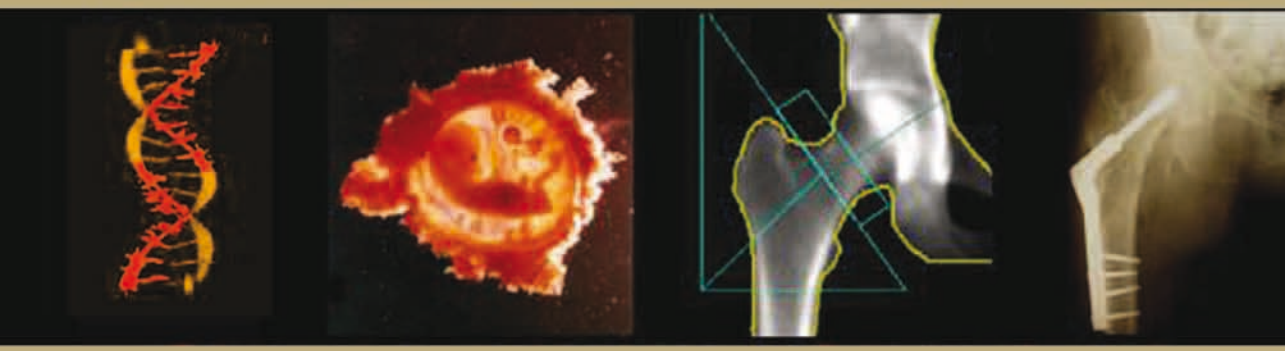
osteoporosis
from genes to phenotypes



Fernando Rivadeneira Ramírez



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Osteoporosis from genes to phenotypes

Ostoporose van genen tot fenotypen

Proefschrift

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Copromotoren	:	Dr. A.G. Uitterlinden Dr. J.J. Houwing-Duistermaat

Questions of science
Science and progress
Do not speak as loud as my heart

The Scientist - Coldplay

Por mis padres,

Carolina, Gabriëlla, Paulina

y toda mi familia

tan lejos pero tan cerca

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Chapter 2

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Chapter 4.1

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Chapter 5

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Chapter 6

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General Introduction

1

1.1 Definition

Osteoporosis is a leading public health problem in our rapidly growing, aging population. It is a systemic skeletal disease characterized by reduced bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (1). Family and twin studies have shown that osteoporosis and its associated phenotypes are under strong genetic control (2). In addition, it has been shown than not one major locus is responsible of the susceptibility to develop osteoporosis; instead, diverse genetic and environmental factors involved with the disease have confirmed its complex multifactorial nature (3).

1.2 Bone physiology

The skeleton is a highly organised and physiologically active system, continuously remodelling through life in order to preserve skeletal integrity, while providing a reliable and constant source of calcium for the circulation and, thus, for all other tissues. There are two types of bone present in the human skeleton, namely *cortical* or *compact* bone and *trabecular* or *spongy* bone, the latter of which is the most metabolically active. The morphology of most human bones consists of an outer surface of cortical bone that surrounds the inner trabecular elements. It is known that both cortical and trabecular bone undergo remodelling, but the frequency of this process is much less in the cortex than in the trabecular components. Bone remodelling comprises the process of bone resorption, which is always followed by bone formation and provides a mechanism for bone self-repair. It represents simultaneous action of bone destroying (resorption) cells or osteoclasts, and bone forming cells or osteoblasts whose combined action on the specific bone surface is expressed in terms of bone remodelling units (BRU) (4). The bone-remodelling process includes five stages: BRU activation, bone resorption, 'coupling' or reversal, bone formation and mineralization of newly formed bone matrix and the resting stage (Figure 1).

The process of bone formation takes several months and is slow compared to the process of bone resorption which is completed within 7-14 days (5). The coupling between bone resorption and bone formation is age dependent. In young individuals (under age 25 years) bone formation normally exceeds bone resorption. During healthy mature adulthood (between 25–35 years of age) the net activity of osteoclasts equals the net activity of osteoblasts. In contrast, in elderly subjects bone resorption is higher than bone formation leading to overall bone loss with aging. The stages in the bone-remodelling process are well co-ordinated by many systemic and locally acting bone factors including numerous hormones, growth factors and

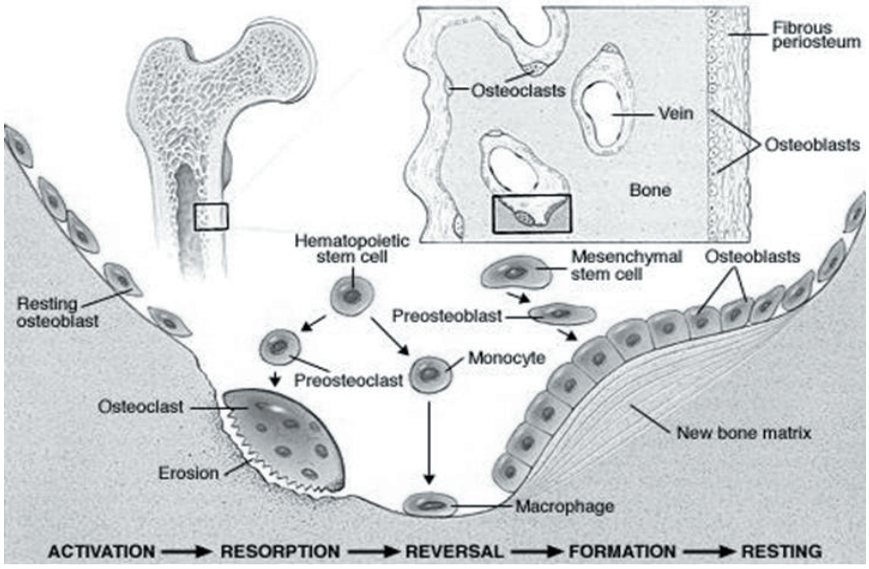


Figure 1. Five stages of the bone-remodeling process.
(Source http:\\www.medscape.com)

cytokines (6). The differences between subjects in bone growth, peak bone mass accrual, bone loss with aging and fracture risk could be due to differences in the action of individual factors but also to their interaction. That is the reason we focus on candidate gene polymorphisms and their interactions.

1.3 IGF-I and bone

IGF-I is a ubiquitous polypeptide that stimulates osteoblast activity, subsequently leading to bone matrix formation and inhibition of bone collagen degradation (7). IGF-I also stimulates osteoclast formation and action, although these effects are less clear (8). It has been determined that skeletal IGF-I originates from two main sources: (a) *de novo* synthesis by bone forming cells (i.e. osteoblasts) where most of IGF-I in bone is derived from and (b) the circulation and/or bone marrow (9). During active bone resorption, as bone matrix is dissolved, significant amounts of IGF-I are released from storage (i.e. bound to IGFBP-2 and IGFBP-5). Considering that IGF-I is stored within the skeletal matrix and is released during bone resorption, IGF-I is one of the critical coupling proteins that keep bone resorption closely linked to formation (10). In addition, it has been determined that the major hormones regulating bone turnover *in vivo* also affect IGF-I expression *in vitro* (10). These include parathyroid hormone (PTH), estrogens, glucocorticoids

and 1,25-dihydroxyvitamin D (9). An important role for IGF-I on bone growth, peak bone mass achievement and posterior bone loss (with aging) has been suggested. IGF-I levels peak during puberty at or about the same time as acquisition of peak bone mass. In addition, serum IGF-I levels decline with ageing along a slope that is similar to that of age-related bone loss. Indeed, IGF-I plasma levels have been associated with low BMD (11-13), osteoporosis (14) and fractures (15), although it is not known if these systemic levels are representative of local (skeletal) concentrations (16-18). Based on this, the *IGF-I* gene (map location 12q22-q24.1) arises as an obvious candidate gene to study in relation to osteoporosis.

1.4 Estrogen receptors and bone

Estrogens are sex hormones responsible for gender dimorphism and reproduction. They are also pleiotropic hormones with numerous biological actions extending beyond non-reproductive tissues, including bone. The biological actions of estrogens are mediated by binding to one of two specific estrogen receptors, ER α (*ESR1* 6q25.1) and ER ß (*ESR2* 14q22-24). Both estrogen receptor isoforms are expressed in osteoblasts, osteoclasts, and in several types of cells from the bone marrow the receptors co-localize in adult bone (19-22). When co-expressed, *ESR2* exhibits an inhibitory action on *ESR1*-mediated gene expression and in many instances opposes the actions of *ERS1* (23). There is also evidence of gender- and age-specific expression of the *ESR2* protein SR2, which can inhibit transcriptional activation by *ESR1*, perhaps through formation of ER α / ER ß heterodimers. In *ESR2,* alternative splicing that removes one or more exons has been observed in osteoblasts, and these alternative splice forms can eliminate or lower ligand binding affinity (23). The characterization of mice lacking *ESR1*, or *ESR2*, or both has revealed that both receptor subtypes have overlapping but also unique roles in estrogen-dependent action *in vivo.* An *ERS1* knockout mouse model has shown that these animals present with decreased bone mass in both genders (24). In contrast, *ERS2* knockout models present with increased cortical bone mass suggesting that *ESR2* is a negative modulator of cortical but not cancellous bone modelling (25).

1.5 Bone Fracture: a Problem of Biomechanics

As described above bone modelling and remodelling occur on the microstructural level, but net effects are evident in whole bone geometry. Previously, it has been shown that fractured individuals possess specific patterns of bone geometry which can significantly discriminate them from non-fractured individuals (26-28). In order to conceptualize bone geometry some understanding of bioengineering is needed. To serve its different functions bone must have antagonist properties: it

must be stiff and able to resist bending forces due to load bearing and/or locomotion, but it should also be flexible enough to deform during impact loading without fracturing (29). With aging this ability to resist fracture is compromised, and is the reason for the prominent increase in the incidence of osteoporotic fracture with senescence (30). But, why do some elderly individuals fracture while others do not? It has been established that those elderly people who suffer osteoporotic fractures have lower bone mass and mechanically weaker bones which are more susceptible to fracture. This weakening can be the result of age-related changes in either tissue properties or in the structural dimensions. Even though tissue changes may not be ruled out, the most remarkable difference between fractured and non-fractured individuals relies on the amount and/or the distribution of bone mass. That is, osteoporotic fracture is structural in nature. BMD assessment using DXA is by far the most used method to assess the risk of osteoporotic fracture. The decline in BMD with aging arises from bone loss in the form of both cortical and trabecular thinning, but also, from an increase in total volume as the bone expands in width with age. Although it is clear that net bone loss within a fixed diameter reduces mechanical strength, the effect on strength is less clear when accompanied by an expanding diameter (caused by periosteal apposition) (Figure 2). The presence of periosteal apposition with aging is the reason the interpretation of BMD should be complemented with indexes of bending strength, such as the section modulus. An important characteristic of the section modulus is that as the diameter of a hollow tube is increased, the same bending strength can be achieved with progressively less material and thus a lower volumetric density. Bones are not hollow tubes, but

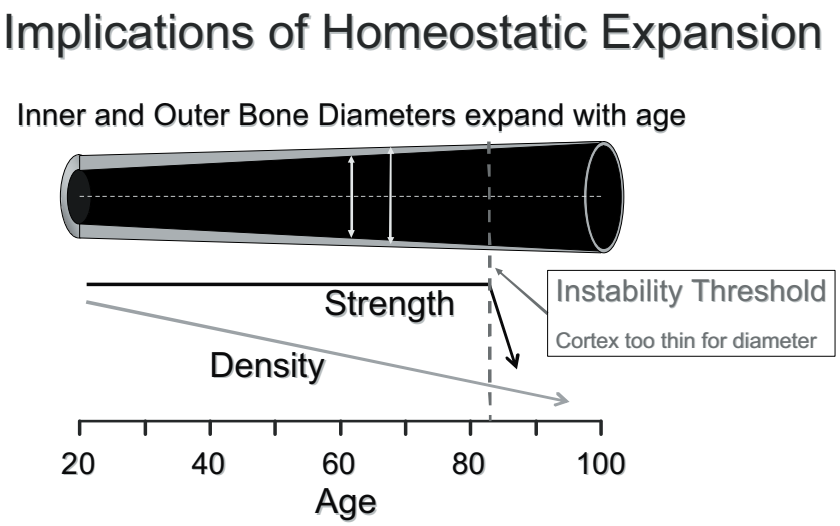


Figure 2. Relation between bone expansion and aging and its consequences over bone density, strength and instability (contribution Thomas J. Beck).

the same biomechanical principles may apply. Although it is acknowledged that low BMD is predictive of fragility fracture, it is not obvious why compensatory expansion should lead to fragility unless expansion leads to cortical instability. If the process of bone expansion is carried too far, the section modulus no longer governs bending strength because the wall becomes unstable and prone to collapse (or buckle). The "buckling ratio" is an index of bone instability which is estimated as (½) the ratio of outer radius to wall thickness. Higher buckling ratios represent higher bone instability and greater susceptibility to fracture. It has not been shown that fragility fractures are due to instability, nevertheless bones should become less stable as cortices thin, diameters increase and as internal support of trabeculae are lost (31). Based on these considerations several parameters of bone geometry can be measured by Hip Structural Analysis (HSA) software developed by Thomas J. Beck to measure hip bone geometry from DXA scans (32,33).

1.6 Approaches in Genetic Studies

In osteoporosis as in other complex diseases in humans, the strategies used to identify genes involved in disease have focused on two main approaches. One includes genome-wide screening using a linkage analysis approach and which is limited to family-based study designs. The other includes candidate gene studies which are commonly but not restricted to association analyses (since linkage strategies may also be used) in population- or family-based studies (3).

Genome-wide screening using linkage mapping is the first step towards positional cloning. It is the process of systematically scanning the entire genomes of interrelated individuals who share a common trait or disease, using regularly spaced, highly variable (polymorphic) DNA segments whose exact position is known. The use of related individuals allows identifying genetic regions "in linkage" with the trait/disease, and to test if they share one or more chromosomal areas identical by descent in those regions more frequently than expected by chance alone. In this way, regions can then be further delineated for further analysis and characterization of the responsible genes. The primary advantage of genome screening is that no prior knowledge of the physiology or biology underlying the disorder being studied is necessary, which may be an advantage when studying complex disorders, such as osteoporosis, with an etiology that is still largely not understood.

The candidate gene approach investigates whether a genetic variant in a particular *already-known* gene is involved with the trait or disease. A major advantage of this approach is that is based on known biology of the gene product. This way, the choice of candidate genes in osteoporosis has been directed towards factors which

regulate bone turnover and proteins that make up normal bone matrix. Using this approach, many genes have been evaluated in osteoporosis, including vitamin D-, calcium-sensing-, calcitonin- and estrogen alpha-receptors, insulin growth factor I, collagen type I alpha 1 and others (2,3,34). In contrast with linkage mapping studies, association studies of candidate genes do not require (a large number of) interrelated individuals with both affected and unaffected members, which are very difficult to collect within the context of a late-onset disease like osteoporosis. Furthermore, it is now believed that association studies of candidate gene studies are better suited to study gene variants underlying common and more complex diseases, where the risk associated with any given candidate gene is relatively small. Nevertheless, a limitation of this approach is that in order to choose a potential candidate gene, some understanding of the mechanisms underlying the disease (i.e., pathophysiology) must already be present.

The first critical step in conducting candidate gene studies is the choice of a suitable candidate gene that may plausibly play a relevant role in the process or disease under investigation. The selection of candidate genes is usually based on knowledge of cellular signalling or metabolic pathways, its location or because it has been examined in previous association studies. Once a candidate gene has been selected, the genetic variants (DNA polymorphisms) within the gene that will be used for testing in an association study must be chosen. There are several types of polymorphisms which include: restriction fragment length (RFLP), minisatellite variable number of tandem repeats (VNTR), microsatellites (mostly CA_n repeats) and single nucleotide (SNP). The SNP is the most suitable and frequently used type of polymorphism in association studies of candidate genes. In the past, the identification of genetic variants in a gene was a laborious process that often involved sequencing the entire gene in 20-50 subjects. Nowadays, we have available databases with more than 5 million SNP's (i.e. dbSNP and Celera) which facilitate the study of polymorphisms in candidate genes. Criteria to select a particular polymorphism for further studies includes determining whether the nucleotide variation is: 1) likely to have functional significance, either because it actually results in amino acid changes in the resulting protein or because it occurs in regulatory regions; 2) with sufficient frequency to allow detection of subtle differences between individuals with and without the trait or disease under investigation. However, variations in non-coding regions may be correlated with altered phenotype in two situations: one, when variations occur in regulatory regions, such as promoters or intron splice sites, or 3'UTR's that could alter mRNA stability and routing; two, when variations are positioned close enough to a functional mutation (usually from 10-250 kb), and are therefore almost always inherited together, which is a phenomenon known as "linkage disequilibrium" (LD). This way, associations can arise due to a) direct effect of the polymorphism (i.e. functional variant), b) LD with a nearby truly functional variant.

One further question in the study of variation in candidate genes is if it is necessary to consider one-SNP-at-a-time or if they should be analyzed as phased haplotypes (35). The rationale behind this approach is that LD in genes appears to have a block-like structure and that a subset of SNP's analyzed through phased haplotypes can identify most of the genetic variation in a genomic region (36). In this way, a good representation of the genetic variation in a gene can be tested by choosing a number of polymorphisms similarly spaced through out the gene and analyzed in the form of haplotypes (35).

1.7 Study Population

1.7.1 Population-Based Setting

Association studies presented in this thesis are based on the Rotterdam Study (37), a prospective population-based cohort study, of determinants of chronic disabling diseases in Caucasian elderly men and women. The Rotterdam Study was approved by the medical ethics committee of the Erasmus Medical Center and written informed consent was obtained from all participants. At baseline, between 1990 and 1993, all inhabitants aged 55 years and over (n=10,275) of the district of Ommoord in Rotterdam were invited to take part in the study. A total of 7,983 subjects (response rate 78%), 4878 of whom were women, entered the study. At baseline, and again at the first and second follow-up visits (1994-1995 and 1997-1999, respectively), information was gathered concerning amongst others lifestyle habits, socio-economic status, medical history and pharmacotherapeutic history. In addition to an interview, all subjects were invited to visit the research center for physical examination and blood drawing. General practitioners (GPs) in the research area (who cover 80% of the cohort) reported all relevant fatal and non-fatal events (such as fractures) through a computerized system. Research physicians verified follow-up information by checking GPs' patient records. This is possible because in The Netherlands the GPs have a gate-keeper function, which means that the only way to access specialist and hospital care is by consulting them. The GPs retain all medical information of his/her patients. For the remaining 20% of the cohort, research physicians regularly visited the GPs and collected data from their records. For hospitalized patients, discharge letters were additionally used for verification. All events were coded independently by two research physicians according to the International Classification of Diseases, 10th revision (ICD-10) (38). If there was disagreement, consensus was reached in a separate session. A medical expert in the field reviewed all coded events for final classification.

1.7.2 Family-Based Setting

Heritability studies of this thesis are based on the Erasmus Rucphen Family (ERF) Study, an ongoing extended-pedigree study of genetic risk factors for neuropsychiatric, cardiovascular, endocrinologic, ophthalmologic and musculoskeletal disorders. The ERF study is part of the Genetic Research in Isolated Populations (GRIP) program which aims to identify genetic risk factors in the development of complex disorders in an isolated community of about 20,000 inhabitants settled around eight adjacent villages in the southwest of The Netherlands. The ERF study population includes living descendants of 50 couples with at least six children and at least one baptized in the community church between 1890 and 1900. Descendants were invited to participate in the study (n=2500) independent of disease status. The Medical Ethics Committee of Erasmus Medical Center Rotterdam approved the ERF study and written informed consent was obtained from every participant. Phenotyping started in June 2002, is currently ongoing and includes a series of medical investigations assessed in a standardized manner (which are comparable to those used in the Rotterdam Study) by trained assistants and physicians during their visit to the research center located in Sprundel, The Netherlands. The assessment included total body, antero-posterior lumbar spine and dual femur DXA scans, anthropometrics, muscle strength and interview about physical activity, medical history and medication use. The current response rate is 78%.

1.8 Aim and Description of Chapters

In this thesis, a genetic epidemiology study of osteoporosis is presented. The objective was to identify genetic determinants of osteoporosis using a candidate gene approach framework. **Chapter 2** presents a detailed epidemiological and biomechanical description of longitudinal changes in BMD and bone geometry in the Rotterdam Study, the setting where the candidate gene studies are conducted. In **Chapter 3** a comprehensive phenotypic analysis of DXA-based osteoporosis traits is performed by determining heritabilities and genetic correlations across BMD and bone geometry measurements. **Chapter 4** focuses on the insulin-like growth factor I gene (*IGF-I*); in **Chapter 4.1** a promoter polymorphism is examined in relation to cross-sectional and longitudinal BMD measurements, and in **Chapter 4.2** the relationship with non-vertebral fractures is examined with respect to bone geometry measurements. In **Chapter 5** polymorphisms in the estrogen receptor beta (*ESR2*) gene are examined in relation to BMD, bone geometry and the risk of osteoporotic fracture. In **Chapter 6**, the interaction between polymorphisms in the *IGF-I*, estrogen receptor alfa (*ESR1*) and *ESR2* genes is examined in relation to osteoporotic fracture and bone parameters. **Chapter 7** consists of a general discussion of the general principles applied and the problems encountered in the process. Finally, the findings of this thesis are placed in perspective of future research.

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Longitudinal Analysis of BMD and Bone Geometry in the Rotterdam Study

2

ABSTRACT

Introduction: Multiple factors are thought to contribute to age-related decline in bone mineral density (BMD). However**,** the underlying mechanisms are still incompletely understood. For this reason we examined epidemiological and biomechanical patterns of longitudinal BMD changes in a large population-based cohort study of elderly men and women age 55 and older.

Methods: Changes in BMD (mean follow-up time 6.5 SD 0.6 years) were studied in 1166 men and 1548 women. Changes in bone geometry were determined by Hip Structural Analysis (HSA) of DXA scans and were assessed in 865 men and 1052 women (mean follow-up time 4.5 SD 0.6 years).

Results: Approximately 45% of the original study population at baseline was lost to follow-up, most likely due to morbidity. As expected, the decline of BMD with age was higher in females and averaged about 0.8% and 0.3 % of baseline BMD per year in females and males respectively. Advanced age (above 75 years), BMD at baseline and weight loss on follow-up were the most prominent factors influencing bone loss in both genders. HSA data showed that the decline of BMD is caused by a combination of bone loss at the cortices (resulting in cortical thinning) and periosteal bone apposition (resulting in an increase in neck width). This process of bone expansion was more pronounced in females. Also with aging, section modulus (an index of bone strength) decreased in both genders, possibly as an adaptive response to changes in body weight. In both genders the buckling ratio (an index of bone instability) increased with age but it was much larger in females.

Conclusion: BMD at baseline and weight loss with follow-up are the most significant determinants of BMD decline in both genders. Greater progression towards bone instability as a result of cortical thinning and periosteal bone apposition might explain the higher susceptibility for hip fracture in elderly women.

INTRODUCTION

Femoral neck fragility in old age has usually been attributed to the "age-related bone loss" or involutional osteoporosis (1,2). Bone mineral density (BMD) measurement by Dual-energy X-ray absorptiometry (DXA) is the most commonly used method to determine the risk of fragility fracture of an individual and to assess loss of BMD with age. Even though the patterns of BMD decline with aging has been matter of intense study for decades now (3-6) the causes (7-11) and underlying mechanisms leading to BMD decline and bone fragility have not been completely elucidated.

The decline in femoral neck BMD with time is not only due to bone loss, but also due to an increase in bone volume caused by bone expansion, the latter being less well studied (12-14). These two processes are interrelated and occur mainly due to cortical thinning and periosteal apposition (bone expansion) with a sexual dimorphism already present early in life (15). These two phenomena have opposing effects on bone strength with bone expansion allowing strength to be maintained with aging even in the presence of considerable bone loss (16). Nevertheless, if a threshold is reached where cortical thinning is excessive in relation to the already expanded femoral neck, cortices are thought to become unstable and prone to collapse (buckle), facilitating the occurrence of fragility fracture(12).

Femoral neck BMD obtained from DXA provides no information regarding the relative contributions of periosteal apposition and endocortical resorption (resulting in cortical thinning) to net bone loss during aging. In this way, persons with similar BMD values may have important underlying differences in bone strength and bone stability and thus, in the risk of fracture.

In this large population-based study we examined the underlying epidemiological and biomechanical patterns of longitudinal BMD decline at the proximal femur, in elderly men and women aged 55 and older. We determined which factors at baseline relate to longitudinal changes in BMD and studied the underlying structural changes in bone geometry with time.

SUBJECTS AND METHODS

Subjects

The Rotterdam Study is a large prospective population-based cohort study of men and women aged 55 and over. The objective is to study the determinants of disease and disability in the elderly, with special focus on neurological, cardiovas-

cular, ophthalmologic and locomotor diseases. The Medical Ethics Committee of the Erasmus Medical Centre has approved the Rotterdam Study. Both the rationale and the study design have been described previously (17). All 10,275 inhabitants of Ommoord, a district in Rotterdam, The Netherlands, were invited to participate. Of these, 7983 (4878 women) participated, resulting in a response rate of 78%.

Baseline data collection was conducted between July 1989 and June 1993 and included an initial home visit and interview by a trained research assistant and an extensive physical examination at the research center. First and second follow-up assessments were performed between July 1993 and January 1996 and between July 1996 and December 1999, respectively, applying the same measurement protocols used at baseline. The third follow-up assessment is currently ongoing.

The present study consists of two separate phases. In the first phase changes in BMD with time were assessed between baseline and second follow-up (6.5 SD 0.6 years) in 2714 individuals. In the second phase, the underlying biomechanical characteristics of BMD decline (changes in bone geometry) were assessed between first and second follow-up (4.5 SD 0.6 years) in 1917 individuals.

BMD Measurements

In each assessment period bone mineral density measurements (g/cm^2) of the proximal femur were performed by dual energy X-ray absorptiometry (DXA) using the same Lunar DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA) and the same standard protocols. Methods, quality assurance, accuracy and precision issues of the DXA measurements have been described previously (3). In contrast to manufacturer recommendation and as reported earlier (18), DPX-L software upgrades did interfere with the serial BMD measurements during follow-up. For this reason, we reanalyzed all existing scans (n=13,391) with the DPX-IQ v.4.7d software. To increase the accuracy in follow-up BMD measurements, the search and template tools in the compare mode of the DPX-IQ software were used to position the femoral neck region-of- interest in scans of the same individual. Additional retrospective calibrations using phantom measurements were performed to adjust for software changes not corrected by the DPX-IQ reanalysis (19). The change in BMD during follow-up was expressed in four different ways: 1) the absolute BMD difference (mg/ cm^2) between assessment periods; 2) the absolute rate of change in BMD (mg/cm^2 per year), calculated as the BMD difference between assessment periods divided by the duration of follow-up in years; 3) percentage rate of change from previous BMD (% per year), calculated as the difference between two assessment periods divided by the earliest BMD measurement and divided by follow-up time in years and 4) the overall relative fraction of baseline BMD (%)

calculated as the BMD difference between two assessment periods divided by the earliest BMD measurement.

Hip Structural Analysis (HSA)

We have used the hip structural analysis (HSA) software developed by Thomas J. Beck to measure hip bone geometry from the DXA scans (12,20). The HSA program differs from the conventional density analyses in the sense that cross-sectional dimensions are derived rather than averaged mass or density. The program uses the distribution of mineral mass in a line of pixels across the bone axis to measure geometric properties for cross-sections in cut planes traversing the bone at that location. Current versions average measurements for a series of 5 parallel "mass profiles" spaced ~1 mm apart along the bone axis. Analysis locations included the narrow-neck *(NN)* region across the narrowest point of the femoral neck. BMD, bone cross-sectional area (CSA), bone width (outer diameter) and cross-sectional moment of inertia (CSMI) were measured directly from mineral mass distributions using algorithms described previously (12). In addition, estimates of cortical thickness were obtained with simple models of the cross-sections which employ measured dimensions and assumptions of cross-section shape. The NN region is modeled as a circular annulus which assumes a proportion of cortical/trabecular bone of 60/40. For the current work, calculations of the section modulus (Z), an index of bending strength, and the buckling ratio (BR), an index of bone instability, were slightly modified from those of previous reports (12) to account for shifts in the center-of-mass. Z was calculated as CSMI $/d_s$, where d_s is the maximum distance from the center of mass to the medial or lateral surface. Buckling ratios were computed as d_s divided by estimated mean cortical thickness. In previous work (12), ½ the outer diameter was used instead of d_s in calculations of Z and BR. As described previously in the section measurements, software updates, which altered scan image acquisition, made follow-up data from the HSA not compatible to baseline measurements. This is the reason why changes in geometry parameters in time were only analyzed between first and second follow-up.

Clinical examination

Height (cm) and weight (kg) were measured in standing position wearing indoor clothes and without shoes. Body mass index or BMI (kg/m^2), was calculated as weight divided by the square of height. Waist and hip circumferences (cm) were measured and waist to hip ratio calculated.

The diagnosis of diabetes was made in individuals with antidiabetic therapy (code A010 of the Anatomical Therapeutical Chemical classification index, WHO 1992) and/or a non-fasting serum glucose level equal to or higher than 11.1 mmol/l. The positive history of myocardial infarction was verified in the EKG by a cardiologist or general practitioner. Blood pressure was measured with the subject in the sitting position at the right upper arm with the use of a random zero sphygmomanometer. The average of 2 measurements was used for analysis and hypertension was defined as a diastolic blood pressure ≥ 100 mm Hg and/or a systolic blood pressure ≥ 160 mm Hg and/or use of antihypertensive medication indicated to treat high blood pressure (grades 2 and 3 of the 1999 World Health Organization criteria). In addition the presence of peripheral arterial disease was assessed as described previously (21,22). Total serum cholesterol was determined by an automated enzymatic procedure.

Home interview

A trained interviewer performed an extensive home interview on medical history, risk factors for chronic disease and food intake. Calcium and vitamin intake was assessed together with total caloric intake as described previously (7,23). In this study, calcium intake was adjusted for total caloric intake. Smoking habits were assessed and categorized as current, former or never. Lower limb disability and use of walking aids was assessed using a modified version of the Standford Health Assessment Questionnaire, as described previously (7,24), and disability was defined as an index of 0.5 or higher. The presence of intermittent claudication was diagnosed according to the criteria of WHO/Rose questionnaire. Age at and reason of menopause was assessed as defined previously (25).

Statistical analysis

Data were analyzed stratified by gender. Multiple linear regression was used to model the patterns of change in BMD and bone geometry across study groups, including presence or absence of follow-up, age groups, tertiles of BMI and tertiles of weight change. Adjustments were done for age, weight, and height. Finally, model assumptions were verified and model residuals were checked for goodness-of-fit. If not stated otherwise, analyses were performed using the SPSS-package V.11 (SPSS Inc. Chicago, IL).

RESULTS

Table 1 compares baseline characteristics across the different assessments performed in the Rotterdam Study, between all individuals with BMD measurements (reference) and those included in the BMD analysis (baseline to second follow-up) and in the bone geometry analysis (first to second-follow-up). The follow-up analyses are based on approximately 45% and 35% individuals, for BMD and bone geometry respectively. When the characteristics are compared at baseline, it becomes evident in both genders that individuals in the groups with follow-up are on average ~ 2 years younger, ~ 1 cm taller, ~1 kg heavier, and with 0.02 higher BMD levels than the overall population with BMD measurements.

Figure 1 illustrates the patterns of BMD change with follow-up. The longest follow-up was on average 6.5 years and was obtained between baseline and second follow-up (Figure 1A). The mean rate of change in BMD was -6.2 mg/cm^2 per year in females and was -3.2 mg/cm^2 per year in males. Figures 1B and 1C compare the two types of BMD measurements (the traditional femoral neck region and the hip structural analysis narrow-neck region, respectively) and were both obtained between first and second follow-up periods on average 4.5 years apart. At the femoral neck region the mean rate of change in BMD was -3.1 mg/cm^2 per year in males and -6.7 mg/cm^2 per year in females. Similarly, at the femoral narrow-neck region the mean rate of change in BMD was -2.5 mg/cm^2 per year in males and -5.1 mg/cm^2 per year in females. In all three analyses the overall trends and patterns are very similar. In men, rates of BMD-loss are increasing with age, while in women, the rates are more stable across age groups and tend to be higher at the youngest (55 to 60 years) and oldest (above 75 years) age categories.

In Table 2 health characteristics are compared between individuals lost to follow-up and across tertiles of BMD change. In both genders approximately 45% of the individuals are lost to follow-up for the BMD measurements. These individuals lost to follow-up are 6.2 years older, 2.4 cm shorter, 2 kg lighter and have 0.04 g/cm^2 lower BMD, than individuals with follow-up BMD assessments. In addition, the presence of morbidity at baseline is higher, and manifested by the presence of disability, disorders of glucose metabolism, cardiovascular disease. Also, men have lower intake of vitamin intake and higher current smoking, but lower past history of smoking. Subjects with the higher rates of BMD loss (lowest tertile in BMD change) were on average 1.6 years older, had the greater weight loss (-0.8 kg per year) and 0.05 g/cm^2 higher BMD levels at baseline. In addition, individuals with the highest rates of BMD decline appeared to have 2 mmHg higher systolic blood pressure (significant in males only) and to be current smokers while they were less often former smokers and had lower intake of folic acid. Women with the highest bone loss appeared to have increased presence of diabetes, although the difference

TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION AT THE DIFFERENT ASSESSMENT PERIODS*

	MALES						
	ALL INDIVIDUALS WITH BMD MEASUREMENTS			BMD ANALYSIS		GEOMETRY ANALYSIS	
	Baseline	1st follow-up**	2nd follow-up**	Baseline	2nd follow-up**	1st follow-up**	2nd follow-up**
n	2452	1867	1287	1157	1157	828	828
Age (years)	67.6 ± 7.7	68.9 ± 7.3	72.2 ± 6.5	65.6 ± 6.5	72.1 ± 6.5	67.5 ± 6.6	72.0 ± 6.5
Height (cm)	174.9 ± 6.8	175.2 ± 6.7	173.9 ± 6.7	175.5 ± 6.6	174.0 ± 6.5	175.4 ± 6.6	173.8 ± 6.5
Weight (kg)	78.8 ± 10.6	79.8 ± 10.7	79.6 ± 11.2	80.1 ± 10.2	79.8 ± 11.1	81.6 ± 10.4	80.8 ± 11.0
BMI (kg/m2)	25.7 ± 3.0	26.0 ± 2.9	26.3 ± 3.2	26.0 ± 2.9	26.3 ± 3.2	26.5 ± 2.9	26.7 ± 3.1
BMD (g/cm2)	0.92 ± 0.137	0.91 ± 0.135	0.90 ± 0.136	0.93 ± 0.131	0.90 ± 0.136	0.93 ± 0.125	0.91 ± 0.131
	FEMALES						
	ALL INDIVIDUALS WITH BMD MEASUREMENTS			BMD ANALYSIS		GEOMETRY ANALYSIS	
	Baseline	1st follow-up**	2nd follow-up**	Baseline	2nd follow-up**	1st follow-up**	2nd follow-up**
n	3375	2514	1725	1543	1543	1007	1007
Age (years)	68.4 ± 8.2	69.6 ± 7.8	72.6 ± 6.8	65.9 ± 6.7	72.5 ± 6.8	67.7 ± 6.7	72.1 ± 6.6
Height (cm)	161.7 ± 6.6	162.1 ± 6.5	160.8 ± 6.3	162.7 ± 6.4	160.9 ± 6.3	162.6 ± 6.2	160.8 ± 6.2
Weight (kg)	69.9 ± 11.0	70.5 ± 11.2	70.3 ± 11.8	70.2 ± 10.9	70.4 ± 11.8	72.3 ± 10.8	72.2 ± 11.3
BMI (kg/m2)	26.7 ± 4.0	26.8 ± 4.0	27.2 ± 4.3	26.6 ± 4.0	27.2 ± 4.3	27.3 ± 3.9	27.9 ± 4.1
BMD (g/cm2)	0.83 ± 0.135	0.82 ± 0.134	0.80 ± 0.133	0.85 ± 0.130	0.80 ± 0.132	0.85 ± 0.123	0.82 ± 0.125

* Data are unadjusted means ± SD

**First and second follow-up measurements were performed on average 1.9 (SD 0.6) and 4.5 (SD 0.6) years after baseline

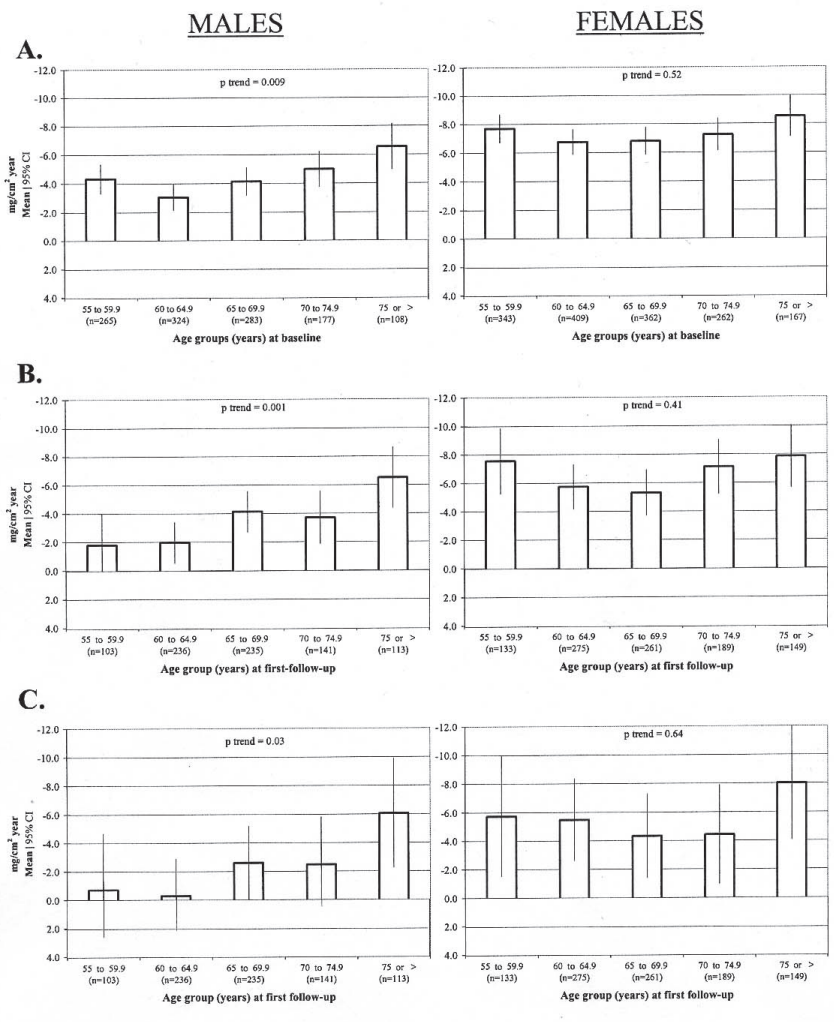


Figure 1. Absolute rates of BMD change in males (left) and females (right) **A.** Measured at femoral neck region between baseline and second follow-up assessment (mean duration 6.5 SD 0.6 years); **B.** measured at femoral neck region between first and second follow-up assessment (mean duration 4.5 SD 0.6 years); **C.** Measured at narrow-neck region between first and second follow-up assessment (mean duration 4.5 SD 0.6 years).

TABLE 2. HEALTH CHARACTERISTICS IN INDIVIDUALS LOST TO FOLLOW-UP AND BY TERTILES OF BMD CHANGE

	MALES				FEMALES			
		Rate of BMD change between baseline and 2nd follow-up				Rate of BMD change between baseline and 2nd follow-up		
	Lost to follow-up	Lowest tertile	Middle Tertile	Highest Tertile	Lost to follow-up	Lowest tertile	Middle Tertile	Highest Tertile
n	889	390	390	386	1265	521	507	520
Age (years)	71.5 ± 7.9†	66.7 ± 7.1	64.9 ± 6.1**	65.1 ± 6.1**	72.4 ± 8.6†	66.0 ± 7.0	65.5 ± 6.8	66.3 ± 6.4
Age at menopause (years)	-	-	-	-	48.7 ± 4.9	49.0 ± 5.4	49.4 ± 4.8	48.5 ± 5.4
Height (cm)	173.5 ± 6.7†	175.0 ± 6.6	175.4 ± 6.8	176.2 ± 6.3	160.3 ± 6.6†	163.0 ± 6.5	162.8 ± 6.6	162.2 ± 6.1
Weight (kg)	76.7 ± 10.9†	80.0 ± 10.1	79.7 ± 10.3	80.4 ± 10.4	69.4 ± 11.3	70.6 ± 11.2	70.5 ± 10.5	69.6 ± 10.9
Rate of weight change (kg per year)	-	-0.9 ± 4.8	0.1 ± 4.7*	0.0 ± 4.4*	-	-0.7 ± 4.9	0.2 ± 4.5**	1.0 ± 4.6**
Body mass index (kg/m2)	25.5 ± 3.1	26.1 ± 2.9	25.9 ± 2.8	25.9 ± 2.9	27.0 ± 4.1	26.6 ± 4.3	26.6 ± 3.8	26.5 ± 3.9
Waist to hip ratio	0.96 ± 0.07	0.96 ± 0.07	0.95 ± 0.07	0.95 ± 0.07	0.88 ± 0.09†	0.86 ± 0.09	0.86 ± 0.09	0.86 ± 0.08
BMD (g/cm2)	0.89 ± 0.142†	0.95 ± 0.136	0.92 ± 0.129**	0.92 ± 0.126**	0.80 ± 0.138†	0.88 ± 0.136	0.84 ± 0.117	0.82 ± 0.131
Lower limb disability (%)	245 (27.7)†	38 (9.8)	42 (10.8)	42 (11.0)	552 (43.9)†	108 (20.9)	111 (22.1)	101 (19.6)
Use of walking aid (%)	62 (7.1)†	7 (1.8)	9 (2.3)	6 (1.6)	115 (9.3)†	12 (2.3)	15 (3.0)	13 (2.5)
Diabetes mellitus (%)	116 (13.5)†	30 (8.0)	29 (7.6)	31 (8.1)	195 (15.7)†	34 (6.7)	30 (6.1)	27 (5.3)
Blood glucose levels (mmol/L)	7.3 ± 2.8†	6.9 ± 2.2	6.8 ± 2.6	6.9 ± 2.3	7.2 ± 3.0†	6.5 ± 2.3	6.5 ± 2.0	6.4 ± 1.9
Hypertension (%)	316 (36.8)†	94 (24.5)	99 (25.6)	98 (25.6)	565 (45.7)†	161 (31.2)	156 (31.0)	147 (28.4)
Systolic blood pressure (mmHg)	142.3 ± 23.7†	137.4 ± 19.4	135.3 ± 19.9*	135.9 ± 20.3*	145.8 ± 22.8†	135.5 ± 21.4	134.2 ± 20.6	135.8 ± 20.7
Diastolic blood pressure (mmHg)	74.1 ± 12.3	74.8 ± 10.4	74.9 ± 10.7	74.5 ± 11.5	74.0 ± 11.7	72.0 ± 10.7	72.8 ± 10.6	73.1 ± 10.7
Myocardial infarction (%)	201 (23.4)†	54 (14.3)	43 (11.2)	61 (16.0)	132 (10.8)†	32 (6.3)	20 (4.0)	35 (6.8)
Intermitent claudication (%)	24 (2.7)	9 (2.3)	4 (1.0)	3 (0.8)	25 (2.0)	0 (0.0)	8 (1.6)	4 (0.8)
Peripheral arterial disease (%)	185 (23.7)†	34 (9.4)	35 (9.6)	30 (8.7)	277 (24.8)†	44 (9.0)	46 (9.7)	49 (10.7)
Total cholesterol (mmol/L)	6.3 ± 1.2	6.4 ± 1.1	6.3 ± 1.1	6.4 ± 1.1	6.9 ± 1.2	6.9 ± 1.2	6.9 ± 1.2	6.9 ± 1.1
Smoking **Current** (%)	300 (33.9)†	116 (30.1)	103 (26.5)	82 (21.4)	265 (21.1)	104 (20.2)	85 (16.9)	90 (17.4)
Past (%)	525 (59.4)†	237 (61.4)	259 (66.6)	261 (68.1)	324 (25.8)	143 (27.7)	156 (31.0)	179 (34.7)
Calcium intake (g/day)	1,105.2 ± 412.7†	1,139.9 ± 484.9	1,165.8 ± 424.3	1,162.5 ± 439.0	1,111.9 ± 394.6	1,104.4 ± 365.4	1,116.0 ± 338.0	1,092.7 ± 382.3
Vitamin D intake (µg/day)	1.7 ± 1.2	1.7 ± 1.1	1.8 ± 1.2	1.9 ± 1.3	1.4 ± 1.0	1.4 ± 0.9	1.3 ± 0.7	1.4 ± 0.9
Folic acid intake (µg/day)	228.9 ± 80.8	229.9 ± 61.4	238.3 ± 87.4	235.8 ± 96.2	205.7 ± 81.1	204.7 ± 66.7	210.7 ± 64.2	212.2 ± 70.3

Data are means ± SD or counts (%) † p < 0.01 significantly different from individuals with follow-up assessments * p<0.05 ** p<0.01 as compared to individuals in lowest tertile

was not statistically significant. Other parameters did not differ across tertiles of BMD change.

The relation between rate of change in BMD, aging and rate of change in body weight between baseline and second follow-up period is shown in Figure 2. In males, weight loss is only significantly higher and related to BMD loss in the older age group ($p=0.05$) while women in the lowest tertile of weight change (greatest weight loss) have the highest rates of BMD loss in all age categories although significantly higher only from age 65 years and older ($p<0.03$).

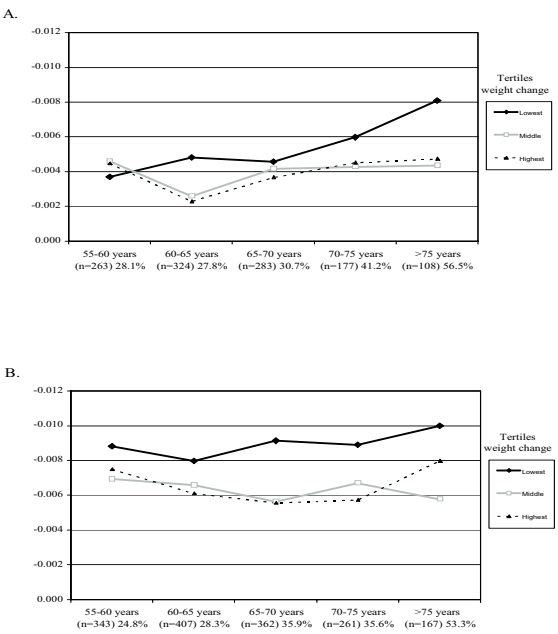


Figure 2. Absolute rates of BMD change with age by tertiles of weight change in: **A.** males and **B.** females. Under each age category group size and percent of individuals in the lowest tertile of weight change (greatest weight loss).

The lowest and highest cut-offs are at -2.0 and 0.7 kg-per-year, respectively. Estimates are adjusted for baseline height and weight and assessed between baseline and second follow-up period with a mean follow-up duration of 6.5 (SD 0.6) years.

In Table 3 the patterns of BMD and bone geometry change are compared across genders between the first and second follow-up periods. Overall, the rates of change are higher in women than in men. In women, NN BMD decreased 2,2% (relative fraction from baseline) while cortical thickness decreased by 2,2% and subperiostal width increased by 0.8%. Thus, BMD decreased most likely due to a combination of cortical thinning and bone expansion. These changes were less apparent in males. The buckling ratio increased by 7.5 % from baseline in females and by 3.9 % in

TABLE 3. PATTERNS OF BMD CHANGE IN MALES AND FEMALES BETWEEN FIRST AND SECOND FOLLOW-UP PERIODS*

	MALES					
	LUNAR Region	**Hip Structural Analysis of the Narrow Neck Region**				
	BMD (mg/cm^2)	**BMD** (mg/cm^2)	**Cortical Thickness** (mm)	**Subperiosteal Width** (mm)	**Section Modulus** (cm^3)	**Buckling Ratio**
Absolute difference	-13.5 ± 2.35	-11.3 ± 4.26	-0.02 ± 0.009	-0.01 ± 0.103	-0.02 ± 0.010	0.29 ± 0.11
Absolute rate per year	-3.1 ± 0.54	-2.5 ± 0.99	-0.005 ± 0.002	-0.01 ± 0.024	-0.01 ± 0.002	0.06 ± 0.03
Relative % rate per year	-0.3 ± 0.06	-0.2 ± 0.14	-0.2 ± 0.14	0.0 ± 0.08	-0.3 ± 0.20	0.9 ± 0.23
Relative fraction (%) from baseline	-1.5 ± 0.27	-1.1 ± 0.58	-1.1 ± 0.62	0.2 ± 0.36	-1.2 ± 0.88	3.9 ± 1.01
	FEMALES					
	LUNAR Region	**Hip Structural Analysis of the Narrow Neck Region**				
	BMD (mg/cm^2)	**BMD** (mg/cm^2)	**Cortical Thickness** (mm)	**Subperiosteal Width** (mm)	**Section Modulus** (cm^3)	**Buckling Ratio**
Absolute difference	-29.8 ± 2.06	-21.9 ± 3.73	-0.04 ± 0.008	0.12 ± 0.090	-0.03 ± 0.008	0.61 ± 0.10
Absolute rate per year	-6.7 ± 0.48	-5.1 ± 0.86	-0.01 ± 0.002	0.02 ± 0.021	-0.01 ± 0.002	0.14 ± 0.02
Relative % rate per year	-0.8 ± 0.06	-0.5 ± 0.12	-0.5 ± 0.13	0.2 ± 0.07	-0.2 ± 0.17	1.7 ± 0.21
Relative fraction (%) from baseline	-3.3 ± 0.24	-2.2 ± 0.51	-2.2 ± 0.55	0.8 ± 0.31	-0.8 ± 0.77	7.5 ± 0.89

* Data are means ± SEM adjusted for age, height and weight with mean follow-up duration of 4.5 (SD 0.6) years

males, pointing towards greater progression towards bone instability in females.

In Table 4A the absolute rates of change in BMD and bone geometry are examined in males across age groups, tertiles of body mass index and by tertiles of rate of change in body weight. It is important to highlight for the tertile groups of weight change, that the highest tertile represents weight gain, the middle static weight and the lowest represents weight loss. As also seen in Figure 1, rates of BMD loss were higher in the older age groups. Consistently, the decrease in cortical thickness and increase in subperiostal width and in buckling ratio (bone instability) is also higher in the older age groups. A similar pattern is seen with bone strength, as section modulus decreases more at older age although not significantly ($p= 0.09$). In contrast, no trend is seen across tertiles of baseline BMI though it appears that the individuals in the lowest BMI tertile have the lowest rates of BMD decrease. Additionally, no trends are seen across tertiles of rate of change in body weight , but consistently, individuals at the lowest tertile of weight change (greatest weight loss) had the highest rates of loss in BMD, cortical thickness and section modulus (strength) and the highest increase in buckling ratio (bone instability). Rates of change in subperiostal width are paradoxically negative but very small in all tertiles of weight change.

In Table 4B the same analysis across age groups, tertiles of baseline body mass index and by tertiles of rate of change in body weight is presented for women. In contrast to men, rates of BMD loss do not follow a linear trend through age groups. Even though the highest rates of BMD loss are seen in the oldest age group, the youngest age group also appears to have increased rates of BMD loss. The pattern of change in bone geometry suggests that cortical thinning is higher in the older age group, while increase in neck width is high in both the youngest and oldest age groups. The loss of bone strength (decrease in section modulus) and progression to instability (increase in buckling ratio) is faster in the older age group. No trends or mean differences in rates of BMD loss or change in geometry is seen across tertiles of baseline BMI. The analysis across tertiles of rate of change in body weight shows that rates of BMD loss increase together with increasing rates of weight loss. Similarly this trend is evident with the parameters of bone geometry, where the rate of cortical thinning, loss of strength (decrease in section modulus) and progression towards bone instability (increase in buckling ratio) is higher with increasing weight loss. No such pattern is seen on changes in neck width.

DISCUSSION

In this population-based study in a large group of elderly men and women aged 55 and over, we examined the epidemiological and biomechanical aspects underly-

TABLE 4A. PATTERNS OF BMD AND GEOMETRY CHANGE BETWEEN FIRST AND SECOND FOLLOW-UP ACROSS AGE, BMI AND WEIGHT CHANGE STRATA IN MALES

	MALES					
	LUNAR Region	Hip Structural Analysis of the Narrow Neck Region				
Age at first follow-up (years)	**BMD** (mg/cm^2)	**BMD** (mg/cm^2)	**Cortical Thickness** (mm)	**Subperiosteal Width** (mm)	**Section Modulus** (cm^3)	**Buckling Ratio**
55 to 64.9 (n = 339)	-1.9 ± 0.62	-0.4 ± 1.11	-0.001 ± 0.0023	-0.033 ± 0.0275	-0.003 ± 0.0026	0.00 ± 0.030
65 to 74.9 (n = 376)	-4.0 ± 0.58	-2.5 ± 1.04	-0.005 ± 0.0021	0.005 ± 0.0258	-0.007 ± 0.0024	0.07 ± 0.028
75 and > (n = 113)	-6.5 ± 1.08	-6.1 ± 1.95	-0.012 ± 0.0040	0.014 ± 0.0482	-0.012 ± 0.0045	0.15 ± 0.052
p-trend	0.0001	0.01	0.02	0.29	0.09	0.01
BMI at first follow-up (kg/m^2)						
Lowest tertile (n = 276)	-2.9 ± 0.67	-0.6 ± 1.22	-0.001 ± 0.0025	-0.076 ± 0.0301	-0.005 ± 0.0030	0.01 ± 0.033
Middle tertile (n = 276)	-3.6 ± 0.67	-3.1 ± 1.22	-0.006 ± 0.0025	0.026 ± 0.0301	-0.007 ± 0.0030	0.08 ± 0.032
Highest tertile (n = 276)	-3.9 ± 0.67	-2.8 ± 1.22	-0.006 ± 0.0025	0.023 ± 0.0301	-0.005 ± 0.0030	0.06 ± 0.032
p-trend	0.27	0.20	0.17	0.02	0.99	0.24
Rate of Weight Change (kg per year)						
Lowest tertile (n = 335)	-4.4 ± 0.68	-3.6 ± 1.22	-0.007 ± 0.0122	-0.015 ± 0.0303	-0.009 ± 0.0028	0.09 ± 0.033
Midle tertile (n = 338)	-3.0 ± 0.68	-1.1 ± 1.23	-0.002 ± 0.0123	-0.001 ± 0.0305	-0.003 ± 0.0029	0.03 ± 0.033
Highest tertile (n = 332)	-3.0 ± 0.68	-1.7 ± 1.22	-0.003 ± 0.0122	-0.011 ± 0.0303	-0.005 ± 0.0028	0.04 ± 0.033
p-trend	0.12	0.27	0.28	0.91	0.24	0.31

Data are estimated means ± SEM from a mean follow-up of 4.5 (SD 0.6) years

* Estimates adjusted for height and weight at first follow-up

** Estimates adjusted for age with lowest and highest BMI tertile cutoffs at 25.2 and 27.5 kg/m^2, respectively.

*** Estimates are adjusted for age, height and weight at first follow-up with lowest and highest tertile cutoffs at -2.0 and 0.7 kg-per-year, respectively.

TABLE 4B. PATTERNS OF BMD AND GEOMETRY CHANGE BETWEEN FIRST AND SECOND FOLLOW-UP ACROSS AGE, BMI AND WEIGHT CHANGE STRATA IN FEMALES

	FEMALES					
	LUNAR Region	Hip Structural Analysis of the Narrow Neck Region				
Age at first follow-up (years)*	**BMD** (mg/cm^2)	**BMD** (mg/cm^2)	**Cortical Thickness** (mm)	**Subperiosteal Width** (mm)	**Section Modulus** (cm^3)	**Buckling Ratio**
55 to 64.9 (n = 408)	-6.3 ± 0.67	-5.6 ± 1.21	-0.011 ± 0.0025	0.042 ± 0.0288	-0.006 ± 0.0026	0.15 ± 0.032
65 to 74.9 (n = 450)	-6.1 ± 0.63	-4.4 ± 1.14	-0.009 ± 0.0024	0.000 ± 0.0271	-0.005 ± 0.0025	0.11 ± 0.031
75 and > (n = 149)	-7.9 ± 1.11	-8.0 ± 2.03	-0.016 ± 0.0042	0.047 ± 0.0481	-0.011 ± 0.0044	0.27 ± 0.054
p-trend	0.36	0.57	0.63	0.76	0.49	0.18
BMI at first follow-up (kg/m^2)**						
Lowest tertile (n = 336)	-6.4 ± 0.73	-5.1 ± 1.33	-0.010 ± 0.0027	-0.006 ± 0.0315	-0.009 ± 0.0029	0.16 ± 0.035
Middle tertile (n = 335)	-6.2 ± 0.73	-5.9 ± 1.32	-0.012 ± 0.0027	0.063 ± 0.0314	-0.005 ± 0.0029	0.17 ± 0.035
Highest tertile (n = 336)	-6.6 ± 0.73	-5.2 ± 1.33	-0.011 ± 0.0027	0.015 ± 0.0314	-0.004 ± 0.0029	0.12 ± 0.035
p-trend	0.85	0.94	0.86	0.64	0.26	0.5
Rate of Weight Change (kg per year)**						
Lowest tertile (n = 335)	-8.3 ± 0.73	-8.9 ± 1.33	-0.018 ± 0.0133	0.015 ± 0.0318	-0.011 ± 0.0029	0.21 ± 0.036
Middle tertile (n = 338)	-5.8 ± 0.72	-5.3 ± 1.32	-0.011 ± 0.0132	0.067 ± 0.0315	-0.005 ± 0.0029	0.18 ± 0.035
Highest tertile (n = 332)	-5.2 ± 0.73	-2.0 ± 1.33	-0.004 ± 0.0133	-0.011 ± 0.0317	-0.003 ± 0.0029	0.06 ± 0.036
p-trend	0.003	0.0002	0.0003	0.55	0.05	0.002

Data are estimated means ± SEM from a mean follow-up of 4.5 (SD 0.6) years

* Estimates adjusted for height and weight at first follow-up

** Estimates adjusted for age with lowest and highest BMI tertile cutoffs at 25.2 and 28.6 kg/m^2, respectively.

*** Estimates are adjusted for age, height and weight at first follow-up with lowest and highest tertile cutoffs at -1.4 and 1.4 kg-per-year, respectively.

ing age-related changes in BMD in one of the longer periods of follow-up reported in the literature (6.5 and 4.5 years, respectively). We have shown that individuals for whom follow-up data are available were younger, taller (~ 2.4 cm), heavier and with higher BMD than those individuals lost to follow-up (~ 45% of all individuals), thus representing a selected and healthy cohort. Accordingly, morbidity was higher in the group lost to follow-up, with increased prevalence of disability, diabetes and cardiovascular disease. They also smoked more often and had lower intake of calcium and folic acid. We found that in both genders, individuals in the lowest tertile of BMD change (representing the highest rates of BMD loss), had higher BMD levels at baseline and greater weight loss with follow-up. In addition, men with greatest BMD loss were older and had higher systolic blood pressure. When examining the rates of change in BMD and bone geometry after 4.5 years of follow-up we found that as compared to men, women have higher rates of BMD loss, with increased cortical thinning and bone expansion and increased loss of strength (decrease in section modulus), with faster progression towards bone instability as manifested by an increase in buckling ratio. In addition, we observed sex-specific patterns of change in bone geometry. In men, an age trend was present where the greatest rates of bone loss and change in geometry were observed in the group of the oldest men (> 75 years). In contrast, rates of change in BMD and geometry were more prominent before age 65 years and after age 75 years, with an increasing linear relationship with increasing weight loss. In women, the rates of change in BMD and bone geometry are more stable across age groups and tend to be higher at the youngest (55 to 60 years) and oldest (above 75years) age categories.

Patterns of BMD Decline and Health Characteristics

Previously, we have estimated rates of change in BMD in both genders, using both a cross-sectional analysis at baseline (3) and a longitudinal analysis with an average follow-up of only 2.0 years between baseline and first follow-up (7). The results from our present study are consistent with these earlier observations in the Rotterdam Study and with those of others (4-6), and show that rates of BMD loss increase with advancing age in both men and women. Also the magnitude of the rates of change per year are comparable with those from previous reports, with rates of loss in women in the range of 1.0% of baseline BMD per year, being almost double that of men (0.5% of baseline BMD per year). Furthermore, we observed in women that rates of BMD loss tend to be higher before the age of 60 years and to rise again after age 75 years. Though this "U" shape pattern did not reach significance, it is biologically plausible that at least some of the women younger than 60 years of age are early post menopausal, and therefore still in the phase of the rapid postmenopausal BMD loss (2,26).

An important observation is that approximately 45% of the individuals evaluated at baseline were lost to follow-up, and as shown by our analysis by health characteristics, this was probably the consequence of increased morbidity, as represented by an increased prevalence of disability, type II diabetes, cardiovascular disease and current smoking. Therefore, the rates of BMD and bone geometry change that we report in this study, although arising in a population-based setting, represent patterns found in a relatively healthy cohort of surviving men and women. This could also be the explanation for the fact that no strong associations were observed between rates of bone loss and morbid conditions. Nevertheless, some relationships with morbidity were seen, although not consistently in both genders. That is, males with higher rates of BMD loss had also higher systolic blood pressure and in women the prevalence of diabetes mellitus was higher in the tertiles with higher BMD loss (although this was not statistically significant). No specific conclusion may be drawn from these unspecific observations, yet these findings are in line with the observation that increased rates of BMD loss are associated to increased risks of diabetes, hypertension, stroke and mortality (7,27,28). In contrast, some cross-sectional studies have shown that the presence of diabetes and hypertension are associated with higher BMD levels (8-11). Even though these results seem paradoxical, they are also in line with our findings that higher rates of BMD loss occurred in individuals with higher BMD. Although regression to the mean could play a role, it is unlikely that it could explain alone the current findings given the long follow-up (6.5 years).

Even though no associations were evident with weight or body mass index (BMI) at baseline, a strong association was seen between loss of body weight and decline in BMD in both genders. Further, our analysis across baseline age groups (Figure 2) showed that, even though the percentage of individuals with higher rates of weight loss increases with aging, higher weight loss in men appeared to be related to higher rates of BMD loss only in the older age groups, while in women this relationship was evident throughout all the age categories. These changes in body weight may be a consequence of underlying morbidity (29), but also they could represent expected decline in body weight with aging (i.e. in the form of sarcopenia) (16).

Patterns of BMD Decline and Bone Geometry

Our findings on bone geometry help to elucidate the way BMD declines with aging and to determine how the aging bone progresses towards fragility. In concordance with previous reports we observe that women have comparatively higher rates of both cortical thinning and bone expansion (increase in femoral neck width)

(13), though the latter remains yet controversial in the light of measurement error (30). We also saw, that despite the presence of periosteal apposition, bone strength declined with time in both genders, with almost equivalent yearly rates in the age group 75 years and over. In contrast to previous reports (12,13), the rate of decline in strength was lower than that observed for BMD in women, but not in men (Table 3). When we examined the patterns of change in bone geometry across tertiles of BMI and weight change, we saw that there is no consistent relation of BMD with BMI categories in any gender. However, there was a clear relationship with weight change in females. Our findings on weight-related decline in BMD are in line with a previous study which explored this relationship (29) and supports the hypothesis that one of the reasons for the BMD decline with aging is mechanical adaptation to changes in skeletal load. When examining the progression towards bone instability, we observed that the buckling ratio increased with aging in both genders. This was only linearly seen in men, while women after age 75 years showed the faster rates (about two times as those observed in men). This is in line with the finding that females had greater increase in neck width than men, and could explain the higher susceptibility for hip fracture in elderly women (31,32).

To further explore the age-related changes in BMD and bone geometry, future studies might specifically look at relations with pharmacologic interventions (e.g. the use of thiaziades, statins), calcium intake and secondary hyperparathyroidism, vitamin supplementation and homocysteine metabolism (33), the growth hormone and insulin-like growth factor I axis (34,35), and sex-steroids (26,36,37).

In summary, this population-based study in elderly men and women age 55 and over, examined the longitudinal patterns of change in BMD and bone geometry. Since almost half of the original study population at baseline was lost to follow-up due to morbidity, the current findings arise in a reasonable healthier cohort and need to be interpreted under the consideration of selection bias. This way, few associations were found with health characteristics at baseline, with increasing age (age 75 years or older), BMD and weight loss with follow-up being the most significant determinants of bone loss in both genders. Bone geometry data showed that BMD declines in the elderly due to bone loss (cortical thinning), but also due to increase in neck width (periosteal apposition), with both processes being more prominent in (postmenopausal) women than in men. Bone strength decreased in both genders possibly as an adaptative response to changes in body weight. Females had greater rates of increase in buckling ratio (index of bone instability) that could explain the higher susceptibility for hip fracture in elderly women. Specific research is needed to elucidate further these relationships between BMD decline, changes in bone geometry, morbidity and weight loss.

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Phenotypic Dissection of DXA-based Traits: Implications for Genetic Studies of Osteoporosis

3

ABSTRACT

In the present study (n=923) we estimated heritabilities (h^2) and genetic correlations of bone mineral density (BMD) and hip geometry measurements, within the Erasmus Rucphen Family study, an extended-pedigree study designed to map disease-related quantitative trait loci. Variance component methodology was used to study age and sex adjusted traits which included BMD of the hip, spine and total body (including body composition) and bone geometry measurements of the hip. An estimate of mechanosensitivity was calculated as the ratio of bone strength to lean mass. We found that heritability across skeletal sites is highest at the head/skull (h^2 ~ 80%), and decreases from the axial towards the appendicular skeleton (h^2 decreasing ~ from 70 to 26%). Consistently, genetic correlations (rho coefficient) of BMD across sites were high and decreased with increasing distance of the measurement sites (rho decreased from 1.00 to 0.50). BMD had significant genetic correlation with height (rho ~ 0.20) and lean mass (rho ~ 0.30). Bone geometry had the highest heritabilities for parameters of size (h^2 ranging ~ from 43 to 75%), followed by cortical factors (h^2 ~ 40%), and was lowest for strength (h^2 ~ 25%). Mechanosensitivity indices were also heritable (h^2 ~ 40%) and genetically correlated (rho ~ 0.40) with bone instability of the femoral neck and to spinal and head/skull BMD. These findings suggest that the heritability of BMD is site-specific and influenced by genes determining height and lean mass. Bone geometry parameters depicting strength and instability are also heritable. Finally, we propose that susceptibility to fragility fracture at older age is heritable partly because they reflect genetically-determined differences in mechanosensitivity.

INTRODUCTION

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. The incidence of hip fracture is high in the elderly (1,2) and its associated morbidity and mortality (3,4) represents enormous costs for health systems (5). Since the completion of the human genome project the study of the genetic basis of complex diseases and hence osteoporosis has gained evolving importance. From the genetic perspective osteoporosis is a multifactorial disease resulting from multiple independent gene effects, and gene-gene and gene-environment interactions (6,7).

The clinically relevant osteoporosis phenotype is the occurrence of low-trauma fracture but, osteoporotic fractures are also rare events that occur late in life. In addition, they are very complex in nature and evidence for their genetic determination is therefore scarce and conflicting (8-10). This difficulty to use osteoporotic fractures as outcome in genetic studies has lead to the use of surrogate traits that assess characteristics of bone health. These include the determination of bone mineral density (BMD), quantitative ultrasound and the use of biochemical markers of bone metabolism.

From these, BMD measured by dual energy X-ray densitometry (DXA) is the most commonly used trait in genetic studies of osteoporosis considering that it is a highly heritable trait with estimates of heritability ranging between 50-70 percent (7) and that is predictive of the risk of fracture (11,12).

However, of the many genetic studies that have used BMD as a surrogate marker for osteoporotic fractures, only some have shown consistent and reproducible associations with candidate genes while few, if any, chromosomal regions have shown consistent linkage (13,14). There are intrinsic limitations underlying the use of BMD as a trait. BMD traits are dynamic, they show gender- and age-specific patterns through life (15,16), and most important, the BMD measurement in itself does not represent invariably the underlying structural integrity of bone (17). The latter also explains why BMD does not fully characterize the risk of fracture (2,12). Interpreting BMD in terms of mass distribution (bone geometry) beyond quantity alone can provide a better understanding of the relation between bone fragility and fracture. Up to now, genetic studies which have looked at bone geometry (18,19) have focused on size-related parameters like hip axis length (20) and neck-shaft angles, while few have looked at properties of mass distribution (21,22).

Considering this, we have made a comprehensive dissection of the DXA-based osteoporosis phenotype to 1) explain what properties of the BMD measurement

makes it such a highly heritable trait, and 2) to identify the properties of BMD that could explain why the susceptibility to fragility fracture at older ages is heritable. To do this, we have studied a genetic isolated population with a broad age range in order to determine site-specific heritabilities and estimate the phenotypic and genetic correlations across BMD and hip bone geometry measurements. Further, we studied the relationship between bone strength and skeletal load by calculating an index of mechanosensitivity, and studied its heritability and genetic correlation with the BMD measurements and the other geometrical parameters.

SUJECTS AND METHODS

Sample Composition and Evaluation of Phenotypes

This study is part of the Erasmus Rucphen Family (ERF) Study, an ongoing extended-pedigree study of genetic risk factors for neuropsychiatric, cardiovascular, endocrinologic, ophthalmologic and musculoskeletal disorders. The ERF study is part of the Genetic Research in Isolated Populations (GRIP) program which aims to identify genetic risk factors in the development of complex disorders in an isolated community of about 20,000 inhabitants settled around eight adjacent villages in the southwest of The Netherlands. The ERF study population includes all living descendants of 50 couples with at least six children and at least one baptized in the community church between 1890 and 1900, who were invited to participate in the study (n=2100) independent of disease status. The Medical Ethics Committee of Erasmus Medical Center Rotterdam approved the ERF study and written informed consent was obtained from every participant. Phenotyping started in June 2002, is currently ongoing and includes a series of medical investigations assessed in a standardized manner by trained assistants and physicians during their visit to the research center located in Sprundel, The Netherlands. The current response rate is 78%. This study is based on the first 1064 participants evaluated until July 31, 2003, who were ascertained independently of disease status. The mean inbreeding coefficient is 0.008 (SD 0.007). The investigation protocols of the phenotypes considered in this study are described below.

Clinical examination

Trained physicians assessed the current use of bone active medication including Vitamin D, calcium, oral glucocorticoids, bisphosphonates and hormone replacement therapy (in women). Further, for women, menopausal status and age at menopause was recorded.

Height (cm) rounded to the closest 0.5 unit and weight (kg) were measured with participants in standing position, wearing light underclothing and without shoes. Body mass index or BMI (kg/m^2), was calculated as weight (kg) divided by the square of height (meters).

Dual-energy x-ray absorptiometry measurements

DXA scans were performed using a Prodigy™ total body fan-beam densitometer and analyzed with the enCORE™ 2002 software V. 6.70.021 (GE Lunar Corporation Madison, WI). Daily quality assurance tests were performed with a calibration block supplied by the manufacturer. Repeated measurements on the calibration block had coefficients of variation less than 1%. In addition a calibration aluminum phantom was measured weekly with coefficients of variation less than 1%.

Dual-femur and antero-posterior lumbar spine scans were performed using standard specific positioning as suggested for DXA measurements. BMD (g/cm^2), BMC (g) and area (cm^2) were obtained from the femoral neck, upper femoral neck, trochanteric, Ward's triangle and L1 to L4 regions of interest. Z-scores (difference in SD from the mean for individuals of same age and gender) and T-scores (difference in SD from the mean of a young adult of same gender) were obtained from the manufacturer database (reference German Caucasian population). In addition hip axis length was also measured by the manufacturer software. When bilateral measurements were available, mean values were computed. Total-body and regional BMD (g/cm^2), fat mass (g), lean mass (g) and bone mineral content (g) were obtained from total body scans and were auto analyzed by the software which employs an algorithm that divides body measurements into areas corresponding to head, trunk, arms and legs. All analyzes were verified by a trained technician who performed adjustments when necessary. Lean mass and fat mass indices were calculated as lean mass (in kg) and fat mass (in kg) divided respectively by the square of height (meters).

Hip Structural Analysis (HSA)

We have used the hip structural analysis (HSA) software developed by Thomas Beck to measure hip bone geometry from the DXA scans (17,23). The HSA program differs from the conventional density analyses in the sense that cross-sectional dimensions are derived density, rather than averaged. Analysis locations included the narrow-neck (NN) region across the narrowest point of the femoral neck and the shaft region located 2 cm distal to the user-located midpoint of the

lesser trochanter. BMD, bone cross-sectional area, bone width (outer diameter) and cross-sectional moment of inertia (CSMI) were measured directly from mineral mass distributions using algorithms described previously (23). Section modulus was calculated as CSMI /d_s, where d_s is the maximum distance from the center of mass to the medial or lateral surface. Buckling ratios were computed as d_s divided by estimated mean cortical thickness.

Mechanosensitivity Indices

Evolutionary and biomechanical theory states that bone strength should scale to skeletal load (24,25), reflecting the intrinsic mechanosensitivity of an individual. In this context, individuals with reduced mechanosensitivity will develop relatively weaker bones for equivalent skeletal loads and hence, show increased susceptibility to fracture. An approximated index of mechanosensitivity is the ratio of bone strength/moment arm to lean mass (used as a crude proxy of skeletal loading). We calculated the following mechanosensitivity indices (MSI):

Shaft MSI as: $$\frac{Z_{shaft} / height}{LM}$$

Femoral neck MSI as: $$\frac{Z_{neck} / (NL \times Sin(180 - NSA)}{LM}$$

where Z=section modulus (index of bending strength), LM=lean mass, NL= neck length and NSA= neck-shaft angle. Estimates were multiplied for a scaling factor of 10 for convenience.

Statistical Analysis

For the genetic modeling we have used the ASreml V.1.10 software (NSW Agriculture and IACR-Rothamsted Hertfordshire,U.K.). ASreml uses residual maximum likelihood methods for fitting linear mixed models, handles large datasets and supports loops. We performed a variance component analysis for quantitative traits (26). This analysis allowed us to distinguish for each trait, the additive polygenic genetic component of the total phenotypic variance, apart from several of its other potential components (dominant polygenic effects, epistatic effects, shared environmental effects, measurement error and residual effects). Since both shared environmental and dominant effects may mask polygenic effects, we have

included in the models a parameter which identified siblings arising from the same mother in order to extract from the polygenic component those shared environmental factors of early life. The narrow-sense heritability (*h2*) was computed as the ratio of the additive polygenic genetic variance to the total variance. Each trait was considered in two different models: a crude one (Model 0) with adjustment for age, sex, and inbreeding coefficients; and a fully adjusted one (Model 1) including age, sex, inbreeding coefficients, height, FMI, LMI and bone active medication, which includes concomitant use of Vitamin D and/or calcium supplements, glucocorticoids, bisphosphonates and hormone replacement therapy (in women).

Bivariate analyses were carried out to determine to what extent the aggregation and co-aggregation in families of a pair of traits may be attributed to shared genetic and environmental factors. Under the principle of Cholesky decomposition (27) genetic and phenotypic correlations were obtained from the formula:

$$\rho_P = \sqrt{h_1^2} \cdot \sqrt{h_2^2} \cdot \rho_G + \sqrt{(1-h_1^2)} \cdot \sqrt{(1-h_2^2)} \cdot \rho_E$$

where, h_1^2 and h_2^2 are the heritabilities of the correlated traits, and ϱ_P, ϱ_G and ϱ_E refer to the phenotypic, genetic and environmental correlations, respectively. This way, $\varrho_P < \varrho_G$, only when there is a negative r_E. Estimates were considered significant if twice the standard errors subtracted (or added) to the estimates did not cross the value of zero.

RESULTS

Of the 1064 participants who visited the research center 923 (87%) had complete phenotypic information. Those with incomplete phenotypic information were significantly older with mean age of 69.7 SD 13.4 years ($p< 0.00001$). Baseline characteristics of the study population are presented in Table 1. About 59% percent of participants are female with no significant differences in mean age across genders. Of all women, approximately 66% were either peri or post menopausal. The average body mass index (BMI) in both men and women was 27 kg/m^2 which corresponds to overweight (BMI>25). As observed from mean Z-scores, BMD is on average within the expected values for age with about 13% of the total population diagnosed with osteoporosis (T-score < -2.5). The use of bone active medication is relatively low in the elderly.

Skeletal BMD Measurements

Heritability (h^2) estimates of BMD measured at the different skeletal sites are shown in Table 2 and Figure 1. Heritabilities of BMD from the total body scan

TABLE 1 CHARACTERISTICS OF THE STUDY POPULATION

	TOTAL	MALES	FEMALES
Assesed participants (n)	923	375	548
Age (years)*	52.2 (14.2)	53.9 (13.7)	51.1 (14.3)
Age at menarche (years)*	-	-	13.1 (1.7)
Age at menopause (years)*	-	-	47.1 (6.3)
Perimenopausal (%)**	-	-	30.1
Postmenopausal (%)**	-	-	35.9
Height (meters)*	1.65 (0.09)	1.72 (0.07)	1.60 (0.06)
Weight (kilograms)*	73.7 (14.5)	81.7 (13.4)	68.3 (12.6)
Body mass index (kg/m2)*	27.0 (4.5)	27.5 (4.1)	26.7 (4.8)
Lean mass index (kg/m2)*	17.0 (2.5)	19.2 (1.9)	15.4 (1.7)
Fat mass index (kg/m^2)*	9.0 (3.6)	7.4 (2.8)	10.2 (3.6)
Fat tissue percent (%)*	33.8 (9.5)	26.9 (7.4)	38.6 (7.6)
Femoral Neck BMD (g/cm2)*	0.91 (0.14)	0.94 (0.14)	0.89 (0.13)
Z-score*	-0.2 (0.9)	-0.3 (0.9)	-0.1 (0.9)
T-score*	-0.9 (1.1)	-1.0 (1.1)	-0.8 (1.1)
L2-L4 Lumbar Spine BMD (g/cm2)*	1.15 (0.19)	1.20 (0.19)	1.12 (0.18)
Z-score*	-0.1 (1.4)	-0.2 (1.3)	0.0 (1.3)
T-score*	-0.5 (1.5)	-0.4 (1.5)	-0.6 (1.5)
Osteopenia **	44.2	47.8	41.7
Osteoporosis **	12.8	13.2	12.6
Use of bone active medication (%)**	6.6	2.4	9.5
Vitamin D or calcium (%)**	2.7	0.3	4.4
HRT or estrogen (%)**	-	-	3.6
Bisphosphonates (%)**	2.1	0.5	3.1
Glucocorticoids (%)**	1.5	2.1	1.1

* Data are unadjusted means (SD) ** Data are percent of participants

varied across skeletal sites, with appendicular segments showing lower h^2 than the axial component, with a total body BMD averaging around 57%. Estimates fluctuated between 27% (ribs) and 81% (head/skull). BMD of the femoral neck and hip regions had an h^2 of about 60% while the h^2 of lumbar spine was approximately 70%. The heritability of bone area at the lumbar spine was 75% while at the femoral neck it was less than 20%. An increase in heritability estimates in the fully adjusted models usually represented a decrease in total variance with a minor change on the genetic additive component. This reduction in total variance could represent either adjustment for environmental factors as well as for measurement error. Of those traits where heritability estimates decreased after adjustment, bone areas of the lumbar spines showed the highest reduction in the additive genetic component suggesting common underlying genetic factors with covariates. Several traits did not yield major changes after adjustment (in particular Z-scores which are BMD

TABLE 2. HERITABILITIES OF DXA BMD-RELATED PHENOTYPES

	Model 0*			Model 1**		
	Variance component			*Variance component*		
Phenotype	*Additive genetic*	*Total*	*% h^2*	*Additive genetic*	*Total*	*% h^2*
FEMORAL NECK						
BMD	0.09 (0.01)	0.15 (0.01)	59.4 (8.7)	0.07 (0.01)	0.12 (0.01)	56.2 (9.1)
BMC	0.25 (0.05)	0.52 (0.03)	47.7 (9.2)	0.14 (0.04)	0.40 (0.02)	34.2 (9.3)
Area	0.04 (0.02)	0.21 (0.01)	16.6 (9.4)	0.01 (0.02)	0.19 (0.01)	7.4 (8.8)
Z-score	0.52 (0.08)	0.83 (0.04)	62.5 (8.7)	0.48 (0.08)	0.80 (0.04)	59.5 (9.0)
T-score	0.58 (0.10)	0.95 (0.05)	61.6 (8.7)	0.47 (0.09)	0.82 (0.04)	57.7 (9.1)
Upper neck BMD	0.09 (0.02)	0.16 (0.01)	54.7 (8.9)	0.07 (0.02)	0.14 (0.01)	47.9 (9.4)
OTHER HIP REGIONS						
Wards triangle BMD (g/cm2)	0.11 (0.02)	0.17 (0.01)	64.8 (7.2)	0.10 (0.02)	0.16 (0.01)	59.3 (8.5)
Throchanter BMD (g/cm2)	0.08 (0.01)	0.15 (0.01)	57.5 (8.5)	0.07 (0.01)	0.12 (0.01)	63.9 (8.4)
Total hip BMD (g/cm2)	0.10 (0.02)	0.17 (0.01)	58.0 (8.4)	0.09 (0.01)	0.13 (0.01)	66.5 (8.1)
LUMBAR SPINE						
L2-L4 BMD (g/cm2)	0.23 (0.03)	0.31 (0.02)	71.7 (7.5)	0.21 (0.03)	0.30 (0.01)	70.3 (7.7)
L2-L4 BMC (g)	79.9 (10.2)	107.6 (4.4)	74.2 (7.3)	61.0 (8.6)	90.1 (4.3)	67.7 (7.7)
L2-L4 Area (cm2)	13.5 (1.71)	17.7 (0.6)	76.0 (7.5)	5.7 (1.1)	10.8 (0.5)	52.4 (9.2)
L2-L4 Z-score	1.51 (0.20)	2.05 (0.10)	73.5 (7.5)	1.52 (0.19)	2.04 (0.11)	74.4 (6.9)
L2-L4 T-score	1.62 (0.21)	2.20 (0.12)	73.6 (6.9)	1.51 (0.20)	2.11 (0.11)	71.8 (7.1)
L1-L4 BMD (g/cm2)	0.20 (0.03)	0.29 (0.01)	69.8 (7.8)	0.187 (0.027)	0.275 (0.013)	68.1 (8.0)
L1-L4 BMC (g)	123.7 (16.0)	166.0 (6.1)	74.5 (7.5)	96.5 (13.5)	139.1 (6.4)	69.4 (7.8)
L1-L4 Area (cm2)	20.9 (2.7)	28.0 (1.1)	74.7 (7.6)	8.9 (1.7)	16.7 (0.8)	53.2 (9.1)
TOTAL BODY						
Total body BMD (g/cm2)	0.05 (0.01)	0.09 (0.00)	57.2 (8.3)	0.04 (0.01)	0.07 (0.00)	63.7 (8.0)
Total body BMC (kg)	0.08 (0.01)	0.13 (0.01)	60.4 (8.4)	0.05 (0.82)	8.3 (0.0)	57.3 (8.5)
Total body Z-Score	0.78 (0.10)	1.10 (0.06)	71.2 (7.0)	0.73 (0.10)	1.05 (0.06)	69.2 (7.1)
Total body T-Score	0.86 (0.14)	1.41 (0.07)	61.5 (8.1)	0.74 (0.10)	1.09 (0.06)	67.4 (7.2)
Head BMD (g/cm2)	0.06 (0.01)	0.07 (0.00)	79.3 (7.4)	0.05 (0.01)	0.07 (0.00)	80.4 (7.5)
Spine BMD (g/cm2)	0.10 (0.02)	0.16 (0.01)	60.6 (8.1)	0.09 (0.01)	0.15 (0.01)	61.2 (8.1)
Ribs BMD (g/cm2)	0.01 (0.00)	0.03 (0.00)	26.6 (9.2)	0.01 (0.00)	0.02 (0.00)	27.6 (8.0)
Arms BMD (g/cm2)	0.02 (0.01)	0.08 (0.00)	30.7 (8.4)	0.02 (0.01)	0.06 (0.00)	35.5 (8.4)
Legs BMD (g/cm2)	0.07 (0.01)	0.14 (0.01)	46.3 (8.9)	0.05 (0.01)	0.11 (0.01)	48.0 (9.0)
Pelvis BMD (g/cm2)	0.06 (0.01)	0.15 (0.01)	42.7 (8.4)	0.05 (0.01)	0.12 (0.01)	43.1 (7.7)

Data are estimates (SE)

* Model 0 Adjustment for Sex, Age, Inbreeding coefficient

**Model 1 Adjustment for Sex, Age, Inbreeding coefficient, height, fat mass index, lean mass index and bone active medication (see text)

measurements adjusted for age and sex).

The phenotypic and genetic correlations between all the BMD measurements and the three most frequent skeletal sites of measurement are shown in Table 3. In general, all genetic correlations are moderately high between sites, with highest correlations occurring at adjacent sites, and decreasing with more distant sites. Overall head/skull BMD was the site observing the lowest genetic correlations with BMD measurement at other sites. Body height and lean mass index were significantly correlated with femoral neck and total body BMD only, and not with L1-L4 lumbar spine BMD. No significant genetic correlation was observed between BMD and fat mass index.

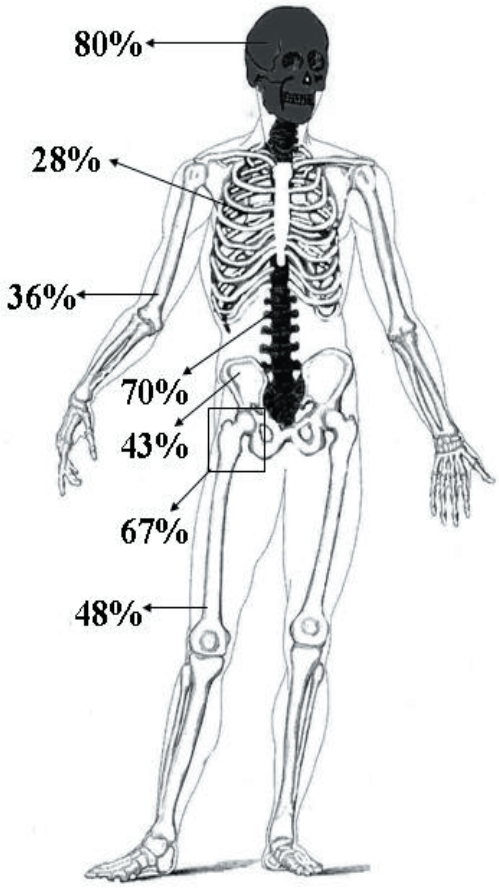


Figure 1. BMD measurements and skeletal traits with heritabilities. In shade the axial skeleton including the head/skull (light shade) and spinal column (dark shade). The square depicts total hip measurement and includes femoral neck (59%), Wards triangle (65%) and trochanter (64%) regions.

Bone Geometry and Size Measurements

The heritability estimates of hip bone geometry and size are shown in Table 4 and Figure 2. Size measurements (like hip axis and neck lengths) and bone diameters (like neck and endocortical width) showed the highest heritability estimates. Hip axis length gave the highest reduction in the additive genetic component after adjustment. Overall, heritability estimates of the geometry parameters in the shaft were higher than at the narrow-neck region. Section modulus (index of bending strength) and cross-sectional moment of inertia (measurement of bending strength) of the femoral neck, had the lowest heritability estimates as compared to the other

TABLE 3. PHENOTYPIC AND GENETIC CORRELATIONS OF BMD AT DIFFERENT SKELETAL SITES

	Femoral neck		L1-L4 spine		Total body	
	Phenotypic	Genetic	Phenotypic	Genetic	Phenotypic	Genetic
Femoral neck	-	-	0.64 (0.028)	0.70 (0.058)	0.82 (0.018)	0.88 (0.039)
Upper femoral neck	0.95 (0.005)	0.98 (0.010)	0.69 (0.072)	0.54 (0.081)	0.75 (0.024)	0.82 (0.059)
Total hip	0.92 (0.008)	0.96 (0.014)	0.69 (0.071)	0.59 (0.076)	0.82 (0.015)	0.79 (0.042)
Trocanther	0.85 (0.014)	0.87 (0.033)	0.69 (0.070)	0.57 (0.075)	0.76 (0.020)	0.72 (0.054)
Wards triangle	0.93 (0.007)	0.97 (0.013)	0.67 (0.027)	0.75 (0.053)	0.83 (0.018)	0.92 (0.040)
L1-L4 spine	0.64 (0.028)	0.70 (0.058)	-	-	0.76 (0.018)	0.84 (0.039)
Head	0.46 (0.030)	0.51 (0.069)	0.59 (0.025)	0.66 (0.054)	0.73 (0.018)	0.78 (0.039)
Spine	0.68 (0.028)	0.75 (0.059)	0.87 (0.012)	0.97 (0.018)	0.79 (0.019)	0.90 (0.038)
Pelvis	0.77 (0.020)	0.81 (0.046)	0.77 (0.020)	0.88 (0.043)	0.84 (0.013)	0.91 (0.031)
Legs	0.79 (0.020)	0.88 (0.043)	0.65 (0.026)	0.71 (0.060)	0.92 (0.007)	0.93 (0.019)
Arms	0.75 (0.025)	0.98 (0.066)	0.66 (0.027)	0.86 (0.073)	0.85 (0.014)	0.92 (0.036)
Total Body	0.82 (0.018)	0.88 (0.039)	0.77 (0.018)	0.85 (0.039)	-	-
Height	0.18 (0.036)	0.24 (0.086)	0.09 (0.037)	0.12 (0.087)	0.18 (0.035)	0.22 (0.089)
Fat mass index	0.22 (0.033)	0.13 (0.137)	0.14 (0.035)	0.12 (0.136)	0.32 (0.031)	0.16 (0.136)
Lean mass index	0.33 (0.033)	0.31 (0.136)	0.09 (0.037)	0.12 (0.087)	0.42 (0.030)	0.35 (0.130)

Data are estimates (SE) and adjusted for Sex and Age

parameters of bone geometry.

Table 5A presents the genetic correlations of the narrow-neck geometry parameters and the most relevant variables used to interpret the risk of fracture: BMD, section modulus (index of bending strength) and the buckling ratio (index of bone instability). Narrow-neck BMD is totally phenotypic and genetically correlated with the average cortical thickness. In contrast, no phenotypic correlation is observed with neck width. In contrast to the traditional femoral neck BMD, no significant correlation is observed between narrow-neck BMD and body height or lean mass index. The section modulus, an index of bone strength, shows phenotypic and genetic correlation with all bone geometry parameters and with body height. Similarly,

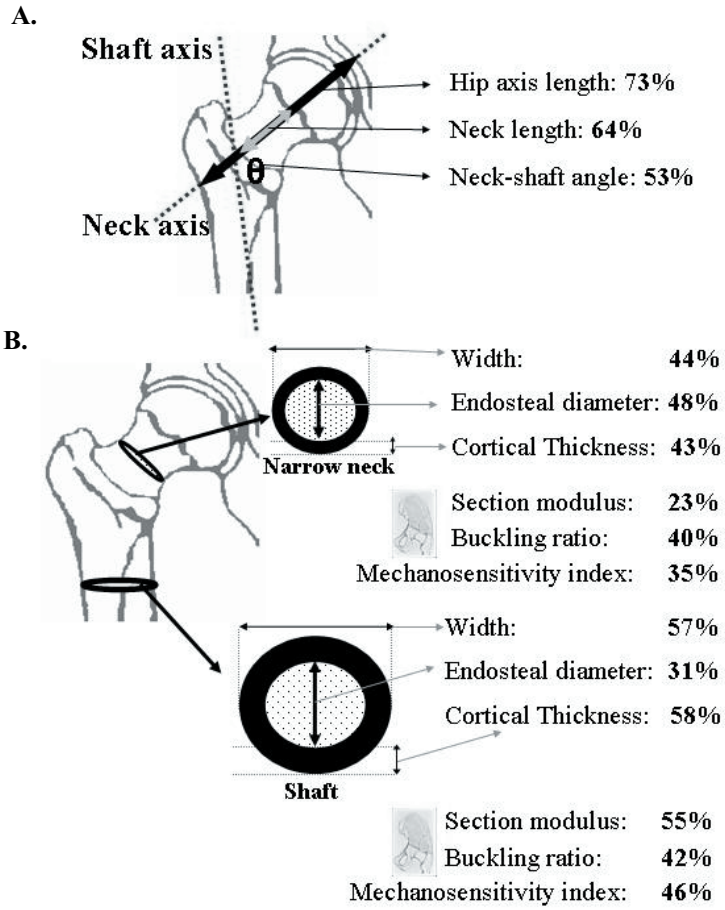


Figure 2. Bone geometry traits with heritabilities. **A.** Measurements of length axis and angles **B.** Bone diameters, cortical thickness, and bone indexes of strength (section modulus), instability (buckling ratio) and mechanosensitivity at the narrow-neck and shaft regions.

TABLE 4. HERITABILITIES OF HIP BONE GEOMETRY

Phenotype	Model 0*			Model 1**		
	Variance component		h^2	*Variance component*		h^2
	Additive genetic	*Total*		*Additive genetic*	*Total*	
HIP						
Hip axis length (cms)	0.28 (0.03)	0.37 (0.02)	75.1 (7.2)	0.18 (0.02)	0.25 (0.01)	72.8 (7.5)
Neck length (cms)	0.22 (0.03)	0.34 (0.02)	62.9 (8.5)	0.20 (0.03)	0.32 (0.02)	63.4 (7.7)
Neck-shaft angle (°)	0.13 (0.02)	0.25 (0.01)	52.4 (8.7)	0.13 (0.02)	0.25 (0.01)	53.2 (8.7)
Narrow neck region						
BMD (g/cm^2)	0.09 (0.20)	0.21 (0.10)	41.6 (9.1)	0.08 (0.02)	0.20 (0.01)	41.6 (9.3)
Cross-sectional area (cm^2)	0.08 (0.02)	0.25 (0.01)	33.8 (8.0)	0.07 (0.02)	0.22 (0.01)	33.6 (9.4)
Cortical Thickness (mm)	0.04 (0.009)	0.09 (0.004)	42.3 (9.1)	0.04 (0.008)	0.08 (0.004)	42.8 (9.3)
Neck width (cm)	0.03 (0.006)	0.05 (0.003)	47.9 (9.5)	0.02 (0.005)	0.05 (0.002)	43.5 (9.9)
Endocortical diameter (cm)	0.03 (0.006)	0.06 (0.003)	50.9 (9.6)	0.02 (0.005)	0.05 (0.003)	48.3 (9.8)
Section modulus (cm^3)	0.02 (0.009)	0.11 (0.005)	22.7 (7.7)	0.02 (0.008)	0.10 (0.005)	19.6 (7.9)
Cross-sectional moment of inertia (cm^4)	0.13 (0.04)	0.53 (0.03)	24.8 (7.9)	0.09 (0.04)	0.46 (0.02)	19.6 (8.0)
Buckling ratio	1.65 (0.42)	4.10 (0.20)	40.2 (9.5)	1.51 (0.40)	3.95 (0.19)	38.1 (9.6)
Shaft region						
BMD (g/cm^2)	0.01 (0.00)	0.03 (0.00)	38.7 (8.1)	0.01 (0.00)	0.02 (0.00)	35.2 (8.1)
Cross-sectional area (cm^2)	0.11 (0.02)	0.25 (0.01)	42.0 (8.1)	0.07 (0.02)	0.21 (0.01)	31.9 (8.2)
Cortical Thickness (mm)	0.27 (0.06)	0.67 (0.03)	40.6 (8.1)	0.25 (0.06)	0.64 (0.03)	38.4 (8.1)
Neck width (cm)	0.02 (0.004)	0.04 (0.002)	57.4 (8.0)	0.02 (0.003)	0.03 (0.001)	57.1 (8.2)
Endocortical diameter (cm)	0.04 (0.008)	0.09 (0.004)	49.9 (8.1)	0.04 (0.007)	0.07 (0.004)	50.9 (8.3)
Section modulus (cm^3)	0.07 (0.011)	0.12 (0.006)	55.3 (7.9)	0.04 (0.008)	0.08 (0.004)	43.5 (8.4)
Cross-sectional moment of inertia (cm^4)	0.32 (0.06)	0.58 (0.03)	54.8 (8.0)	0.19 (0.04)	0.42 (0.02)	45.2 (8.5)
Buckling ratio	0.11 (0.03)	0.27 (0.01)	41.8 (9.4)	0.11 (0.02)	0.26 (0.01)	42.0 (8.5)

Data are estimates (SE)

* Model 0 Adjustment for Sex, Age, Inbreeding coefficient

** Model 1 Adjustment for Sex, Age, Inbreeding coefficient, height, fat mass index, lean mass index and bone active medication (see text)

the buckling ratio (index of bone stability) has a very high genetic and phenotypic correlation with all bone geometry parameters but not with body height.

In Table 5B the genetic correlations between the size measurements of the hip and the lumbar spine L1-L4 area are presented. In line with the observed reduction in the additive genetic component of the heritability after adjusting for covariates (including body height), all the size measurements possess high genetic correlation with body height, with the highest correlation observed with the lumbar spine L1-L4 area.

Mechanosensitivity Indices

As shown in Table 6 after adjusting for covariates the mechanosensitivity indices of the femoral neck and shaft have significant heritabilities of 35 and 46%, respectively. When looking at the variance components after adjustment, total variances of both estimates decreased, but the additive genetic component increased in the narrow-neck index in contrast to a relative decrease in the additive component of the shaft index (suggesting common genetic factors with covariates).

The phenotypic correlation between the two mechanosensitivity indices was 0.45 (SE 0.04) while there was no significant genetic correlation between them 0.24 (SE 0.17).

Trait phenotypic and genetic correlations between both mechanosensitivity indices with BMD, bone geometry and covariates are also shown in Table 6. The narrow-neck mechanosensitivity index had significant genetic correlation of 0.40 with L1-L4 lumbar spine BMD but not with femoral neck BMD. Genetic correlation was also significant with total body and head/skull BMD. Similarly, there was significant correlation with all components of bone geometry including bone size (hip axis length), strength (section modulus) and instability (buckling ratio). There was no signification correlation with body height, lean and fat mass parameters. In contrast, the mechanosensitivity index at the shaft had significant correlations only with the bone instability parameter of the femoral neck (buckling ratio), and with body height and lean mass index.

In Table 7A mean trait values are compared across tertiles of the narrow-neck mechanosensitivity index in men and women. As compared to men, women had higher mechanosensitivity index of the narrow neck (mean 8.9 SD 2.4 versus mean 7.7 SD 2.3 $p<0.00001$) and of the shaft (mean 3.4 SD 0.5 versus mean 3.0 SD 0.5 $p<0.000001$).Also, in contrast to all the other BMD measurements of the skeleton,

TABLE 5A. PHENOTYPIC AND GENETIC CORRELATIONS OF HIP STRUCTURAL ANALYSIS MEASUREMENTS

	Narrow-neck BMD		Narrow-neck Section Modulus		Narrow-neck buckling ratio	
	Phenotypic	Genetic	Phenotypic	Genetic	Phenotypic	Genetic
Lunar Femoral Neck Region						
BMD	0.69 (0.018)	0.83 (0.049)	0.38 (0.030)	0.56 (0.117)	-0.65 (0.020)	-0.76 (0.060)
Narrow-neck Region						
BMD	-	-	0.76 (0.016)	0.67 (0.094)	-0.87 (0.011)	-0.94 (0.027)
Neck width	0.01 (0.037)	-0.25 (0.135)	0.60 (0.031)	0.55 (0.120)	0.36 (0.031)	0.54 (0.105)
Cross-sectional area	0.90 (0.007)	0.88 (0.034)	0.60 (0.031)	0.55 (0.120)	-0.64 (0.020)	-0.67 (0.085)
Endocortical Diameter	-0.24 (0.035)	-0.45 (0.115)	0.40 (0.042)	0.36 (0.146)	0.57 (0.024)	0.70 (0.075)
Cortical thickness	1.00 (0.000)	1.00 (0.000)	0.74 (0.016)	0.66 (0.096)	-0.88 (0.008)	-0.95 (0.024)
Section modulus	0.76 (0.016)	0.67 (0.094)	-	-	-0.46 (0.027)	-0.41 (0.141)
Cross-sectional moment of inertia	0.59 (0.030)	0.40 (0.138)	0.96 (0.003)	0.94 (0.021)	-0.24 (0.033)	-0.10 (0.163)
Buckling ratio	-0.87 (0.011)	-0.94 (0.027)	-0.46 (0.027)	-0.41 (0.141)	-	-
Height	0.09 (0.036)	0.08 (0.106)	0.30 (0.033)	0.50 (0.126)	0.07 (0.036)	0.11 (0.106)
Fat mass index	-0.22 (0.035)	-0.15 (0.160)	-0.23 (0.032)	-0.18 (0.196)	0.16 (0.035)	0.21 (0.159)
Lean mass index	-0.03 (0.055)	0.04 (0.168)	-0.06 (0.048)	0.06 (0.204)	0.14 (0.058)	0.07 (0.184)

TABLE 5B. PHENOTYPIC AND GENETIC CORRELATIONS OF SIZE MEASUREMENTS

	Narrow-neck width		Hip Axis Length		L1-L4 Spine Area	
	Phenotypic	Genetic	Phenotypic	Genetic	Phenotypic	Genetic
Neck width	-	-	0.47 (0.034)	0.58 (0.090)	0.36 (0.032)	0.59 (0.101)
Hip axis length	0.47 (0.034)	0.58 (0.090)	-	-	0.50 (0.028)	0.58 (0.000)
Neck length	0.13 (0.042)	0.26 (0.115)	0.64 (0.022)	0.73 (0.049)	0.20 (0.036)	0.32 (0.089)
Neck-shaft angle	0.16 (0.045)	0.08 (0.129)	0.30 (0.048)	0.30 (0.100)	0.09 (0.037)	0.20 (0.103)
L1-L4 area	0.36 (0.032)	0.59 (0.101)	0.50 (0.028)	0.58 (0.062)	-	-
Height	0.39 (0.032)	0.49 (0.087)	0.54 (0.027)	0.56 (0.057)	0.62 (0.023)	0.74 (0.047)
Fat mass index	-0.07 (0.035)	0.00 (0.157)	-0.02 (0.035)	-0.12 (0.127)	0.03 (0.035)	-0.10 (0.136)
Lean mass index	0.05 (0.055)	0.22 (0.160)	0.19 (0.039)	0.21 (0.127)	0.13 (0.041)	0.15 (0.133)

Data are estimates (SE) and adjusted for Sex and Age

TABLE 6. HERITABILITIES AND TRAIT CORRELATIONS OF THE MECHANOSENSITIVITY INDEXES

	Heritabilities			
	Narrow-neck mechanosensitivity index		**Shaft mechanosensitivity index**	
Variance component	**Model 0***	**Model 1****	**Model 0***	**Model 1****
Additive genetic component	1.31 (0.50)	1.52 (0.04)	0.08 (0.017)	0.06 (0.013)
Total variance	5.46 (0.26)	4.30 (0.21)	0.18 (0.009)	0.13 (0.007)
% h^2	24.0 (8.9)	35.3 (9.5)	42.6 (8.3)	46.1 (8.4)
	Trait correlations***			
	Narrow-neck mechanosensitivity index		**Shaft mechanosensitivity index**	
	Phenotypic	Genetic	Phenotypic	Genetic
Bone mineral density				
Femoral neck	0.15 (0.043)	0.23 (0.154)	0.04 (0.039)	0.18 (0.131)
L1-L4 lumbar spine	0.25 (0.046)	0.40 (0.138)	0.09 (0.041)	0.09 (0.124)
Total body	0.08 (0.041)	0.32 (0.150)	-0.05 (0.038)	0.03 (0.133)
Head/Skull	0.19 (0.035)	0.33 (0.127)	0.04 (0.037)	0.02 (0.114)
Bone geometry				
Hip axis length	-0.16 (0.036)	-0.29 (0.124)	0.01 (0.037)	-0.12 (0.115)
Section modulus narrow neck	0.66 (0.020)	0.36 (0.176)	0.37 (0.031)	0.19 (0.171)
Buckling ratio narrow neck	-0.29 (0.050)	-0.43 (0.172)	-0.27 (0.042)	-0.38 (0.142)
Height	-0.07 (0.035)	-0.21 (0.131)	-0.19 (0.035)	-0.25 (0.108)
Fat mass index	-0.35 (0.030)	-0.06 (0.189)	-0.32 (0.032)	-0.16 (0.169)
Lean mass index	-0.41 (0.029)	-0.09 (0.189)	-0.41 (0.037)	-0.33 (0.154)

Data are estimates (SE)
* Model 0 Adjustment for Sex, Age, Inbreeding coefficient
** Model 1 Adjustment for Sex, Age, Inbreeding coefficient, height, fat mass index, lean mass index and bone active medication (see text)
*** Adjustment for Sex and Age

women had higher head/skull BMD than men (mean 2.07 SD 0.30 versus mean 1.96 SD 0.26 g/cm^2 $p<0.00001$). In males, there is no difference of mean age across tertiles while in women we see that mechanosensitivity decreases with age. In general height, weight and BMI are lower with increasing mechanosensitivity while BMD is higher at increasing mechanosensitivity. The latter relationship is especially evident when looking at BMD Z-scores. In concordance, buckling ratios (indices of bone instability) are lower with increasing mechanosensitivity.

Table 7B shows the same analysis across tertiles of femoral shaft mechanosensitivity. In both genders mean age is higher at increasing mechanosensitivity values while height, weight, BMI and BMD values decrease with increasing mechanosensitivity at the shaft. In contrast, BMD Z-scores do increase and buckling ratios (bone stability) are lower with increasing mechanosensitivity (significantly only in males).

TABLE 7A. CHARACTERISTICS OF INDIVIDUALS BY NARROW-NECK MECHANOSENSITIVITY INDEX (MSI)

	MALES				FEMALES			
	Tertiles of mechanosensitivity index				Tertiles of mechanosensitivity index			
	Lowest	Middle	Highest	p-trend	Lowest	Middle	Highest	p-trend
n	124	123	124	-	181	182	181	-
Mechanosensitivity index	5.4 (0.9)	7.4 (0.5)	10.3 (1.7)	-	6.3 (1.2)	8.9 (0.6)	11.5 (1.6)	-
Age (years)	54.1 (12.4)	53.7 (13.1)	53.8 (15.7)	0.83	57.9 (11.9)	50.0 (13.8)	45.5 (14.3)	<0.00001
Height (m)	1.73 (0.1)	1.73 (0.1)	1.71 (0.1)	0.11	1.59 (0.1)	1.61 (0.1)	1.61 (0.1)	0.01
Weight (kg)	87.7 (13.2)	82.7 (12.2)	74.7 (11.1)	<0.00001	74.3 (12.5)	67.0 (11.3)	63.4 (11.5)	<0.00001
Body mass index (kg/m^2)	29.4 (4.0)	27.8 (3.7)	25.5 (3.5)	<0.00001	29.5 (4.9)	26.0 (4.0)	24.5 (4.0)	<0.00001
Femoral neck BMD (g/cm^2)	0.93 (0.13)	0.94 (0.15)	0.96 (0.15)	0.15	0.84 (0.11)	0.89 (0.13)	0.93 (0.14)	<0.00001
Z-score (SD)	-0.5 (0.9)	-0.4 (0.9)	0.0 (1.0)	0.0002	-0.4 (0.8)	-0.1 (0.8)	0.2 (1.0)	<0.00001
Lumbar spine BMD (g/cm2)	1.17 (0.18)	1.18 (0.19)	1.24 (0.19)	0.003	1.06 (0.16)	1.13 (0.17)	1.18 (0.19)	<0.00001
Z-score (SD)	-0.6 (1.4)	-0.4 (1.4)	0.4 (1.6)	<0.00001	-0.5 (1.3)	0.0 (1.2)	0.4 (1.4)	<0.00001
Total body BMD (g/cm2)	1.19 (0.10)	1.19 (0.12)	1.20 (0.10)	0.59	1.06 (0.09)	1.09 (0.10)	1.11 (0.10)	<0.00001
Z-score (SD)	-0.6 (1.1)	-0.4 (1.2)	0.1 (1.1)	<0.00001	-0.4 (0.9)	-0.1 (0.9)	0.3 (1.0)	<0.00001
Head / skull BMD (g/cm2)	1.94 (0.25)	1.95 (0.26)	2.00 (0.26)	0.05	1.95 (0.28)	2.08 (0.28)	2.17 (0.29)	<0.00001
Narrow-neck buckling ratio	11.3 (2.2)	10.6 (2.5)	9.8 (1.9)	<0.00001	10.9 (2.6)	8.9 (1.8)	8.3 (1.8)	<0.00001
Shaft buckling ratio	2.9 (0.4)	2.8 (0.6)	2.5 (0.5)	<0.00001	3.1 (0.5)	2.8 (0.5)	2.6 (0.5)	<0.00001

Data are unadjusted means (SD)

TABLE 7B. CHARACTERISTICS OF INDIVIDUALS BY SHAFT MECHANOSENSITIVITY INDEX (MSI)

	MALES				FEMALES			
	Tertiles of mechanosensitivity index				Tertiles of mechanosensitivity index			
	Lowest	Middle	Highest	p-trend	Lowest	Middle	Highest	p-trend
n	120	120	120	-	176	178	176	-
Mechanosensitivity index	0.25 (0.02)	0.29 (0.01)	0.35 (0.03)	-	0.29 (0.02)	0.34 (0.01)	0.39 (0.03)	-
Age (years)	45.9 (13.6)	53.9 (12.5)	61.0 (10.9)	<0.00001	45.7 (13.9)	51.0 (13.9)	56.6 (12.8)	<0.00001
Height (m)	1.75 (0.1)	1.72 (0.1)	1.69 (0.1)	<0.00001	1.62 (0.1)	1.60 (0.1)	1.58 (0.1)	<0.00001
Weight (kg)	89.2 (14.6)	80.7 (11.4)	75.9 (10.0)	<0.00001	73.7 (14.5)	68.2 (11.0)	63.2 (9.8)	<0.00001
Body mass index (kg/m^2)	29.1 (4.5)	27.2 (3.8)	26.5 (3.4)	<0.00001	28.0 (5.6)	26.7 (4.4)	25.5 (4.0)	<0.00001
Femoral neck BMD (g/cm^2)	0.98 (0.15)	0.93 (0.13)	0.92 (0.13)	0.0003	0.91 (0.12)	0.89 (0.14)	0.86 (0.14)	0.005
Z-score (SD)	-0.4 (1.0)	-0.4 (0.8)	-0.2 (0.9)	0.05	-0.3 (0.8)	-0.1 (0.9)	0.0 (0.9)	0.0001
Lumbar spine BMD (g/cm2)	1.20 (0.18)	1.19 (0.19)	1.20 (0.19)	0.83	1.14 (0.16)	1.12 (0.20)	1.12 (0.19)	0.22
Z-score (SD)	-0.5 (1.4)	-0.2 (1.5)	0.1 (1.6)	0.003	-0.3 (1.1)	-0.2 (1.4)	0.3 (1.4)	0.00001
Total body BMD (g/cm2)	1.22 (0.11)	1.18 (0.10)	1.17 (0.10)	0.0001	1.11 (0.09)	1.09 (0.10)	1.07 (0.10)	0.00005
Z-score (SD)	-0.3 (1.2)	-0.4 (1.0)	-0.2 (1.2)	0.67	-0.2 (0.9)	-0.1 (1.0)	0.1 (1.0)	0.007
Head / skull BMD (g/cm2)	2.00 (0.24)	1.95 (0.27)	1.93 (0.26)	0.03	2.10 (0.28)	2.08 (0.30)	2.03 (0.32)	0.04
Narrow-neck buckling ratio	10.9 (2.5)	10.6 (2.1)	10.2 (2.3)	0.03	9.5 (2.4)	9.3 (2.6)	9.4 (2.3)	0.92
Shaft buckling ratio	2.8 (0.4)	2.8 (0.6)	2.7 (0.5)	0.58	2.8 (0.5)	2.9 (0.6)	2.9 (0.6)	0.09

Data are unadjusted means (SD)

DISCUSSION

To our knowledge this is the first study to make such a comprehensive dissection of the DXA-based osteoporosis phenotype. We have found that the heritability across skeletal sites is highest at the head/skull (h^2 ~ 80%), and decreases as it departs progressively from the axial skeleton to the appendicular skeleton (h^2 decreasing ~ from 70 to 26%). Consistently, genetic correlations of BMD across sites were high and also decreased with decreasing vicinity of the measurement sites (rho decreased from 1.00 to 0.50). BMD measurements at the femoral neck and total body observed significant genetic correlation with height (rho ~ 0.20) and lean mass index (rho ~ 0.30) while lumbar spine BMD did not. The hip structural analysis showed that the parameters with highest heritability estimates were those of size and dimension (h^2 ranging ~ from 48 to 26%), followed by factors related to cortical thickness (h^2 ~ 40%), with the measures of bone strength showing the lowest yet significant heritability estimates (h^2 ~ 25%). BMD showed high genetic correlations with all the geometry parameters, being highest with cortical factors (rho between 0.88 and 1.00), followed by strength factors (rho between 0.40 and 0.67) and least and inverse with size and dimension factors (rho between -0.25 and -0.45). The genetic correlation between size geometric parameters and body height ranged from 0.30 (hip axis length) to 0.75 (lumbar spine area). Mechanosensitivity indices determined at the femoral neck and shaft were found to be significantly heritable (~ 35 and 46% respectively) with no significant genetic correlation between them. Both were genetically correlated (rho ~ 0.40) with the index of bone instability (buckling ratio) of the femoral neck, suggesting this could be one of the properties explaining how susceptibility to fragility fracture at older age might be heritable.

Why are Heritabilities Different at Skeletal Sites?

The strong differences in heritability estimates observed across the different BMD sites of measurement (Figure 1), are in line with the hypothesis that the genetic control of bone mass and morphology, even within a given bone, is highly site-specific (28). Considering the fact that head, spine and limbs arise from distinct embryological origins (29), the observed genetic correlations could probably be reflecting differential genetic regulation across skeletal sites, but most probably, differences in heritability are a consequence of differential adaptation to different types of locomotive strains (environmental influences). In contrast to the previous suggestion that the use of BMD measured at multiple sites does not provide additional information for the clinic since they are highly correlated (30), we have shown that for genetic studies, multiple site measurements do reflect different genetic backgrounds.

What Properties of BMD Reflect Bone Fragility and Which Ones Are Heritable?

Equivalent BMD values may concur with different underlying structural configurations. For this reason, we have focused further on hip bone geometry parameters, which arise as interesting traits for genetic studies considering their varying biomechanical nature and that they are heritable (Figure 2). There use though, requires some understanding of bioengineering. BMD can decrease due to a reduction in mineralization, a reduction on bone mass and due to an increase in bone volume. Using DXA there is no way to distinguish between changes in bone mineralization and mass, yet osteoporosis by definition is a disorder of structure and not material. Some rare alterations in mineralization are heritable (i.e. hereditary rickets or osteomalacia), predispose to fracture, but are not osteoporosis. This way, osteoporosis and low BMD are mainly the result of a decrease in bone mass and/or an increase in bone volume, phenomena which have opposing effects on bone strength and consequently on the risk of fracture. That is, if two bones have equal diameters the one with less mass is weaker. In contrast, two bones with equal mass, the one with greater diameter is stronger but less dense. This way, bone expansion can improve bone strength and compensate for net bone loss. Yet, thinning of cortices with consequent expanding diameters will also progress with aging towards bone instability making bones prone to buckle and fracture. This gradual process towards instability with aging would also explain why fractures tend to occur at old age.

Section modulus (index of bending strength) has lower heritability than the other geometrical parameters, a finding which is in line with the concept that bone strength is the consequence of dynamic and adaptative geometrical configurations which preserve bone strength through life. In addition, the buckling ratio (index of cortical instability) has a similar heritability ($h2 \sim 40\%$) and a high genetic correlation ($rho \sim 0.94$) with BMD measured at the same region, probably reflecting a property (bone instability) included in the BMD measurement which predicts fracture and is heritable.

Nevertheless, there are properties underlying the BMD measurement which may be heritable but may not influence the process towards bone fragility directly. The adjustments for and the analyses of genetic correlations we have done with body height, fat mass and lean mass indices provided some insight into intermediate pathways which can influence the heritabilities of our bone traits. In our variance component analysis we observed that the additive genetic component of BMD measurements decreased after adjustment for covariates (including height and lean mass index), suggesting possible common genetic factors. Genetic correlations also suggested that most BMD measurements of the appendicular skeleton are to some extent influenced by genes acting influencing size (body height). This

is illustrated when the heritability of BMD measurements at the traditional femoral neck region (h2 ~ 60%) is compared to that observed in the narrow-neck (h2 ~ 40%). The standard BMD measurement of the femoral neck has greater bone area and comparatively higher heritability. However, it also has significant genetic correlation with body height (rho ~ 0.20) which lacks in the narrow-neck BMD. These findings suggest that a considerable proportion of the high heritability in BMD measurements could be explained by skeletal size. Similarly, we observed that lean mass is also significantly correlated to BMD measurements at appendicular sites (rho between ~ 0.30 and 0.40), suggesting that genes related to muscle mass may indirectly be influencing the heritability of BMD.

The concept of mechanosensitivity

If we accept that bone strength is adaptative in response to the demands on skeletal loading (stress and strains), even in the presence of external and internal (hormonal) environmental regulation, the occurrence of weaker or stronger bones in presence of a given skeletal load would be reflecting the genetically-determined mechanosensitivity of an individual. This is in essence, the degree of bone sensitivity to mechanical stimuli (skeletal load) and has been proposed as a potential osteoporosis phenotype (31). The crude mechanosensitivity indices of the femoral neck and shaft proposed in this manuscript not only showed to be highly heritable (~40%) but were consistent with the fragility concept. Both indices had significant genetic correlation with buckling ratios (index of bone instability) and as shown in the analyses by mechanosensitivity tertiles, decreased mechanosensitivity resulted in increased bone instability (buckling ratios) and lower BMD Z-scores. Mean BMD levels appear to decrease with higher mechanosensitivity of the shaft, but this is an artifact produced by the difference in mean age across tertiles. This bias is a result of the secular trend in height (older individuals are significantly shorter, data not shown), which is part of the equation of the shaft mechanosensitivity index.

Skull/head BMD useful in genetic studies of osteoporosis?

We have shown that head/skull BMD is the measurement with the highest heritability and the lowest genetic correlation with BMD measurements at other skeletal sites. This is understandable considering that the skull is the skeletal site receiving the lowest strains (absence of mechanical loading) and thus less environmental influences. It has been shown that the skull is responsive to low strains (32) and with greater sensitivity than in other skeletal sites in order to be strong enough to exert its protective function on the brain (33). Our study did not only show that head/skull BMD was genetically correlated to the narrow neck mechanosensitivity index but

also, head/skull BMD increased with mechanosensitivity. Further, in contrast to the other BMD measurements, head/skull BMD is higher in women than in men, which is in concordance with the observation that mechanosensitivity is also higher in women than in men. A possible explanation for this gender differences is that higher mechanosensitivity in women is a compensatory response to the estrogen-mediated restrain on bone apposition which results in less biomechanical adaptation (34,35). Alternatively, mechanosensitivity in women could be higher due to less strain (bodyweight and/or lean mass) over the skeleton as compared to men. In line with these hypotheses we observed that narrow-neck mechanosensitivity decreased with aging only in women. In addition, it is important to note that monogenic disorders with abnormal regulation of bone formation, such as sclerosteosis and van Buchem's disease (36), have phenotypes which compromise the skull as well as other skeletal sites like clavicles, ribs, and diaphysis of the long bones. Considering this and our present findings, more attention should be directed to the genetics of head/skull BMD where further insight into the pathophysiology of osteoporosis may be found.

There are some limitations in our study. We have presented combined estimates for men and women, which is not an ideal analytical setting even after adjustment for gender differences. We acknowledge that in addition to differences in bone size, hormonal differences after menopause play a center role in the determination of bone phenotypes. We have also done gender- and age-stratified determination of trait heritabilities and correlations, but confidence limits in our gender stratified analysis became wider and always overlapped. In view of contradictory evidence for gender-specific effects (37), our future work will be focused on addressing gender-specific effects over BMD, bone geometry and body composition.

Another issue to consider is the isolated nature of our population, which may compromise the extrapolation of our phenotypes to other populations, but baseline characteristics of our study population do not characterize a very distinct or special Caucasian population. One aspect in which our population differs is inbreeding, therefore, we have adjusted all our estimates for inbreeding coefficients. In addition since participants of our study were not ascertained through individuals with extreme osteoporosis phenotypes, inferences about the relevance of genetic factors can be made at the population level. Similarly, as compared to twin studies heritability estimates of our population are less prone to be influenced by shared environment given the wider spectrum of familial relationships (38,39).

Previous studies have also used bivariate analysis as a tool to determine genetic correlations of BMD measurements across skeletal sites in different populations (40), between BMD measurements and other osteoporosis traits including quantitative ultrasound (QUS) measurements (41) and also between BMD and the oc-

currence of fracture (42). As shown in the study by Livshits et al. and in our study, the degree of genetic correlation is high across skeletal sites (ranging between 0.50 to 0.80). Yet, the study by Knapp et al. suggests that the low genetic correlation between BMD measured by DXA and the QUS measurements could be explained by differences in bone composition (cortical versus trabecular), types which are not adequately discriminated by the DXA measurement. Even though our findings on genetic correlations could suggest several degrees of pleiotropy and independence across traits, it is important to note that genetic correlation may also be caused by close linkage and linkage disequilibrium of genes with similar functions. Finally, as shown by Deng et al. there is low genetic correlation between fracture susceptibility and variations in BMD measurements, finding which supports our concept that bone geometry should be preferred over BMD in genetic studies.

In summary, we have made a comprehensive evaluation of DXA-based traits with implications for genetic studies of osteoporosis. Our analyses show different heritabilities through the skeleton, with higher heritabilities in the axial segment with progressively lower estimates towards the appendicular segment. Measurement of bone geometry parameters of the hip explain underlying biomechanical properties of BMD and showed diverse degrees of heritability. Genetic correlations of the studied traits provide further understanding of the traits, the intermediate pathways and their underlying genetic factors. Mechanosensitivity indices were shown to be heritable traits genetically independent of size and genetically correlated to bone instability and head/skull BMD which might be regarded as a possible indicator of mechanosensitivity. We propose that susceptibility to fragility fracture at older age is heritable partly because they reflect genetically-determined differences in mechanosensitivity. Rather than using isolated BMD measurements, future genetic studies should target bone geometrical parameters and indices of mechanosensitivity.

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The Insulin-like Growth Factor I Gene Promoter Polymorphism and Osteoporosis

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Association between an Insulin-Like Growth Factor I Gene Promoter Polymorphism and Bone Mineral Density in the Elderly: The Rotterdam Study

4.1

ABSTRACT

Studies of the role of variants of the *IGF-I* gene in the regulation of bone mineral density (BMD) have yielded conflicting results. We examined the role of a microsatellite repeat polymorphism in one of the promoter regions of the *IGF-I* gene in relation to femoral BMD in elderly women and men from the Rotterdam Study. We studied 5648 and 4134 individuals at baseline and follow-up (approximately 2 years later), respectively. Femoral BMD measurements were performed using dual energy X-ray absorptiometry (DXA). In women, baseline BMD levels were on average 0.02 g/cm^2 lower (95%CI for difference -0.03, -0.00 g/cm^2) without the *192-bp* allele as compared with the homozygotes for the allele (p=0.03). The mean rate of BMD change from baseline to follow-up was -6.9 mg/cm^2 (95%CI -10.8, -3.0), -4.5 mg/cm^2 (95%CI -6.4, -2.5) and -2.3 mg/cm^2 (95%CI -4.2, 0.3) in non-carriers, heterozygotes and homozygotes for the *192-bp* allele, respectively (p *trend*=0.03). Adjustment for age and body mass index did not essentially change this relation. No such effects were observed in men. Our findings suggest that this promoter polymorphism or another functional polymorphism in linkage disequilibrium may be a genetic determinant of BMD levels and rate of bone loss in postmenopausal women.

INTRODUCTION

Osteoporosis has been defined as a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (1). From the genetic perspective, BMD is a complex trait determined by the peak bone mass achieved during adulthood and the subsequent rate of bone loss with age. Heritability estimates for BMD have been reported to be high, ranging between 50-80% (2, 3). The contribution of genetic factors to the regulation of bone loss has been much less well studied and is yet conflicting (4). While Christian *et al.* (5) found no evidence of a genetic effect on radial (cortical) bone in aging male twins, Kelly *et al.* (6) reported a significant genetic effect on changes in axial bone density in adult twins. It has become clear that there is not one major single gene responsible for the risk of osteoporosis (3). Rather, an individual's susceptibility to develop osteoporosis is determined as in most common diseases, by several common gene variants with modest but real genetic effects (7). The regulation of bone mass depends on several factors, including the balance between the amount of bone resorbed by osteoclasts and the amount of bone formed by osteoblasts. Genes involved in the mechanisms that control the differentiation and the function of these cells may be determinants of BMD and osteoporosis. One approach to identify individual genetic factors is the so-called *candidate gene approach*. So far, several genes have been investigated including the VDR, COL1α1, IL-6, and TGF-β genes among others. Of these, only the COL1α1 gene has been found associated to BMD and fracture risk in a consistent way as illustrated by two meta-analyses (8, 9). The insulin-like growth factor I (IGF-I) gene has also been considered a candidate gene based on its important role in bone metabolism.

IGF-I is a ubiquitous polypeptide that stimulates osteoblast activity, subsequently leading to bone matrix formation and inhibition of bone collagen degradation (10). IGF-I also stimulates osteoclast formation and action (11). Plasma levels of IGF-I decrease with age both in males and females. Reduced plasma levels have been associated with low BMD (12-14), osteoporosis (15) and fractures (16), although it is not known if these systemic levels are representative of local skeletal concentrations (17-19).

Several polymorphisms (20-22) have been identified in the *IGF-I* gene (map location 12q22-q24.1) and in the 5' flanking promoter region extending up to 1630 bp upstream of the transcription initiation site of exon 1 (23-26). On position –684 of this promoter region lies a $(CA)_n$ microsatellite repeat polymorphism (27). Earlier, we have found that birth weight (28, 29), body height and serum levels of IGF-I after age 55 years (30) increased with the number of *192-bp* alleles in the genotype for this polymorphism. Further, subjects without this "wild-type" allele

had increased risk for type 2 diabetes and myocardial infarction (30).

Results of genetic studies regarding the relation of this polymorphism to osteoporosis and BMD have been controversial. Rosen *et al.* (31) reported that the *192-bp/192-bp* genotype was more prevalent in 25 Caucasian men with Idiopathic Osteoporosis than in controls. In the same study, healthy men with this genotype tended (p=0.15) to have lower BMD T scores. In 314 healthy postmenopausal Japanese women, Miyao *et al.* (32) found no association between BMD and the *IGF-I* promoter genotypes. In contrast, Kim *et al.* (33) found that a genotype based on one of the major alleles of the polymorphism was related to spinal and femoral BMD in 300 postmenopausal Korean women. In a study in 542 female sib pairs and 363 pre-menopausal women, Takacs *et al.* (34) found no evidence for a relation between femoral or spinal BMD and the *IGF-I* gene locus or the $(CA)_n$ microsatellite repeat polymorphism.

Given these inconsistent reports and our previous findings on the relation of this *IGF-I* gene promoter polymorphism to serum IGF-I levels, height, type 2 diabetes mellitus (30) and birth weight (28), we examined the role of the *192-bp* allele in relation to bone mineral density and rate of bone loss in a large population-based cohort.

EXPERIMENTAL SUBJECTS

Subjects were derived from the Rotterdam Study, a single-center prospective population-based study of determinants of chronic disabling diseases in the elderly (age 55 years and over). Written informed consent was obtained from every participant. The design of the study has been described previously (35). In an attempt to evaluate all 7983 participants from the Rotterdam study, 7012 (87.9%) subjects were genotyped for the polymorphism (27) in the promoter region of the human *IGF-I* gene. From the 971 individuals not genotyped, 848 had no blood sample to isolate DNA for the analysis and in the remaining 123 we failed to obtain a genotype after multiple attempts.

MATERIALS & METHODS

The analysis was performed in two phases. In the first phase (baseline), all individuals with complete BMD and antropometric measurements (n=5648), were used for a cross sectional analysis of femoral BMD levels. In the second phase (follow-up), subjects that had complete BMD measurements both at baseline and on the follow-up visit (n=4134), were used to study the yearly rate of change in BMD.

Measurements

Age was calculated for each individual from the date of birth and the date of BMD bone scan. Three 10-year strata were defined starting at age 55 years. Height (cm) and weight (kg) were measured in standing position wearing indoor clothes and without shoes. Body mass index or BMI (kg/m^2), was calculated as weight divided by the square of height. Bone mineral density measurements (g/cm^2) of the proximal femur were performed by dual energy X-ray absorptiometry (DXA) using a Lunar DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA). Methods, quality assurance, accuracy and precision issues of the DXA measurements have been described previously (36).

Approximately two years (mean 23.8 months, SD 7.0) after the baseline scan, follow-up BMD measurements were performed using identical procedures. The rate of change in BMD (mg/cm^2 per year) was calculated as the difference between baseline and follow-up BMD divided by the time (in years) elapsed between measurements (and multiplied by a factor of 1000 for scale convenience).

Genotyping

Polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to amplify the polymorphic $(CA)_n$ repeat 1 kb upstream of the human *IGF-I* gene. The reaction was carried out in a final volume of 7.5 µl containing 25 ng of genomic DNA obtained from peripheral white blood cells and extracted by standard proteinase K digestion and salting-out procedure (37), 5 pmol forward primer (5'-ACCACTCTGGGAGAAGGGTA-3'), 0.5 pmol reverse primer (5'-GCTAGCCAGCTGGTGTTATT-3'), 25 mM dNTP, 2.2 mM $MgCl_2$, 0.01% W1 (Invitrogen), and 0.4 U Taq DNA Polymerase (Invitrogen). PCR was performed in 384 well plates (94°C 5 min; 35 PCR cycles of 30 sec at 94°C, 30 sec on 55°C, and 30 sec on 72°C; 72°C 7 min; 4°C hold). Forward primers were labeled with FAM, HEX, or NED to determine the size of PCR products by fragment analysis on an automated sequencing apparatus (ABI 377 Genescan Software V.3.1, 6.25% longranger gel, filter set D, predefined categories according to size and labeling of peak height between 100 and 2000 bp, each lane containing three samples). The size of the PCR products was determined in comparison with internal ROX 500-size standard (Perkin Elmer). The two highest peaks were labeled (binned) with Genotyper Software V.2.5. The automatic binning was reviewed by two independent observers in separate files from the same gel, which were subsequently cross-checked. Discordant binned samples were genotyped again. From sequence analysis it is known that the allele with length *192-bp ("wild-type"* in our population) corresponds to 19 CA-repeats ($CA_{(19)}$). Based on the relationship between the polymorphism and serum IGF-I levels, genotypes were assembled from two

allele categories as described by Vaessen *et al.* (30): the *192-bp* allele and all other alleles pooled as "*non-192-bp*" alleles. This resulted in three groups of individuals: homozygotes for the *192-bp* allele, heterozygotes for the *192-bp* allele and non-carriers of the *192-bp* allele.

Statistical analysis

Genotype and allele frequencies of the *IGF-I* promoter polymorphism were determined, and the genotype frequencies were tested for Hardy-Weinberg equilibrium proportions using the ARLEQUIN-package (38). Analyses specifying risk genotypes with other alleles showed no significant associations, but were consistent with the *192-bp* allele approach used.

In both the baseline and follow-up analyses, means and standard deviations were computed for all measurements, and compared with those of the same gender in the reference population using student T-tests. Subsequently, stratified analyses by gender (and age groups) were performed. Multiple linear regression was used to model the relation with BMD and rate of BMD change adjusted for age, BMI, and baseline BMD (in the follow-up analysis). Possible interactions between genotypes and covariates were explored in plots and tested in the linear regression models including product terms. Trend analysis assuming an underlying additive genetic model (39) was done for the presence of zero, one or two copies of the associated allele, incorporating the genotype variable as a continuous term in the multiple linear regression models. Finally, model assumptions were verified and model residuals were checked for goodness-of-fit. If not stated otherwise, all analyses were performed using the SPSS-package V.10 (SPSS Inc. Chicago, IL).

RESULTS

Allele and *192-bp* genotype frequencies are shown in Table 1. No significant deviations of the frequencies were observed among the baseline and follow-up groups. All genotype frequencies were in Hardy-Weinberg equilibrium proportions. In men and women, allele frequencies were stable over age categories (data not shown).

Table 2 compares the characteristics of women and men of the two analytical phases with all women and men of the Rotterdam study (reference population n=7983). Overall, women showed lower BMD at baseline and a higher rate of BMD loss than men. The BMD levels of the group used for the baseline analysis were slightly lower than those observed in the group used for the follow-up analysis. The use of concurrent estrogen replacement therapy (HRT) or bone modulators in women is very low.

TABLE 1. ALLELE AND 192-BP GENOTYPE FREQUENCY DISTRIBUTIONS OF THE STUDY POPULATIONS

		Reference*	Baseline Analysis			Follow-up Analysis		
POLYMORPHISM		**TOTAL**	**TOTAL**	**FEMALES**	**MALES**	**TOTAL**	**FEMALES**	**MALES**
Length PCR product	**(CA)n**	n = 7012	n = 5648	n = 3265	n = 2383	n = 4134	n = 2341	n = 1793
198 bp	**22**	1.6%	1.5%	1.4%	1.6%	1.5%	1.4%	1.5%
196 bp	**21**	6.8%	6.8%	6.9%	6.8%	6.8%	6.7%	7.0%
194 bp	**20**	19.1%	19.4%	19.4%	19.4%	19.5%	19.8%	19.1%
192 bp	**19**	65.9%	65.7%	65.5%	66.1%	65.7%	65.3%	66.3%
190 bp	**18**	4.4%	4.4%	4.5%	4.2%	4.3%	4.4%	4.1%
188 bp	**17**	1.7%	1.7%	1.7%	1.7%	1.7%	1.8%	1.6%
Other rare alleles[ç]	**-**	0.5%	0.5%	0.5%	0.3%	0.4%	0.4%	0.4%
Genotypes								
Homozygous 192 bp		43.7%	43.5%	43.2%	44.0%	43.7%	43.3%	44.1%
Heterozygous 192 bp		44.3%	44.4%	44.6%	44.2%	44.1%	44.0%	44.3%
No 192 bp homozygous		12.0%	12.1%	12.2%	11.8%	12.2%	12.7%	11.6%

* *All Rotterdam Study participants genotyped for the IGF-I promoter polymorphism* [ç] *174-bp* CA_{10} *, 176-bp* CA_{11} *, 186-bp* CA_{16} *and 200-bp* CA_{23}

TABLE 2. CHARACTERISTICS OF THE STUDY POPULATIONS

MEASUREMENTS	Reference TOTAL n = 7983	Reference FEMALES n = 4878	Reference MALES n = 3105	Baseline Analysis FEMALES n = 3265	Baseline Analysis MALES n = 2383	Follow-up Analysis FEMALES n = 2341	Follow-up Analysis MALES n = 1793
Age (years)	70.6 (9.8)	71.7 (10.3)	69.0 (8.7)	68.3[c] (8.2)	67.5[c] (7.6)	69.2[c] (7.7)	68.6 (7.3)
Height (cms)**	166.6 (9.5)	161.1 (6.8)	174.6 (6.8)	161.7 (6.5)	174.9 (6.8)	162.0 (6.4)	175.2 (6.6)
Weight (kgs)**	73.7 (11.6)	69.9 (10.9)	78.7 (10.6)	69.9 (10.9)	78.7 (10.6)	70.1 (10.7)	79.4 (10.9)
BMI (kg/m^2)**	26.3 (3.6)	26.8 (4.0)	25.7 (3.0)	26.8 (4.0)	25.7 (2.9)	26.7 (3.9)	25.9 (2.9)
Time since menopause (years)	-	21.2 (11.0)	-	18.7[c] (9.5)	-	19.4[c] (9.1)	-
HRT* **or bone modulators** (%)	-	2.0	-	2.7	-	2.8	-
BMD (g/cm^2) *Femoral neck****	0.84 (0.14)	0.81 (0.13)	0.88 (0.13)	0.81 (0.13)	0.88 (0.13)	0.82 (0.13)	0.88 (0.13)
*Ward Triangle****	0.70 (0.15)	0.67 (0.15)	0.73 (0.15)	0.67 (0.15)	0.73 (0.15)	0.68 (0.15)	0.74 (0.14)
*Trochanter****	0.78 (0.15)	0.72 (0.13)	0.85 (0.14)	0.72 (0.13)	0.85 (0.14)	0.73 (0.13)	0.85 (0.13)
BMD change *Femoral neck*****							
Absolute *(g/cm^2 - year)*	-0.004 (0.03)	-0.004 (0.03)	-0.003 (0.03)	-	-	-0.004 (0.04)	-0.003 (0.03)
Relative *(% of baseline - year)*	-1.2 (10.3)	-1.2 (12.2)	-1.1 (7.3)	-	-	-1.1 (12.3)	-1.0 (7.2)

Data are unadjusted means (SD) or (%)

* *Hormone replacement therapy*

** *Based on 6917 individuals with present antropometric measurements*

*** *Based on 5823 individuals with present BMD measurements*

**** *Based on 4331 individuals with present BMD measurements*

[c] $p < 0.01$

When analyzing cross-sectional baseline BMD measurements in females, the *IGF-I* polymorphism accounted for 0.2% of the variance (R^2) in BMD levels. Mean femoral neck BMD adjusted for age and BMI (Figure 1) increased with the number of *192-bp* alleles in the genotypes (*p* for trend =0.02). BMD was on average 0.02 g/cm^2 (95%CI for difference –0.03, -0.00 g/cm^2) lower in women without the *192-bp* allele as compared to the homozygotes for the allele (*p*=0.03). This effect was also consistent at the other femoral sites (*p* for trend=0.01 and <0.01 for the trochanter and Ward's triangle respectively).

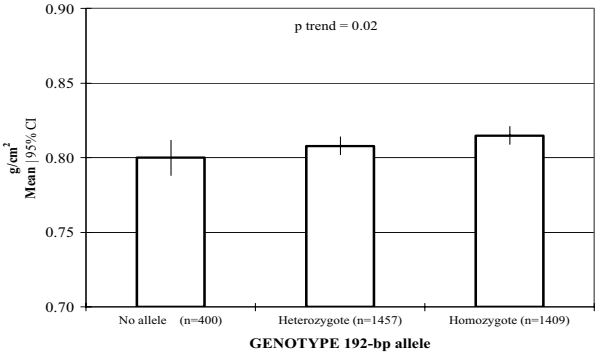


Figure 1. Baseline mean* BMD of the femoral neck within *192-bp* genotype groups in women *Data adjusted for age and BMI

When analyzing in age strata (Figure 2), this effect was only observed in women older than age 65 years: the differences between homozygous women and non-carriers of the *192-bp* allele was -0.02 g/cm^2 (95%CI -0.04, -0.00 g/cm^2) and -0.04 g/cm^2 (95%CI -0.07, 0.01 g/cm^2), for the 65-75 years and 75 years-and-over age categories, respectively.

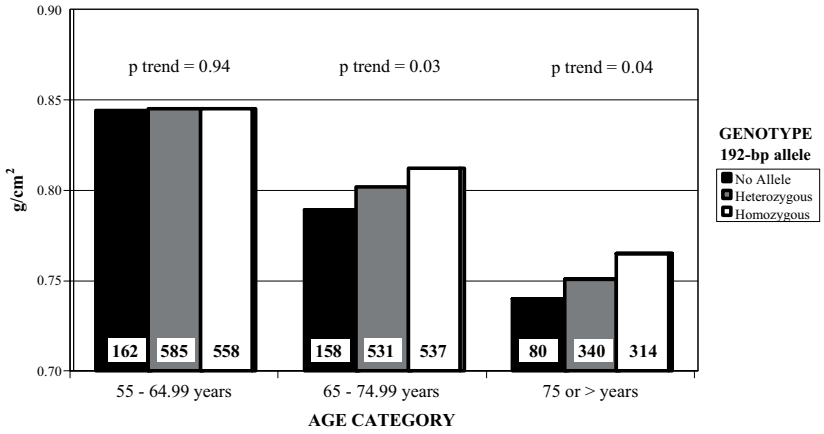


Figure 2. Baseline mean* BMD of the femoral neck within *192-bp* genotype groups and age categories in women. *Data adjusted for age and BMI

The interaction between *IGF-I* genotype and age was borderline significant (p for interaction=0.06). There was no significant interaction of *IGF-I* genotype and BMI (data not shown). No such dose effect on baseline BMD was observed in males overall (Figure 3) or within age groups (data not shown).

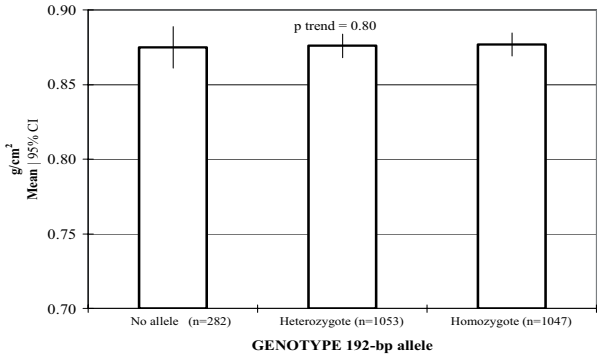


Figure 3. Baseline mean* BMD of the femoral neck within *192-bp* genotype groups in men *Data adjusted for age and BMI

At follow-up, the rate of change in mean BMD observed in the period between the baseline and follow-up measurements was analyzed. In women, the *IGF-I* promoter genotype accounted for 0.1% of the variance (R^2) in BMD change. The mean rate of BMD change per year showed a significant inverse trend (p=0.03) according to the number of *192-bp* alleles in the genotype, -6.9 mg/cm^2 (95%CI -10.8, -3.0) in women carrying zero, -4.5 mg/cm^2 (95%CI -6.4, -2.5) in women carrying one (heterozygotes) and -2.3 mg/cm^2 (95%CI -4.2, 0.3) in women carrying two copies (homozygotes) of the *192-bp* allele, respectively (Figure 4). Also in women, this trend was consistent through all age strata (data not shown). Adjustment for baseline BMD did not essentially modify the results (data not shown). In males, no such trend effect was observed in the follow-up analysis, overall or within age groups (data not shown).

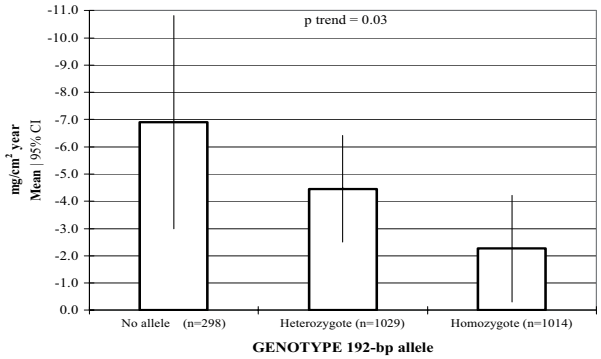


Figure 4. Mean* femoral neck BMD change of the femoral neck within *192-bp* genotype groups in women *Data adjusted for age, BMI and baseline BMD

DISCUSSION

This population based study in elderly individuals, showed that in women, the absence of the wild type (*192-bp*) allele in a $(CA)_n$ repeat polymorphism in the promoter region of the *IGF-I* gene is associated with lower BMD levels and higher rates of bone loss at the different femoral sites. No associations were observed in men at any femoral site of BMD measurement.

Our findings of decreased BMD levels and faster rate of bone loss in the absence of the *192-bp* allele are in agreement with the association reported earlier, where the absence of the *192-bp* allele was associated with lower total IGF-I serum levels (30). Results from that study may be extrapolated to our present study, since it was performed in a random subset of our current study population. Further, we have recently reported that this promoter polymorphism influences the age-related decline in IGF-I levels (40).

Strengths of our study design include its population-based nature, ethnic homogeneity, large sample size, gender-stratified analysis and defined age range (elderly/postmenopausal). BMD characteristics in this study are similar to those reported previously in our population (36). Since individuals were selected on the basis of having complete BMD measurements either at baseline or at follow-up, we recognize the possibility of selection bias. Individuals whose BMD was not measured in the Rotterdam Study, were older and probably had higher morbidity, and, if measured, would presumably have had lower BMD than those in the study. However, there is no evidence for genotype selection in our population as allele frequencies were virtually the same in the various study groups and in the genotypic reference population, and since they remained stable with age. Similarly, the bias arising from the individuals not genotyped for the polymorphism seems to be random from the genetic perspective, since our allele frequencies are similar to those reported in other Caucasian populations (27, 34).

The short follow-up time (approximately 2 years) used to evaluate the rate of BMD change limits the power to assess differences among individuals. However, the *192-bp* allele dose effect of our follow-up analysis is in agreement with our cross-sectional analysis in the sense that the presence of the *192-bp* allele relates to higher BMD levels and lower rates of bone loss.

The gender specificity of our findings is in concordance with the association between IGF-I levels and BMD reported earlier by Barrett-Connor *et al.* (14). It is not explained by differential survival between sexes in our population, since genotype and allele frequency distributions were similar in men and women in different age categories. Most likely, our findings seem to identify differential responses of

the genotypes to the postmenopausal bone loss caused by estrogen deficiency (41). Since differences in estrogen levels influence the IGF-I regulatory axis (42-45), the hypo-estrogenic state inflicted by the menopausal process in women, may be (in the absence of concurrent osteoporotic treatment i.e. hormone replacement therapy) modulating directly the observed *IGF-I* genotype effect on the rate of bone loss and BMD level. Anyway, it is unlikely that estrogen deficiency explains most of the decline in IGF-I levels with age, since IGF-I levels begin to fall in both genders long before the age of menopause (14). Alternatively, genetic determined variations in body composition with aging could also be indirectly related to the observed genotype effect. Although the genotype-dependent differential rate of bone loss is observed from age 55 years, the genotype effect on BMD levels is only evident after age 65 years. This may reflect the time it takes for these genotype effects (during post-menopausal estrogen deficiency) to become detectable on BMD differences. Evaluation of possible biologic interactions of the *IGF-I* gene with genes and proteins related to estrogen metabolism might provide further insight.

Not observing the *192-bp* allele dose effect in males may be attributed to various mechanisms. In elderly men, estrogen levels are higher as compared to postmenopausal women (46), which might also affect the rate of bone loss, making age-related increases in bone turnover less pronounced in men (47). Similarly, from the perspective of bone size and architecture (48), both genders have an age-related decline in bone material properties, but men exhibit greater compensating bone-remodeling patterns (subperiosteal expansion and bone apposition at the femoral diaphysis). These differences in bone geometry are reflected in BMD measurements, since DXA adjusts for the scanned area but does not correct for the fact that wider bones are also thicker, resulting in greater BMD even if the actual density of bone tissue is not different (49). This way, our analysis could not discriminate changes in BMD due to variations in bone mineral from those caused by changes in bone geometry. Interestingly, Looker *et al.* postulated earlier that IGF-I could be related to the gender differences evidenced in bone geometry with aging (48).

Previous studies have either failed to identify associations between this polymorphism and BMD, or are conflicting. There are differences in study design that may explain this. Takacs *et al.* (34) included in their association analysis a population of pre-menopausal women, as compared to the post-menopausal population studied by us. Furthermore, the failure to identify linkage of this polymorphism to BMD in their sib-pair analysis may be explained by lack of power given the complexity of the trait and the relatively small effect size of the *IGF-I* polymorphism. Although Miyao *et al.* (32) and Kim *et al.* (33) included postmenopausal women in their study, we face caveats to compare our findings to theirs. Differences exist between the Dutch and both Japanese and Korean populations in allele and genotype frequencies, in linkage disequilibrium (LD) and in racial phenotypes. In contrast to

our findings, Rosen *et al.* (31) associated the presence of the *192-bp* allele to lower total IGF-I serum levels and to male osteoporosis. Male patients with Idiopathic Osteoporosis represent a very distinct phenotypic trait compared to the estrogen-deficient osteopenia inflicted by menopause (41), which together with differences in power, might contribute to apparent contradictory results.

Given the population-based approach of our study, we cannot distinguish if this polymorphism itself is involved in the regulation of *IGF-I* expression or merely flags another polymorphism in the promoter region functionally involved in *IGF-I* expression. If the latter is true, this may be an explanation for inconsistent findings in the association studies of this polymorphism with BMD (31-34) and other outcomes (50), since LD can differ between populations.

In summary, we found in postmenopausal women a small, but significant effect between an *IGF-I* gene promoter polymorphism, and both BMD and (short term) rates of bone loss. The presence of the wild type (*192-bp*) allele in the genotype was associated with higher BMD and lower rate of bone loss. This genotypic effect on BMD in women may therefore suggest a relation between IGF-I activity and bone loss due to estrogen deficiency. In men, no effect was observed probably due to gender differences in the age-related hormonal changes affecting bone turnover rates, bone size and architecture. This population-based study provides substantial evidence to implicate genetic determined levels of IGF-I to bone mineral density in Caucasian postmenopausal women.

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The Influence of an Insulin-Like Growth Factor I Gene Promoter Polymorphism on Hip Bone Geometry and the Risk of Nonvertebral Fracture in the Elderly: The Rotterdam Study

4.2

ABSTRACT

Introduction: Previously, we found a CA-repeat promoter polymorphism in the *IGF-I* gene associated with IGF-I levels and BMD in post-menopausal women, but the relationship with fractures is unclear. In this large population-based study of elderly men and women we examined the association between this *IGF-I* promoter polymorphism with parameters of bone geometry and the occurrence of fractures.

Material and methods: Within the Rotterdam Study, a prospective population-based cohort, the *IGF-I* polymorphism was analyzed in relation to incident non-vertebral fractures in 2799 men and 4212 women followed on average for 8.6 years. Further, we estimated structural parameters of hip bone geometry indirectly from DXA outputs of the femoral neck in 2372 men and 3114 women. We studied *neck width*, *cortical thickness*, and the *cortical buckling ratio* and the *section modulus* as indexes of bone stability and bending strength.

Results: Women heterozygotes and non-carriers of the allele had respectively 1.2 (95%CI 1.0-1.5) and 1.5 (95%CI 1.1-2.0) increased risk of having a fragility fracture at older age as compared to homozygotes for the *192-bp* allele (p trend=0.0007). In men fracture risk was not influenced by the polymorphism. As compared to homozygotes for the *192-bp* allele, non-carrier males had ~1% narrower femoral necks and 2.2% lower section moduli (p-trend<0.05). Non-carrier females had 1.7% thinner cortices, and 1.6% higher *buckling ratios* (p-trend<0.05) but no significant differences in femoral neck widths and section moduli. In women with low BMI, genotype differences in bone strength (section modulus) and fracture risk were accentuated (p-interaction=0.05). The genotype dependent differences in hip bone geometry did not fully explain the genotype dependent differences in fracture risk.

Conclusions: The CA-repeat promoter polymorphism in the *IGF-I* gene is associated with the risk for fragility fracture at old age in women and with bone structure in both genders.

INTRODUCTION

The ultimate clinical outcome of osteoporosis is fracture; its occurrence is mainly determined by the strength of bone and the risk of falling. While strength depends on bone geometry and bone material quality, the risk of falling determines the type of fall, the force of impact and the likelihood of fracture (1,2). In addition, the specific site of fracture follows specific epidemiological patterns which are influenced by different environmental factors (2).

Family history of osteoporotic fracture is a risk factor for future fractures (3), indeed heritability of risk has been estimated to be between 25 and 35% (4,5). For these reasons, the search for genetic determinants of fracture risk has gained evolving importance, especially after the completion of the Human Genome Project (6-8). In the general population, an individual's genetic susceptibility for fracture has been found to be associated with several common gene variants that have modest but real genetic effects (9,10). Examples are polymorphisms in the COL1α1 (11,12), VDR (13) and ERα (14) genes, all of which have been associated with the risk of fracture. The insulin-like growth factor I (IGF-I) has been shown to have an important role in bone metabolism (15). Plasma levels of IGF-I decrease with age both in males and females and reduced levels have been associated with low BMD (16-18), osteoporosis (19) and fractures (20,21). Consequently, the *IGF-I* gene has been considered a strong candidate to explain part of the genetic susceptibility to osteoporotic fracture.

A $(CA)_n$ microsatellite repeat polymorphism located in the 5' promoter region of the *IGF-I* gene has been described previously (22-24). Earlier, we have studied this polymorphism in relation to several traits and diseases in the Rotterdam study. Birth weight (25,26), body height and serum levels of IGF-I after age 55 years (27) have been shown to increase with the number of *192-bp* alleles in the genotype for this polymorphism. With regard to osteoporosis we found a small, but significant gender-specific effect: the presence of the wild-type (*192-bp*) allele in the genotype was associated with higher BMD and lower rate of BMD decline in postmenopausal women (28). This genotypic effect on BMD suggested a relation between IGF-I activity and BMD decline due to menopausal estrogen deficiency. Not observing this effect in men could be explained by gender differences in age-related hormonal changes affecting bone turnover rates, bone size and geometry.

Given our previous findings on the relation of this *IGF-I* gene promoter polymorphism to BMD, we analyzed the polymorphism in relation to the risk of non-vertebral fracture in elderly men and women. Further, we studied several parameters of hip bone geometry, in relation to the risk of hip fracture and in relation to the *IGF-I* promoter polymorphism.

MATERIAL AND METHODS

Subjects

Individuals were derived from the Rotterdam Study (n=7983), a single-center prospective population-based study of determinants of chronic disabling diseases in the elderly (age 55 years and over). Written informed consent was obtained from every participant. The design and rationale of the study has been described earlier (29). As described previously (28), we have genotyped 87.9% of the participants (n=7012) for the polymorphism in the promoter region of the human *IGF-I* gene. Genotyping was not possible in the remaining participants due for technical reasons (1.5%) or the absence of blood samples for DNA isolation (10.6%).

The analysis was performed in two phases. In the first phase, we analyzed *IGF-I* promoter genotypes (n=7012) in relation to non-vertebral fracture follow-up data in all genotyped individuals with complete fracture follow-up information (n=1219 individuals with at least one fracture and 5793 individuals without fracture). In the second phase, we examined at baseline all genotyped individuals with complete BMD and anthropometric measurements for a cross-sectional analysis of hip bone geometry and fracture (n=5506).

Genotyping

Polymerase chain reaction (PCR) using genomic DNA obtained from peripheral white blood cells was performed using oligonucleotide primers designed to amplify the polymorphic $(CA)_n$ repeat, located 1 kb upstream of the human *IGF-I* gene. Specific genotyping methods and quality control procedures have been described earlier (28). From sequence analysis it is known that the allele with length *192-bp ("wild-type"* in our population) corresponds to 19 CA-repeats ($CA_{(19)}$) (22). Based on the relationship between the polymorphism and serum IGF-I levels, genotypes were assembled from two allele categories as first described by Vaessen *et al.* (27): the *192-bp* allele and *all-other* alleles pooled as "*non-192-bp*" alleles. Individuals were classified in three genotypes groups: homozygotes for the *192-bp* allele, heterozygotes for the *192-bp* allele and non-carriers of the allele (*non-192-bp* homozygotes).

Fracture Follow-up

Information on incident non-vertebral fractures was collected from baseline (1990-1993) until December 31st 2002, comprising an average follow-up period of 8.6 (SD 3.4) years. Fracture events were retrieved from computerized records of the general practitioners (GP) in the research area (covering 80% of the cohort). Research physicians regularly followed participant information in GP's records

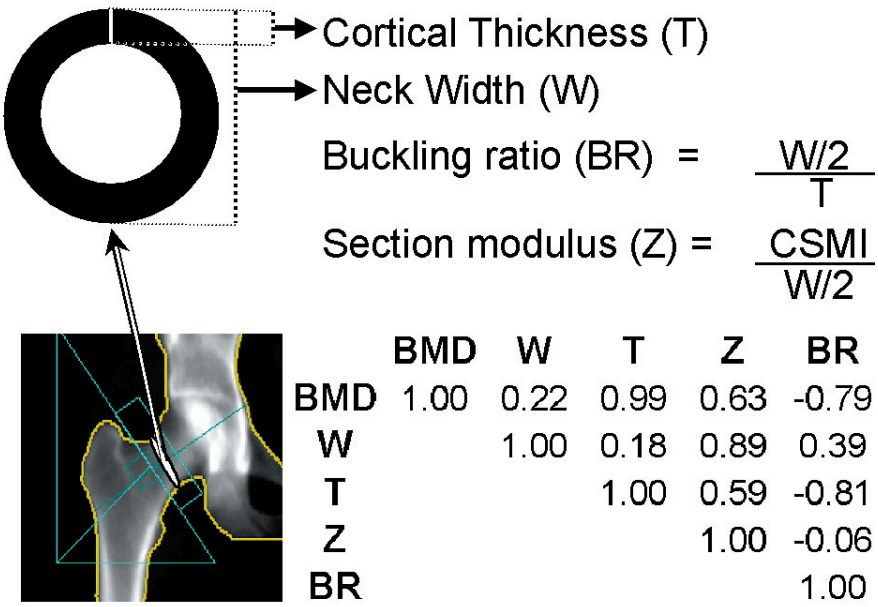
outside the research area and made an independent review and encoding of all reported events. Subsequently, a medical expert in the field reviewed all coded events for final classification. Additional information on hip fractures was gathered through the Dutch National Hospital Registration. We studied the relationship of the polymorphism to "all types" of fracture. Subsequently, we considered "all fragility" fractures including hip, pelvic, proximal humerus and wrist fractures. Further, since wrist fractures occur at a considerable younger age with specific falling patterns (2), we excluded them to study the group with fragility fractures occurring at old age (mean age > 75 years). We also independently analyzed groups with fractures occurring at the hip, pelvis, proximal humerus or wrist. Finally, we studied groups with "other" non-vertebral fractures including fractures of the rib (n=59), sternum (n=9), hand (n=120), lower leg (n=63), ankle (n=55), metatarsus (n=55) and foot (n=58). Vertebral fractures were not considered in this study.

Measurements

Between 1990 and 1993, participants were invited to come to the research centre for a baseline clinical examination. Height (cm) and weight (kg) were measured in standing position wearing indoor clothes and without shoes. Body mass index or BMI (kg/m^2), was calculated as weight divided by the square of height. Bone mineral density measurements (g/cm^2) of the proximal femur were performed by dual energy X-ray absorptiometry (DXA) using a Lunar DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA). Methods, quality assurance, accuracy and precision issues of the DXA measurements have been described previously (30).

Bone geometry computational method

Parameters of structural geometry of the hip were calculated indirectly from conventional DXA outputs obtained from the femoral neck region of interest (ROI) and are illustrated in Figure 1. Geometry parameters can be estimated from the BMD and the bone outer diameter using models of the cross-section represented by the ROI (31). The average diameter of the femoral neck was obtained by dividing the bone area by the width of the region of interest (usually 1.5 cm). To assess the reliability of this approximation of bone geometry, we compared the femoral neck diameter obtained from this calculation with that measured with the DXA ruler tool as recently described by Duan *et al.* (32), in 30 scans randomly selected from all 5674 available DXA scans. Femoral neck diameter approximated from bone area had a single measured percent coefficient of variation of 10.6 (n=30) and 10.8 (n=5674), as compared to 9.6 obtained when measured directly (n=30) with the DXA ruler tool.



	BMD	W	T	Z	BR
BMD	1.00	0.22	0.99	0.63	-0.79
W		1.00	0.18	0.89	0.39
T			1.00	0.59	-0.81
Z				1.00	-0.06
BR					1.00

FIGURE 1. Graphical correspondence and correlations of the hip bone geometry parameters. All Pearson's correlations are significant ($p<0.00001$). CSMI=cross-sectional moment of inertia

We used BMD and femoral neck diameter to approximate femoral neck geometry parameters that are conventionally extracted directly from the mass distribution using hip structural analysis (33). The mathematical calculation and the underlying assumptions regarding the geometry and structure of bone have been reported earlier (32,34,35). In summary, the approximation method assumes that the bone within the femoral neck region has the configuration of a uniform right circular cylinder (tube), with a proportion of cortical mass of 60% and an effective density of hydroxyapatite in fully mineralized bone tissue of ~1.05 g/cm^3(31). As shown in Figure 1, since the specific parameters considered in the analysis are obtained from a computational algorithm, by definition they are not independent from each other. In addition to BMD, we considered the *neck width* (cm) and the estimated *cortical thickness* (mm), the *section modulus* (cm^3) an index of bending strength related to the radial distribution of the bone mass (computed as the ratio of the cross-sectional moment of inertia CSMI to the outer radius of the cross-section), and the *cortical buckling ratio* (BR) an index of bone geometrical instability (computed as the ratio of the radius of the cross-section to the cortical thickness). In engineering this instability threshold is reached when buckling ratios exceed a factor of ~10 (36). Both geometry estimates and genotypes were obtained in a total of 5506 individuals.

Statistical Analysis

Genotype and allele frequencies of the *IGF-I* promoter polymorphism were determined, and the genotype frequencies were tested for Hardy-Weinberg equilibrium proportions using the GENEPOP-package (37).

In both phases we stratified the analyses by gender. For the analysis of fracture follow-up data we estimated incident rates overall and by genotype. For the time-to-event or fracture-free survival analysis we specified age (in years) as the underlying time variable instead of follow-up time to analyze the effect of chronological age on fracture. To do this we took into account delayed entry (left truncation) by using the counting process notation of S-PLUS V.6.0. We estimated cumulative probabilities of fracture events after age 55 years using the Kaplan-Meier method. Crude and adjusted hazard ratios were estimated using COX proportional-hazards models. The assumption of proportionality of hazards was verified for all covariates. Interactions between genotype and covariates were examined. In addition, the risk of fracture was analyzed in BMI strata defined by tertiles and by the following BMI definition: low (or underweight with BMI less than 20 kg/m2), normal (with BMI between 20 and 25 kg/m2) and high (or overweight including obesity with BMI greater than 25 kg/m2). For the analysis of hip bone geometry parameters, multiple linear regression was used to compare adjusted means between individuals with and without hip fracture, and to model the relation of genotype with bone geometry and anthropometric variables. Bone geometry parameters were also studied through cross-sectional strata across age decades starting at age 55 years and pooling together all individuals older than age 75 years to preserve sufficient power for the analysis. The influence of BMI on the relation was analyzed in strata as described previously for the risk of fracture. Adjustments were done for age, weight, and height. Trend analysis assuming an underlying additive genetic model (38) was done for the presence of zero, one or two copies of the associated allele, incorporating the genotype variable as a continuous term in a multiple linear regression model. Finally, model assumptions were verified and model residuals were checked for goodness-of-fit. If not stated otherwise, analyses were performed using the SPSS-package V.11 (SPSS Inc. Chicago, IL).

RESULTS

Genotype frequencies of women and men in the bone geometry study (n=5506) were very similar to those observed in all the 7012 genotyped individuals of the Rotterdam Study (44% in homozygotes, 44% in heterozygotes and 12% in non-carriers of the *192-bp* allele) and were in Hardy-Weinberg equilibrium (HWE) proportions.

TABLE 1 CHARACTERISTICS OF THE STUDY POPULATIONS

	Rotterdam Study Reference			IGF-I genotype analysis	
	TOTAL	**MALES**	**FEMALES**	**MALES**	**FEMALES**
MEASUREMENTS	n = 7983	n = 3105	n = 4878	n = 2800	n = 4212
Age (years)*	71.3 (10.1)	69.4 (8.8)	72.5 (10.6)	68.3 (8.3)	70.5 (9.7)
Height (cms)*	166.6 (9.5)	174.6 (6.8)	161.1 (6.7)	174.7 (6.8)	161.2 (6.7)
Weight (kgs)*	72.9 (12.0)	78.2 (10.8)	69.3 (11.4)	78.3 (10.7)	69.4 (11.3)
BMI (kg/m^2)*	26.3 (3.7)	25.6 (3.0)	26.7 (4.1)	25.7 (3.0)	26.7 (4.1)
Years since menopause*	-	-	21.2 (11.0)	-	20.6 (12.4)
Use of HRT or bone active agents (%)	-	-	2.0	-	2.3
BMD Femoral Neck (g/cm^2)*	0.84 (0.14)	0.88 (0.13)	0.81 (0.13)	0.88 (0.13)	0.81 (0.13)
Hip fracture**	5.7 (369)	3.2 (78)	7.3 (291)	2.8 (65)	6.8 (245)
Pelvic fracture**	1.0 (65)	0.4 (10)	1.4 (55)	0.4 (10)	1.2 (44)
Proximal humerus fracture**	2.3 (149)	1.0 (24)	3.1 (125)	1.1 (24)	3.2 (114)
Wrist fracture**	5.4 (346)	1.3 (33)	7.8 (313)	1.4 (33)	7.8 (281)
Other fracture**	7.5 (481)	5.0 (122)	9.0 (359)	5.0 (114)	9.1 (328)

* Data are unadjusted means (SD)

** Data are incident rates per 1000 person years (Number of fractures)

Anthropometric measurements based on 6917 individuals and BMD measurements based on 5823 individuals

Table 1 compares baseline characteristics and fracture incident rates observed during the follow-up period in women and men included in the *IGF-I* genotype analysis (n=7012), to those observed in the reference population (n=7983). On average, individuals included in the *IGF-I* genotype analyses are approximately two years younger. All other characteristics did not show significant differences, including BMD levels and use of concurrent estrogen replacement therapy (HRT) or bone active agents in women. *IGF-I* genotypes were available for more than 92% and 88% of men and women with fracture. Fracture incident rates in individuals with genotypes are not significantly different from those observed in the reference population.

IGF-I Genotype and Fracture Risk

The influence of genotype on fracture rates is shown in Table 2. In women, non-carriers of the *192-bp* allele have approximately 1.2 times increased risk of "all-type" and "fragility fracture" as compared to homozygotes for the *192-bp* allele. When pooling hip, pelvic and proximal humerus fractures together as "fragility fractures occurring at older age", non-carrier women have 1.5 times increased risk as compared to homozygous for the *192-bp* allele. In Figure 2 the fracture-free survival analysis shows that this higher proportion of non-carrier women with "fragility fracture at older age" is consistent through age with a significant allele-dose effect (p=0.007).

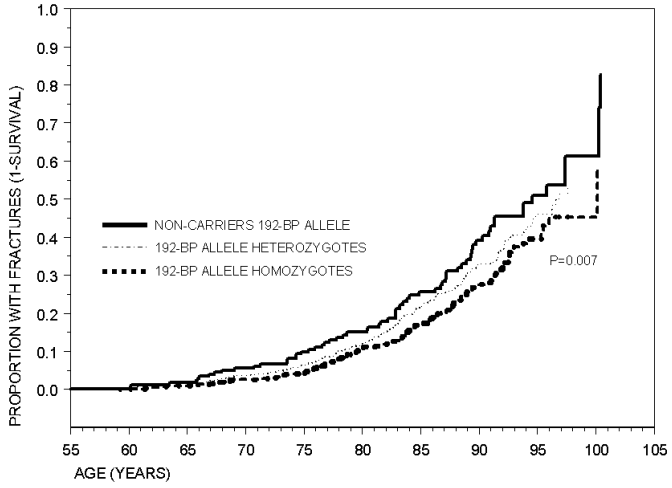


FIGURE 2. Proportion of fragility (hip, pelvis and proximal humerus) fractures with age according to *IGF-I* promoter genotypes in women

TABLE 2A. FRACTURE RATES AND FRACTURE-FREE SURVIVAL BY IGF-I PROMOTER GENOTYPES IN FEMALES

FEMALES

	192-bp ALLELE						Cox analysis of *fracture-free* survival				
	Homozygotes		*Heterozygotes*		*Non-carriers*		192 bp allele heterozygotes vs homozygotes		192 bp allele non-carriers vs homozygotes		Trend
	Subjects	Person-years	Subjects	Person-years	Subjects	Person-years					
	1840	14923	1858	14989	514	4131					
Type of fracture	Cases	Fracture rate	Cases	Fracture rate	Cases	Fracture rate	HR	95%CI	HR	95%CI	*p*
All types	389	0.0261	421	0.0281	128	0.0310	1.1	[0.9-1.2]	1.2	[1.0-1.4]	0.13
All fragility	260	0.0174	287	0.0191	90	0.0218	1.1	[0.9-1.3]	1.2	[1.0-1.6]	0.10
Fragility at older age	143	0.0096	179	0.0119	59	0.0143	1.2	[1.0-1.5]	1.5	[1.1-2.0]	0.01
Hip	98	0.0066	102	0.0068	35	0.0085	1.0	[0.8-1.3]	1.2	[0.8-1.8]	0.42
Pelvis	17	0.0011	21	0.0014	6	0.0015	1.2	[0.6-2.3]	1.3	[0.5-3.2]	0.54
Proximal Humerus	29	0.0019	57	0.0038	21	0.0051	1.9	[1.2-3.0]	2.7	[1.5-4.7]	0.0002
Wrist	120	0.0080	111	0.0074	34	0.0082	0.9	[0.7-1.2]	1.0	[0.7-1.5]	0.83
Other	139	0.0093	139	0.0093	39	0.0094	1.0	[0.8-1.2]	1.0	[0.7-1.4]	0.94

TABLE 2B. FRACTURE RATES AND FRACTURE-FREE SURVIVAL BY IGF-I PROMOTER GENOTYPES IN MALES

MALES

	192-bp ALLELE						Cox analysis of *fracture-free* survival				
	Homozygotes		*Heterozygotes*		*Non-carriers*		192 bp allele heterozygotes vs homozygotes		192 bp allele non-carriers vs homozygotes		Trend
	Subjects	Person-years	Subjects	Person-years	Subjects	Person-years					
	1228	9527	1248	9488	324	2448					
Type of fracture	Cases	Fracture rate	Cases	Fracture rate	Cases	Fracture rate	HR	95%CI	HR	95%CI	*p*
All types	100	0.0105	117	0.0123	23	0.0094	1.2	[0.9-1.5]	0.9	[0.6-1.4]	0.88
All fragility	57	0.0060	59	0.0062	16	0.0065	1.1	[0.7-1.5]	1.1	[0.6-1.9]	0.91
Fragility at older age	48	0.0050	37	0.0039	14	0.0057	0.8	[0.5-1.2]	1.1	[0.6-2.1]	0.87
Hip	31	0.0033	22	0.0023	12	0.0049	0.7	[0.4-1.3]	1.5	[0.8-2.9]	0.63
Pelvis	6	0.0006	3	0.0003	1	0.0004	0.5	[0.1-2.1]	0.6	[0.1-5.4]	0.44
Proximal Humerus	11	0.0012	12	0.0013	1	0.0004	1.1	[0.5-2.5]	0.4	[0.0-2.7]	0.54
Wrist	9	0.0009	22	0.0023	2	0.0008	2.5	[1.2-5.4]	0.8	[0.2-3.9]	0.33
Other	43	0.0045	64	0.0067	8	0.0033	1.3	[0.9-2.0]	0.6	[0.3-1.4]	0.91

* IGF-I genotype hazard ratios (HR) have homozygotes for the *192-bp* allele as reference group.
Adjustment for BMD, height or weight did not essentially modify the estimates.

In these three types of fracture (hip, pelvis and proximal humerus), non-carriers tend to have higher incidence rates than their heterozygous and homozygous counterparts, with a significant allele dose effect at the proximal humerus (Table 2A). In men, there were no significant difference in fracture rates among *IGF-I* genotypes, as shown in Figure 3 for fragility fractures at older age, though the genotype coefficient was not significantly different to that observed in women (p=0.80). In both genders, no consistent fracture rate differences were observed for wrist and other-type of fractures. Whether or not high impact fractures (i.e., skull fractures n=40) were included in the latter group did not modify the risk estimates. Adjustment for BMD, height and weight, BMI or hip geometry parameters did not essentially change the risk estimates (i.e. in women the risk of fragility fractures at older age was 1.45 (95%CI 0.9, 2.1) and 1.30 (95%CI 0.9, 1.7) for non-carriers and heterozygotes as compared to homozygotes for the *192-bp* allele, respectively). When evaluating interactions between the *IGF-I* genotype and BMI in women (Table 3), the allele-dose effect for the risk of hip and fragility fracture at older age was accentuated in the lower tertile and "low" BMI strata. The interaction between "low" BMI (less than 20 kg/m2) and the non-carrier genotype was significant both for hip (p=0.04) and fragility (p=0.05) fractures. This means that women with low BMI and who are non-carriers have an increased risk of hip and fragility fracture at older age, which is greater than that observed by the sum of having very low BMI or being non-carrier alone. In this BMI stratum (which represents only ~5% of the women), as compared to homozygote carriers, women who don't carry any *192-bp*

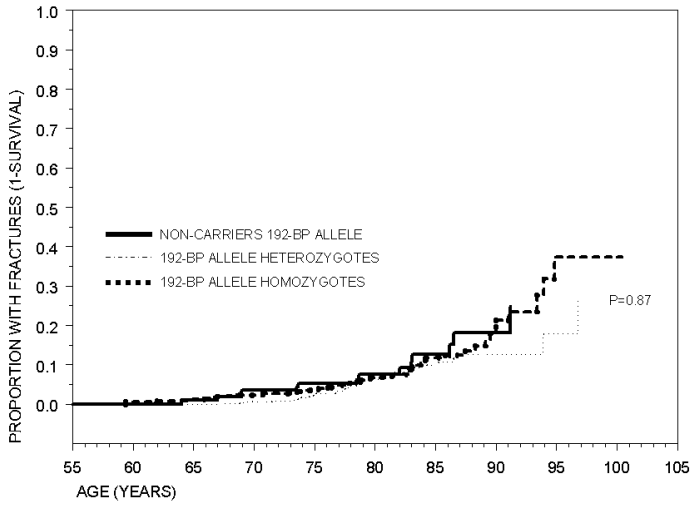


FIGURE 3. Proportion of fragility (hip, pelvis and proximal humerus) fractures with age according to *IGF-I* promoter genotypes in men

TABLE 3. RISK OF HIP AND FRAGILITY FRACTURE BY IGF-I PROMOTER GENOTYPE IN DIFFERENT BMI STRATA IN WOMEN

	1st tertile <24.8 kg/m² n=1677		**2nd tertile 24.8 to 28.1 kg/m² n=1580**		**3rd tertile >28.1 kg/m² n=1600**		**Low <20.0 kg/m² n=136**		**Normal 20.0 to 25.0 kg/m² n=1668**		**High >25.0 kg/m² n=3053**	
					HIP FRACTURE							
Genotype 192-bp allele	**HR**	**95%CI**	**HR**	**95%CI**	**HR**	**95%CI**	**HR**	**95%CI**	**HR**	**95%CI**	**HR**	**95%CI**
Non-carriers	1.5	[0.8-2.8]	1.4	[0.7-2.6]	1.1	[0.4-2.6]	5.9	[1.1-32.7]	1.1	[0.6-2.1]	1.4	[0.9-1.9]
Heterozygotes	1.0	[0.7-1.7]	0.7	[0.4-1.3]	1.3	[0.8-2.3]	2.0	[0.4-11.0]	0.9	[0.6-1.4]	1.1	[0.5-3.2]
Homozygotes	1.0	[Reference]	1.0	[Reference]	1.0	[Reference]	1.0	[Reference]	1.0	[Reference]	1.0	[Reference]
Trend	*p*=0.25		*p*=0.65		*p*=0.59		*p*=0.04		*p*=0.95		*p*=0.31	
					FRAGILITY FRACTURE							
Genotype 192-bp allele	**HR**	**95%CI**	**HR**	**95%CI**	**HR**	**95%CI**	**HR**	**95%CI**	**HR**	**95%CI**	**HR**	**95%CI**
Non-carriers	1.7	[1.0-2.7]	1.4	[0.8-2.4]	1.4	[0.7-2.6]	4.5	[1.3-16.2]	1.3	[0.8-2.1]	1.5	[0.9-1.9]
Heterozygotes	1.3	[0.9-1.8]	1.0	[0.7-1.5]	1.6	[1.0-2.3]	1.0	[0.2-4.0]	1.2	[0.8-1.7]	1.3	[0.5-3.2]
Homozygotes	1.0	[Reference]	1.0	[Reference]	1.0	[Reference]	1.0	[Reference]	1.0	[Reference]	1.0	[Reference]
Trend	*p*=0.04		*p*=0.35		*p*=0.10		*p*=0.03		*p*=0.32		*p*=0.03	

allele have 5.9 (95%CI 1.1-32.7) and 4.5 (95%CI 1.3-16.2) times greater increased risk of hip and fragility (at older age) fracture, respectively (table 3). In men, the evaluation of such interactions was limited by the small number of fractures.

Hip Bone Geometry and Fracture Risk

When hip bone geometry was examined in relation to the occurrence of hip fracture (Table 4), in both genders, individuals with hip fracture had on average approximately 8 % significantly lower BMD levels ($p<0.00001$), 6% lower section moduli ($p=0.003$), 9% thinner cortices ($p<0.00001$), and 10% higher buckling ratios ($p<0.00001$) as compared to non-fractured individuals. No differences were observed in neck width. When analyzing the overall effect of age on bone geometry (data not shown), there was no apparent change in femoral neck width across age strata, while there was a decrease in section modulus ($p<0.00001$), a decrease in cortical thickness ($p<0.00001$) and an increase in cortical buckling ratio ($p<0.00001$).

Hip Bone Geometry by IGF-I Genotype

Anthropometric and bone geometry parameters were then evaluated across *IGF-I* promoter genotypes (Table 5). Men who do not carry the *192-bp* allele had significantly shorter femoral neck widths and lower section moduli with evidence for an allele dose effect. Non-carrier women had significant lower height, BMD and cortical thickness, and increased buckling ratios, also with evidence for an allele dose effect. In both genders, section modulus was 2-3% lower in non-carriers as compared to homozygotes for the *192-bp* allele, with mean differences that were borderline significant ($p<0.10$).

Bone geometry estimates across age strata and by *IGF-I* genotype are presented for men and women in Table 6. In men, the allele dose effects observed for femoral neck width and section modulus are driven by the group older than 75 years. In women, differences in cortical thickness and buckling ratio are suggested after age 65 years, but significant trends are present only in the group older than 75 years.

When evaluating bone geometry parameters in relation to different BMI levels in women, a substantial genotype effect on bone strength was observed. Genotype differences in section modulus decreased with increasing BMI (data not shown) and are accentuated in the "low" BMI stratum (p-trend=0.0003). In this stratum ($n=67$), non-carriers and heterozygotes for the *192-bp* allele had 30.7% ($p=0.002$) and 20.0% ($p=0.02$) lower section modulus as compared to homozygotes for the *192-bp* allele (mean$\pm$SEM = 0.93$\pm$0.05). Although not significant, a similar pattern was observed in men (data not shown).

TABLE 4. HIP GEOMETRY IN RELATION TO HIP FRACTURE

	MALES				FEMALES			
	HIP FRACTURE				HIP FRACTURE			
	Absent	Present			Absent	Present		
	n=2323	n=49	*Difference*	*p*-value	n=2999	n=135	*Difference*	*p*-value
BMD (g/cm^2)	0.88 ± 0.003	0.80 ± 0.018	↓ 8.7%	<0.0001	0.81 ± 0.002	0.75 ± 0.010	↓ 7.5%	<0.00001
Femoral neck width (cm)	3.03 ± 0.005	3.06 ± 0.031	↑ 0.8%	0.42	2.69 ± 0.004	2.69 ± 0.020	↓ 0.0%	0.98
Section modulus (cm^3)	1.35 ± 0.005	1.26 ± 0.036	↓ 6.1%	0.03	0.98 ± 0.004	0.92 ± 0.019	↓ 6.0%	<0.01
Cortical thickness (mm)	0.17 ± 0.001	0.15 ± 2.689	↓ 9.3%	<0.0001	0.16 ± 0.001	0.15 ± 0.002	↓ 8.0%	<0.00001
Cortical buckling ratio	9.17 ± 0.033	10.16 ± 2.689	↑ 10.8%	<0.0001	8.76 ± 0.023	9.63 ± 0.132	↑ 9.9%	<0.00001

Estimated means are adjusted for age, height and weight

TABLE 5. CHARACTERISTICS OF THE STUDY POPULATION BY IGF-I PROMOTER GENOTYPES

	MALES				FEMALES			
	192-bp allele				*192-bp allele*			
MEASUREMENTS	**Homozygotes** n =1041	**Heterozygotes** n = 1049	**Non-carriers** n = 282	**p-trend**	**Homozygotes** n = 1355	**Heterozygotes** n = 1392	**Non-carriers** n = 387	**p-trend**
Age (years)	67.6 ± 0.2	67.2 ± 0.2	68.1 ± 0.5	0.83	68.3 ± 0.2	68.3 ± 0.2	68.1 ± 0.4	0.78
Height (cms)*	174.8 ± 0.2	174.9 ± 0.2	174.9 ± 0.4	0.71	162.1 ± 0.2	161.6 ± 0.2	161.2 ± 0.3	0.005
Weight (kgs)*	79.0 ± 0.3	78.8 ± 0.3	78.0 ± 0.6	0.20	70.2 ± 0.3	70.0 ± 0.3	69.7 ± 0.6	0.43
BMI (kg/m^2)*	25.8 ± 0.1	25.7 ± 0.1	25.4 ± 0.2	0.07	26.7 ± 0.1	26.8 ± 0.1	26.8 ± 0.2	0.63
Age at menopause* (years)	-	-	-	-	47.7 ± 0.2	47.4 ± 0.2	47.7 ± 0.5	0.63
BMD Femoral Neck (g/cm^2)**	0.88 ± 0.004	0.88 ± 0.004	0.88 ± 0.007	0.70	0.82 ± 0.003	0.81 ± 0.003	0.80 ± 0.006	0.03
Neck width (cm)**	3.04 ± 0.007	3.03 ± 0.007	3.01 ± 0.013	0.03	2.68 ± 0.006	2.70 ± 0.006	2.67 ± 0.011	0.97
Section modulus (cm^3)**	1.36 ± 0.008	1.34 ± 0.008	1.32 ± 0.015	0.04	0.97 ± 0.006	0.98 ± 0.006	0.95 ± 0.011	0.30
Cortical Thickness (mm)**	16.9 ± 0.079	16.9 ± 0.079	16.9 ± 0.153	0.87	15.8 ± 0.065	15.7 ± 0.064	15.5 ± 0.122	0.03
Buckling ratio**	9.2 ± 0.049	9.2 ± 0.049	9.1 ± 0.094	0.55	8.7 ± 0.041	8.9 ± 0.040	8.9 ± 0.077	0.03

Data are MEAN ± SEM * Adjusted for age ** Adjusted for age, height and weight

TABLE 6. HIP GEOMETRY PARAMETERS BY IGF-I PROMOTOR GENOTYPES AND AGE STRATA

	MALES				FEMALES			
	192-bp ALLELE				*192-bp* ALLELE			
	Homozygotes n=1041	**Heterozygotes** n=1049	**Non-carriers** n=282	**Trend**	**Homozygotes** n=1355	**Heterozygotes** n=1392	**Non-carriers** n=387	**Trend**
Femoral neck width (cm)								
Age 55-64.9 years	3.04 ± 0.011	3.04 ± 0.010	3.02 ± 0.021	0.57	2.66 ± 0.011	2.66 ± 0.011	2.69 ± 0.021	0.30
Age 65-74.9 years	3.04 ± 0.011	3.03 ± 0.011	3.03 ± 0.021	0.54	2.65 ± 0.012	2.68 ± 0.012	2.63 ± 0.022	0.98
Age > 75 years	3.07 ± 0.018	3.01 ± 0.019	2.98 ± 0.032	0.007	2.67 ± 0.017	2.66 ± 0.016	2.68 ± 0.033	0.96
Section modulus (cm^3)								
Age 55-64.9 years	1.37 ± 0.012	1.38 ± 0.011	1.35 ± 0.023	0.53	0.98 ± 0.010	0.99 ± 0.010	1.01 ± 0.018	0.20
Age 65-74.9 years	1.34 ± 0.013	1.33 ± 0.013	1.32 ± 0.025	0.39	0.95 ± 0.010	0.96 ± 0.010	0.91 ± 0.019	0.40
Age > 75 years	1.35 ± 0.024	1.27 ± 0.025	1.28 ± 0.042	0.04	0.92 ± 0.014	0.90 ± 0.013	0.91 ± 0.027	0.39
Cortical thickness (mm)								
Age 55-64.9 years	1.73 ± 0.012	1.75 ± 0.011	1.74 ± 0.023	0.47	1.64 ± 0.010	1.64 ± 0.010	1.64 ± 0.019	0.94
Age 65-74.9 years	1.67 ± 0.013	1.67 ± 0.013	1.65 ± 0.026	0.50	1.57 ± 0.011	1.55 ± 0.011	1.54 ± 0.020	0.07
Age > 75 years	1.64 ± 0.019	1.60 ± 0.020	1.66 ± 0.035	0.99	1.48 ± 0.013	1.45 ± 0.013	1.43 ± 0.026	0.04
Cortical buckling ratio								
Age 55-64.9 years	8.95 ± 0.073	8.87 ± 0.071	8.91 ± 0.143	0.61	8.30 ± 0.063	8.27 ± 0.062	8.40 ± 0.117	0.65
Age 65-74.9 years	9.31 ± 0.078	9.34 ± 0.078	9.36 ± 0.155	0.75	8.66 ± 0.070	8.87 ± 0.071	8.79 ± 0.130	0.12
Age > 75 years	9.62 ± 0.120	9.60 ± 0.126	9.24 ± 0.215	0.21	9.27 ± 0.095	9.42 ± 0.091	9.70 ± 0.189	0.04

Estimated means ± SEM are adjusted for age, height and weight
In males n=1002, 943 and 437 and in females n=1304, 1225 and 734 in the 55-64.9 years, 65-74.9 years and >75 years strata, respectively.

DISCUSSION

This population based study in elderly individuals showed that the absence of the wild-type (*192-bp*) allele in a $(CA)_n$ repeat polymorphism in the promoter region of the *IGF-I* gene is associated with increased risk of non-vertebral fracture in women, in particular with "fragility" fractures occurring at older age (hip, pelvis and proximal humerus). Although no evidence for an effect of the polymorphism on fracture risk was seen in men, an effect cannot be completely ruled out since risk effect sizes were not significantly different between genders. In addition, the *IGF-I* promoter polymorphism was associated with hip bone geometry in gender and age specific ways, but the genotype dependent differences in hip bone geometry did not fully explain the genotype dependent differences in fracture risk.

These results on fracture and bone geometry in women are in line with our previous findings where the absence of the *192-bp* allele was associated with lower BMD levels and greater rate of bone loss in women (28). In the present study female non-carriers had thinner (estimated) cortices and higher buckling ratios especially in women older than 75 years. This pattern is consistent with the observation that non-carriers were more likely to suffer fragility. Nevertheless, the *IGF-I* genotype-dependent fracture risk does not seem to be mediated by genotype-dependent differences in hip bone geometry, considering that adjustment for either baseline BMD or bone geometry parameters did not essentially modify the genotype effect on the risk of fragility fracture. This could suggest that other IGF-dependent differences extrinsic to bone (i.e., effects on lean mass, cushion adiposity, neurological fitness) may be involved in the complex pathway leading to fracture. Differences in hip bone geometry are observed in men older than 75 years. Non-carriers of the *192-bp* allele show wider femoral necks with higher section moduli, but consistent with the observation that buckling ratios were not higher in non-carriers, the genotype did not discriminate fragility fractures in men. This suggests that the presence of the *192-bp* allele appears to cause men to have comparative wider and (resulting) stronger necks, but with no increase in cortical thickness and no (resulting) increase in stability.

One important question deals with the mechanism for achieving these effects on bone geometry. Genetically determined differences in IGF-I levels could be an explanation. As shown previously, decreased IGF-I levels are associated with decreased BMD in both genders (16-18), with gender differences that could partially be explained by IGF-I mediated changes in sex steroid bioavailability (18,39,40). Similarly, it has been postulated that IGF-I levels could be involved in the sexual dimorphism observed for the changes in bone geometry with aging (41,42). In addition, we have reported earlier that this promoter polymorphism influences the age-related decline in IGF-I levels (43). Further, we have found that the presence

of the *192-bp* allele was associated with increased total IGF-I serum levels and with body height in a random sample of the Rotterdam Study (27). However, given the population-based approach of our association study, we cannot determine if the *192-bp* promoter polymorphism is directly involved in the regulation of *IGF-I* expression, or if it is in linkage disequilibrium with another variant in the *IGF-I* gene locus that affects *IGF-I* gene function. Therefore, the specific mechanisms underlying the functionality of the polymorphism merit further research.

Even though we observe that height in women increases with the presence of the *192-bp* allele as already reported (27), differences in bone strength and instability are not attributable to differential body size since the *IGF-I* genotype-dependent differences in bone geometry remained after adjustment for height (in addition to age and weight).

Our findings in women with low BMI (less than 20 kg/m2), where differences in fracture risk and bone strength (section modulus) across genotypes become more evident, suggest that differences in body weight could influence the IGF-I mediated adaptation of bone to mechanical stimuli. This so-called "mechanosensitivity" has been thought to play a role in the development of postmenopausal osteoporosis (44,45) and is thought to be genetically determined (46). Further, this relationship of decrease loading affecting IGF-I action on bone formation is in concordance with findings recently reported in mouse models where skeletal unloading was found to induce resistance to IGF-I on bone formation (47) by inhibiting the activation of the IGF-I signaling pathways (48). In addition, differential production and/or activity of IGF-I will be more evident in this extreme stratum of frail women with expected low IGF-I , estrogen levels and/or increased morbidity.

In a recent report (35), Ahlborg *et al.* found a relation between the extent of endocortical resorption and periosteal apposition in the distal radius of postmenopausal women using a crude approximation of bone geometry similar to the one we have used. Interestingly, their results suggest that (postmenopausal) estrogen deficiency removes a constraint on increasing periosteal apposition (i.e., bone expansion), a phenomenon which compensates for endocortical bone loss by maintaining the section modulus (41). It could be hypothesized that if IGF-I mediates bone expansion directly, differential IGF-I bioavailability across genotypes will influence bone instability and consequent fracture susceptibility in postmenopausal women. That is, after menopause, non-carriers of the *192-bp* allele will reach instability faster as a consequence of rapidly expanding diameter in bones with already thin cortices. However, the structural data do not appear to support this hypothesis because femoral neck width was not detectably greater in women without the *192-bp* allele, but this could be explained by measurement limitations as discussed below.

There are some limitations to our study. Although the approximation method for estimating femoral neck geometry has been used previously (32,35) it is crude and relies on several assumptions. Nevertheless, with the exception of neck width, our bone geometry estimations show expected patterns for sex, age and weight (41). We also show that differences in geometric parameters significantly discriminate fractured individuals. Except for neck width, our findings are consistent with previous reports using DXA (49), film radiography (50) and computed tomography (51) which show wider necks, thinner cortices, lower strength (section moduli or cross-sectional moments of inertia) and increased instability (buckling ratios). Yet, it is important that our findings on bone geometry using the approximation method are verified by more direct measurements of geometry either by Hip Structure Analysis (33) or by a high-resolution 3-D method.

Another limitation of our study is that the analysis of bone geometry was cross-sectional. Thus, the relationship is weakened by the fact that most fracture events occurred on average 8.2 years later than baseline geometry measurements. Further, age-specific effects on bone geometry parameters should be interpreted cautiously since this type of analysis is prone to age cohort effects. Our inability to detect subtle increases in femoral neck width through age strata (widely observed elsewhere (32,35,49,52,53)) suggests lack of precision. This was also indicated by our sub-study on 30 random scans, where we found the femoral neck width derived from bone area to be less precise than the ruler method. This lack of precision may also be the reason why we were unable to observe a relationship between femoral neck width and the occurrence of hip fracture. Similarly, a direct measurement of bone geometry within a longitudinal study design should allow a better assessment of how changes in bone geometry relate to fracture incidence.

A further limitation might be information and selection biases. We expect the bias in fracture follow-up information to be small since fracture incidence rates of our study are similar to those reported earlier in our population within shorter follow-up periods (54). Moreover, genotypes were absent in only about 10% of all fracture cases. With regard to the BMD and geometry analysis there could be selection bias since individuals were selected on the basis of having complete BMD measurements and *IGF-I* genotypes. However, as discussed earlier (28) the effect seems to be random, since *IGF-I* allele frequencies were similar to those observed in other Caucasian populations (55).

Considering than we have evaluated multiple endpoints in our study, multiple testing concerns could arise. Nevertheless a more stringent significance threshold ($p<0.01$) in that respect, would not affect the interpretation of our fracture analysis. An argument against considering our findings prone to a type I error is the fact that the direction of the effect seems invariably consistent, in that non-carriers of the

192-bp allele show unfavorable patterns of bone geometry as well as greater fracture risk as compared to homozygotes for the allele. As reported earlier for the *IGF-I* genotype effect on BMD (28) the relationship between the *IGF-I* genotype effect on bone geometry parameters is expected to be real, but modest as for most genetic effects in complex diseases (9).

In summary, this population-based study provides evidence to implicate genetically determined levels of (local or systemic) IGF-I in the complex pathway leading to fragility fracture. We found an *IGF-I* gene promoter polymorphism associated with increased risk of "fragility" fractures occurring at older age (hip, pelvis and proximal humerus). Non-carriers of the *192-bp* allele had 1.5 (95%CI 1.1-2.0) times increased risk of having this type of fragility fracture. In males, no association was observed with the risk of non-vertebral fracture. Hip bone geometry was associated to the polymorphism in gender-specific ways but does not explain the *IGF-I* genotype effect on fracture risk. Women non-carriers of the *192-bp* allele had femoral necks with thinner cortices and higher instability (buckling ratios). In men, femoral neck width and section modulus increased with the number of *192-bp* (wild-type) alleles in the genotype, but no apparent differences in bone instability were observed.

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Estrogen receptor β polymorphisms and BMD, bone geometry and the risk of osteoporotic fracture

5

ABSTRACT

Introduction: In this large population-based study we examined the role of polymorphisms in the *ESR2* gene in relation to changes in BMD, hip bone geometry and the occurrence of fractures.

Methods: Two polymorphisms of the *ERS2* gene were genotyped in 6417 Caucasian subjects of the Rotterdam Study, a population based prospective cohort of men and women aged 55 and over. One polymorphism located in the second intronic region, and the other in the 3'UTR. Four haplotypes were constructed from the two SNP's and results are presented for *haplotype 4*, the most frequent haplotype. BMD was obtained by DXA and bone geometry parameters were measured by Hip Structural Analysis (HSA) in 4418 individuals at baseline. For follow-up (mean 4.5 ± 0.6 years), 1765 individuals with complete BMD and geometry measurements were analyzed. Baseline parameters and change with follow-up were compared across *haplotype 4* genotypes, and adjusted for age, height and weight, using ANCOVA. The *ESR2* genotypes were analyzed in relation to incident non-vertebral fractures (mean folllow-up 7.1 ± 3.4 years) in 2588 men and 3829 women using Cox's regression, and in relation to prevalent and incident vertebral fractures in 1498 men and 1971 women usin (multiple) logistic regression.

Results: At baseline, homozygous female carriers of *haplotype 4* had significant wider femoral necks (p = 0.02), thinner cortices (p = 0.01), increased buckling ratios (p = 0.001) and lower vertebral area (p = 0.08) compared to non-carrier women. The follow-up analyses showed that women homozygous for *haplotype 4* had significantly lower rates of BMD loss per year (p-trend = 0.01). Changes in HSA measurements were consistent with changes in BMD, where female non-carriers of *haplotype 4* had higher rates of cortical thinning, increase in neck width (bone expansion) and buckling ratios (bone instability), and decrease in section modulus (bone strength) than *haplotype 4* homozygotes. In addition, women homozygous for *haplotype 4* had 1.4 (95% CI, 1.0-1.8, p = 0.08) increased risk of non-vertebral fragility fractures, and 1.8 (95 % CI, 1.2-2.6, p = 0.01) increased risk of all types of vertebral fractures, compared with non-carriers of *haplotype 4*. In contrast, male homozygous carriers of *haplotype 4* had reduced risk for "other types" (not hip, pelvis, proximal humerus or wrist) fractures (HR 0.5, (95% CI, 0.3-1.0), p = 0.04). Also in men, a small effect on increased mortality risk (HR 1.2, (95% CI, 1.0-1.4) was seen in homozygous carriers of *haplotype 4*.

Conclusion: Carriership of *haplotype 4* in the *ESR2* gene is associated with increased risk for both vertebral and fragility fracture in Caucasian postmenopausal women. In males, the decreased risk for "other types" of non-vertebral fracture (oposing effect) can possibly be explained by selection on male survival.

INTRODUCTION

Osteoporosis is characterized by low bone mass and structural deterioration of bone tissue, leading to increased bone fragility and susceptibility to fracture (1). A major predictor of this process is bone mineral density (BMD), which can be assessed by dual-energy X-ray absoptiometry (DXA) (2). Osteoporosis is considered to be a multifactorial disease with a strong genetic influence. Heritability estimates for BMD have been reported to be high, varying between 50 and 80% (3,4). Nevertheless, bone fracture risk will not only depend on BMD, but also on bone geometry and the risk of falling.

Estrogens (and their receptors) play an important role in bone metabolism throughout life (5). Not only are they involved in adolescent growth and bone acquisition in young adulthood, but also estrogen deficiency (as seen after menopause) leads to bone loss and a subsequent increase in bone fragility (6). Genes from the estrogen endocrine pathway such as the estrogen receptors are therefore potential candidate genes for osteoporosis. Although two forms of estrogen receptors exist (ERS1 with chromosome map location on 6q25 and ERS2 on 14q22-24), most studies have focused on ERS1. Even though some studies have shown in women associations between polymorphisms in the ERS1 gene with BMD, vertebral bone area, and fracture risk (7-11), others have not (12-15).

Until now, three association studies have been done on the *ESR2* gene in relation to femoral neck or lumbar spine BMD (16-18). One of these studies showed significant association in men with femoral neck BMD and a dinucleotide (CA) repeat polymorphism (D14S1026) (17). This study also showed an association with femoral neck BMD in men and two common SNP's, rs1256031 (with allelic frequencies of: C = 52.2%, T = 47.8%) and rs1256059 (with allelic frequencies of C = 54.8 %, T = 45.2 %), which are located in intron 2 and intron 6, respectively (Figure 1) (17). No associations were seen with lumbar spine BMD or in women. In contrast with these results, Scariano et al. (16) found significant associations in postmenopausal women with lumbar spine and femoral neck BMD and the dinucleotide (CA) repeat polymorphism. Accordingly, associations with BMD remain conflicting and the relationship with fractures has not yet been studied.

The aim of our study was to study genetic variants in the ERS2 gene suitable to examine in association with osteoporosis traits, including BMD, hip bone geometry and risk of fracture. We have done this in the Rotterdam Study, a large prospective population-based cohort study of diseases in elderly Caucasian men and women.

MATERIAL AND METHODS

Study Population

Subjects were participants of the Rotterdam Study, a large prospective population based cohort study of elderly men and women aged 55 years and over. The study was designed to investigate the occurrence of disease in the elderly in relation to several potential determinants. In summary, all 10275 inhabitants of Ommoord, a district in Rotterdam, the Netherlands, were invited to participate. Of those, 7983 participated, bringing the overall response rate to 78 percent. Both the rationale and the design of the study have been described previously (19). The Medical Ethics Committee of Erasmus University Medical School has approved the Rotterdam Study and participants provided written informed consent. Baseline assessment took place between 1990 and 1993, while first and second follow-up assessments were performed between July 1st 1993 and January 1st 1996, and between July 1st 1996 and January 1st 2000, respectively. Baseline and follow-up examinations, including a home interview and an extensive physical examination at the research center was performed

We performed the analysis in two phases. In the first phase we searched for single nucleotide polymorphisms (SNP's) in the *ESR2* gene and made a selection for the analysis. In the second phase, we determined haplotypes from the selected polymorphisms and analyzed them at baseline (n = 4418) and follow-up (n = 1765) in relation to BMD and bone geometry parameters. We also analyzed incident non-vertebral fractures (n = 1010 individuals with at least one fracture, and 5407 individuals without fracture) and prevalent vertebral fractures (n = 381 screened in 3469 individuals).

Genotyping

Genomic DNA was isolated from peripheral leucocytes by using salting out methodology (20). For *ESR2* we genotyped initially six SNP's scattered across the gene. Taqman allelic discrimination assay was used, while primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands). For details see http://store.appliedbiosystems.com. Reactions were performed on the Taqman Prism 7900HT 384-well format. We determined linkage disequilibrium (LD) among

these six SNP's using PHASE (v.2.0) software (University of Washington, Seattle, WA) (21,22). After selecting SNP's for the analysis, PHASE was also used to construct haplotype alleles.

Measurements

In each assessment period, height and weight were measured in standing position wearing indoor clothing without shoes. BMI was computed as weight in kilograms divided by height in meters squared (kg/m²). During the home interview female participants were asked to recall their age at menopause and responses were validated as described previously (23). Age was calculated from the date of birth and the date of the DXA-scan.

BMD (g/cm²) of the femoral neck was measured by dual energy X-ray absorptiometry (DXA, Lunar DPX-L densitometer, Madison, WI, USA) as described previously (24). Due to different software updates during the follow-up period all available scans were reanalyzed using the Lunar DPX-IQ V.4.4. software in order to allow comparison between longitudinal BMD measurements (25). Follow-up measurements were done using identical procedures. The first follow-up started after approximately 2 years (mean: 23.8 months; SD: 7.0), the second after approximately 6.5 years (mean: 78.1 months; SD: 4.5). The rate of change in BMD was calculated as the difference between baseline and second follow-up (approximately 6.5 years) divided by the time elapsed between the measurements. The rate of change in bone geometry parameters was calculated as the difference between first and second follow-up divided by the time elapsed between the measurements. The relative change from baseline (or first follow-up) was estimated as the difference in BMD (or bone geometry parameters) between assessment periods divided by the BMD at baseline (or bone geometry parameters at 1st follow-up).

Hip Structural Analysis (HSA)

We have used the hip structural analysis (HSA) software developed by Thomas J. Beck (Johns Hopkins University, Baltimore, Maryland) to measure hip bone geometry from the DXA scans (26,27). The HSA program differs from the conventional density analyses in that cross-sectional dimensions are derived mass or density. The program uses the distribution of mineral mass in a line of pixels across the bone axis to measure geometric properties for cross-sections in cut planes traversing the bone at that location. Current versions average measurements for a series of 5 parallel mass profiles spaced ~1 mm apart along the bone axis. Analysis location included the narrow-neck (NN) region across the narrowest point of the femoral neck. BMD, bone cross-sectional area (CSA), bone width (outer diameter) and cross-sectional moment of inertia (CSMI) were measured directly from mineral mass distributions using algorithms described previously (26). In addition, estimates of cortical thickness are obtained with simple models of the cross-sections, which employ measured dimensions and assumptions of cross-section shape. The NN region is modeled as a circular annulus, assuming a proportion

of cortical/trabecular bone of 60/40 percent. For the current work, calculations of section moduli (Z), an index of bending strength, and buckling ratios (BR), an index of bone instability, were slightly modified from those of some early reports to account for shifts in the center-of-mass. Z was calculated as CSMI /d_s, where d_s is the maximum distance from the center of mass to the medial or lateral surface. Buckling ratios were computed as d_s divided by estimated mean cortical thickness. In earlier work ½ the outer diameter was used instead of d_s in calculations of Z and BR (28). As described previously in the measurements section, software updates, which altered scan image acquisition, made follow-up data from the HSA not comparable to baseline measurements. This is the reason why changes in geometry parameters over time were only analyzed between first and second follow-up.

Non-vertebral Fracture Follow-up

The non-vertebral fracture analysis is based on follow-up data collected from baseline (1990-1993) until December 31, 2001, comprising a follow-up period of 7.1 SD 3.4 years. Fracture events were reported either by general practitioners (GP's) in the research area by means of a computerized system (covering 80% of the cohort) or through hospital records. Research physicians regularly checked participant information in GP's records outside the research area and independently reviewed and coded the information. Subsequently, for final classification, a medical expert reviewed all coded events. Additional information on hip fractures was gathered through the Dutch National Hospital Registration. We studied the relationship of the polymorphism to "all types" of non-vertebral fracture. Subsequently, we considered "fragility" fractures, including hip, pelvic and proximal humerus fractures. We also independently analyzed groups with fractures occurring at the hip, pelvis, proximal humerus, or wrist. Finally, we studied groups with "other" non-vertebral fractures including fractures of the rib (n=53), sternum (n=8), hand (n=102), lower leg (n=57), ankle (n=53), metatarsus (n=51), and foot (n=53).

Vertebral Fracture Assessment

Both at baseline and at follow-up visit, between 1997 and 1999, thoracolumbar radiographs of the spine were obtained. The follow-up radiographs were available for 3469 individuals, who survived on average 9.7 years after baseline center visit and who were still able to come to our research center. All follow-up radiographs were scored for presence of vertebral fracture using the McCloskey/ Kanis method, as described previously (29,30). If a vertebral fracture was detected, the baseline radiograph was evaluated as well. If the vertebral fracture was already present at baseline, it was considered a baseline prevalent fracture. If it was not present at baseline, the fracture was defined to be incident.

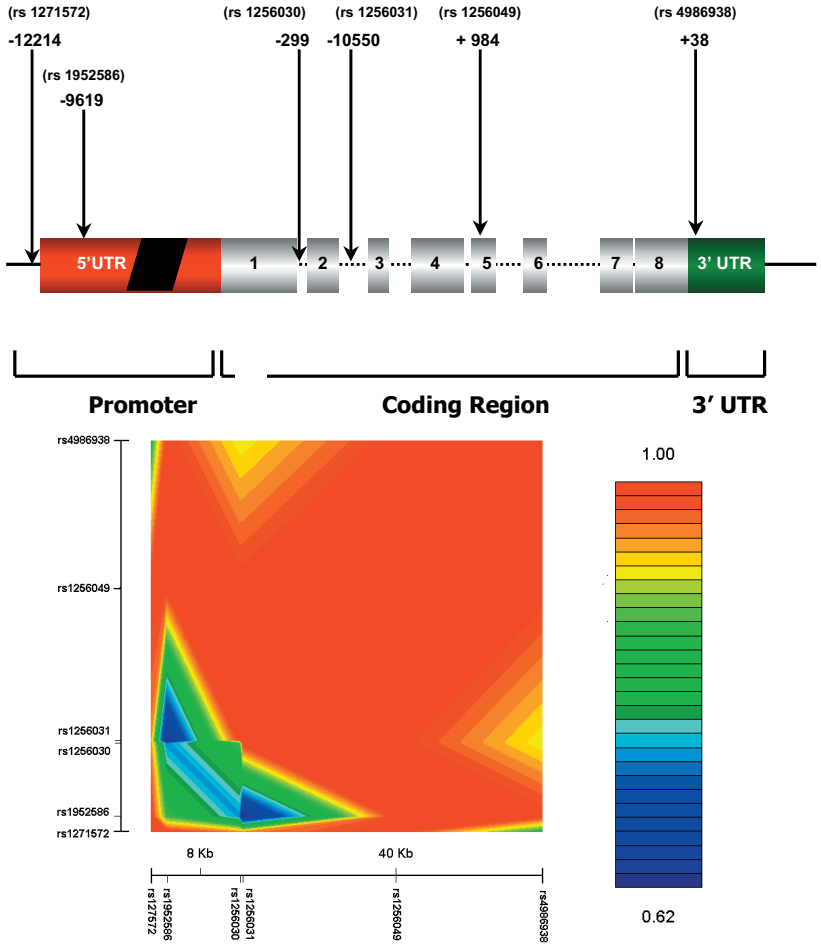
Statistical Analysis

All analyses were performed for men and women separately. Genotype and haplotype frequencies were tested for Hardy-Weinberg equilibrium proportions using the GENEPOP-package (31).

For haplotypes constructed from the intron 2 and the ’3UTR polymorphisms, means and standard deviations were computed for all measurements and compared with those of the same gender in the complete Rotterdam Study using t-tests. To study the relation with bone geometry parameters and aging, three 10 year strata were defined for age at baseline, starting at age 55 years and pooling together all individuals older than 75 years (n = 816) to maintain sufficient power for analysis.

Multiple linear regression was used to model the relation of haplotype carrier status with BMD and bone geometry parameters, adjusted for age, height and weight at baseline and in the follow-up analysis to test the relation with rate of change in BMD or geometry parameter, adjusted for age, height and weight at baseline or at first follow-up. We analyzed the rate of change in mean BMD by *haplotype 4* carrier ship between baseline and second follow-up and we measured geometry parameters in the period between the first follow-up and the second follow-up. Trend analysis assuming an underlying additive genetic model (32) was done for the presence of zero, one or two copies of the associated haplotype, incorporating the haplotype variable as a continuous term in a multiple linear regression model. Finally, model assumptions were verified and model residuals were checked for goodness-of-fit.

For the analysis of mortality and incident non-vertebral fractures, we estimated incident rates overall and by haplotype carrier status. For the time-to-event or fracture-free survival analysis, we specified age (in years) as the underlying time variable instead of follow-up time to analyze the effect of chronological age on fracture. To do this we took into account delayed entry (left truncation) by using the counting process notation of S-PLUS V.6.0. We estimated cumulative probabilities of fracture events after age 55 years using the Kaplan-Meier method. Crude and adjusted hazard ratios were estimated using COX proportional-hazards models. The assumption of proportionality of hazards was verified for all covariates. Interactions between haplotypes and covariates were examined. To estimate the risk of vertebral fractures, odds ratios with 95 % confidence intervals (95% CI) were calculated using (multiple) logistic regression models. We performed all vertebral fracture analysis separately for both prevalent and incident fractures and combined for prevalent and incident vertebral fractures, called “all types” of vertebral fractures. Significance of p-values was set at the 0.05 level. If not stated otherwise, SPSS 11.0 (SPSS, Chicago, IL, USA) was used for the statistical analyses.



Standardized Linkage Disequilibrium coefficients D' between the studied ESR2 polymorphisms

	rs 1271572	rs 1952586	rs 1256030	rs1256031	rs1256049	rs 4986938
rs 1271572	-	1.000	0.956	0.955	1.000	0.854
rs 1952586	1.000	-	0.742	0.618	1.000	1.000
rs 1256030	0.956	0.742	-	1.000	1.000	0.912
rs 1256031	0.955	0.618	1.000	-	1.000	0.913
rs 1256049	1.000	1.000	1.000	1.000	-	1.000
rs 4986938	0.854	1.000	0.912	0.913	1.000	-

Figure 1. Localization of the analyzed polymorphisms within a schematic representation of the *ESR2* gene (top), with LD map between polymorphisms (middle) and table of LD coefficients (D') estimated using the Phase v.2.0 software (bottom).

RESULTS

Genotype and Linkage Disequilibrium Analysis

Genotype frequencies in men were out of Hardy Weinberg equilibrium (HWE) proportions (p = 0.01). No significant deviations of the frequencies were observed among any other baseline or follow-up groups (data not shown).

All of the available single nucleotide polymorphisms (SNP's) were selected from the NCBI (http://www.ncbi.nlm.nih.gov/SNP) and Celera (http://www.celera.com/) databases. We chose to genotype six validated SNP's scattered over the gene according to previous publications and with allele frequencies (f) sufficiently high to preserve power in an analysis (f > 0.10). Location, LD map and LD estimations for the six SNP's are shown in Figure 1 (n = 90). The SNP rs1256031, located 10550 basepairs upstream from the start of exon 2, was in strong LD (D' of 1.00) with two other single nucleotide polymorphisms, scattered through the gene, namely rs1256030 and rs1256049. In addition, SNP rs4986938, located 38 basepairs downstream from the 3'UTR showed somewhat lower LD with the intron 2 SNP (D'= 0.91). Therefore, we selected the intron 2 (rs1256031) and the 3'UTR (rs4986938) SNP's for the final analysis considering the previously reported associations (17) and the somewhat lower LD. Both SNPs are T/C substitutions with a frequency of the T allele of 54.3 and 37.9% for the intron 2 and the 3'UTR polymorphisms, respectively. Haplotype alleles were constructed from the two SNP's and coded as numbers 1 through 4 in order of increasing frequency in the population (1 $=C_{int}C_{utr}$ 2 = $T_{int}T_{utr}$, 3 = $T_{int}C_{utr}$, 4 =$C_{int}T_{utr}$). Table 1 shows the frequencies of the haplotypes. We focused our analysis on the presence of the risk *haplotype 4*, and classified individuals into three genotype groups: non-carriers (with frequency of 31%), heterozygous carriers (with frequency of 49%) and homozygous carriers (with frequency of 20 %) of *haplotype 4*.

TABLE 1. *ESR2* HAPLOTYPE FREQUENCIES

	Haplotype			
Subjects	**1**	**2**	**3**	**4**
All	0.7	17.1	37.1	45.1
Women	0.8	17.1	37.1	45.0
Men	0.4	17.3	37.2	45.1

Data are percent frequencies

Baseline Characteristics

Table 2 shows the baseline characteristics of the reference study population (n = 7983) and of the four study populations (Table 2A of BMD and geometry analyses and Table 2B of the fracture analyses). As compared to the total study population individuals in the baseline group were on average 1.1 years younger, while individuals in the follow-up group were 4.9 years younger. At baseline, individuals of the follow-up group were 1.1 cm taller, 2.7 kg heavier, and had a 0,6 kg/m² higher BMI. BMD at baseline was on average ~ 0,025 g/cm² higher in the follow-up group. For fracture analysis we used all genotyped individuals. Compared to the total study population genotyped individuals followed for the presence of non-vertebral fracturtes were on average 1.1 years younger. Indicviduals who were screened for the presence of vertebral fractures were on average 4.8 years younger than the reference population. No significant differences were observed in other chracteristics.

Baseline Characteristics and Bone Geometry Parameters by Haplotype 4 Carrier Status

Table 3 shows age, anthropometric characteristics and bone geometry parameters for the Haplotype 4 carrier status at baseline. Women, who were homozygous carriers of *haplotype 4* were the youngest with no differences in age at menarche or menopause. No differences were seen in body height, weight, BMI or (femoral neck or lumbar spine) BMD. Female homozygous carriers had 1.0 % higher femoral neck widths (p = 0.02), 3.8 % increased buckling ratios (p = 0.001) and 2.3 % thinner cortices (p = 0.01) as compared to non-carriers. A significant trend was seen for increased buckling ratio in this group (p-trend = 0.01) and a borderline significant trend was seen for higher femoral neck width (p-trend = 0.06) in homozygous carriers of *haplotype 4*. Although not significant, non-carrier males tended to have the thinnest cortices, lowest section modulus (strength) and highest buckling ratio (bone instability) as compared to the other genotype groups.

Longitudinal Changes in BMD and Bone Geometry Parameters by Haplotype 4 Carrier Status

Table 4 shows parameters of change in BMD and bone geometry with follow-up by *haplotype 4* carrier status. Overall, women show higher rates of bone loss than men (0.6-0.9 % versus 0.4 % respectively), after 6.6 years (SD = 0.4) of follow-up. In female homozygous carriers lower rates of loss is seen for the absolute (g/cm²-yr) and relative (% of baseline) BMD (p = 0.04 and p = 0.02 respectively), with a significant trend for both. Although not significant, changes in geometry were consistent with the changes in BMD, where female non-carriers of *haplotype 4* had higher rates of cortical thinning, increase in neck width (bone expansion) and buckling ratios (bone instability), while section modulus (bone strength) was decreased more as compared to homozygous carriers of haplotype.

TABLE 2. CHARACTERISTICS OF THE STUDY POPULATION

	Rotterdam Study			Baseline Analysis		Follow-up Analysis	
	Total (n = 7983)	Women (n = 4878)	Men (n = 3105)	Women (n = 2494)	Men (n = 1924)	Women (n = 953)	Men (n = 812)
Age (years)	70.6 ± 9.8	71.7 ± 10.3	69.0 ± 8.7	67.8 ± 8.1*	67.1 ± 7.5*	65.5 ± 6.6*	65.4 ± 6.5*
Height (cm)	166.6 ± 9.5	161.1 ± 6.7	174.6 ± 6.8	161.9 ± 6.5	175.1 ± 6.8	162.5 ± 6.4	175.4 ± 6.5
Weight (kg)	72.9 ± 12.0	69.3 ± 11.4	78.2 ± 10.8	70.1 ± 11.7	79.3 ± 10.3	71.8 ± 10.5	81.0 ± 10.0
BMI (kg/m²)	26.3 ± 3.7	26.7 ± 4.1	25.6 ± 3.0	26.7 ± 4.1	25.8 ± 2.8	27.2 ± 3.9	26.3 ± 2.8
Age at menopause (years)	-	48.8 ± 5.0	-	49.0 ± 5.3	-	49.0 ± 5.0	-
Age at menarche (years)	-	13.7 ± 1.8	-	13.7 ± 1.8	-	13.6 ± 1.8	-
BMD femoral neck (g/cm²)	0.86 ± 0.14	0.83 ± 0.14	0.92 ± 0.14	0.84 ± 0.13	0.92 ±0.13	0.87 ± 0.13	0.93 ± 0.12

	Rotterdam Study			Incident Fracture Analysis		Vertebral Fracture Analysis	
	Total (n = 7983)	Women (n = 4878)	Men (n = 3105)	Women (n = 3829)	Men (n = 2588)	Women (n = 1790)	Men (n = 1404)
Age (years)	70.6 ± 9.8	71.7 ± 10.3	69.0 ± 8.7	70.4 ± 9.6	68.1 ± 8.2	65.8 ± 6.8*	65.3 ± 6.4*
Height (cm)	166.6 ± 9.5	161.1 ± 6.7	174.6 ± 6.8	161.2 ± 6.7	174.7 ± 6.8	162.5 ± 6.4	175.7 ± 6.6
Weight (kg)	72.9 ± 12.0	69.3 ± 11.4	78.2 ± 10.8	69.4 ± 11.3	78.4 ± 10.8	70.4 ± 10.8	80.1 ± 10.2
BMI (kg/m²)	26.3 ± 3.7	26.7 ± 4.1	25.6 ± 3.0	26.7 ± 4.1	25.7 ± 3.0	26.6 ± 4.0	25.9 ± 2.8
Age at menopause (years)	-	48.8 ± 5.0	-	48.9 ± 5.4	-	49.0 ± 5.4	
Age at menarche (years)	-	13.7 ± 1.8	-	13.7 ± 1.8	-	13.6 ± 1.8	
BMD femoral neck (g/cm²)	0.86 ± 0.14	0.83 ± 0.14	0.92 ± 0.14	0.83 ± 0.14	0.92 ± 0.14	0.85 ± 0.13	0.93 ± 0.13

Data are means ± SD

* Significant difference from reference group ($p<0.01$)

TABLE 3. CHARACTERISTICS OF THE STUDY POPULATION BY HAPLOTYPE 4 CARRIER STATUS

	WOMEN (N = 3806)				**MEN** (N = 2576)			
	ESR2 HAPLOTYPE 4				***ESR2 HAPLOTYPE 4***			
Measurements	**Non-carriers** (29.6 %)	**Heterozygotes** (50.8 %)	**Homozygotes** (19.6 %)	*p*	**Non-carriers** (31.4 %)	**Heterozygotes** (47.1 %)	**Homozygotes** (21.5 %)	*p*
Age (years)	70.3 ± 0.3	70.6 ± 0.2	70.0 ± 0.4	0.34	68.1 ± 0.3	67.9 ± 0.2	68.7 ± 0.3	0.11
Height (cm) [a]	161.2 ± 0.2	161.2 ± 0.1	161.1 ± 0.2	0.81	174.8 ± 0.2	174.6 ± 0.2	174.9 ± 0.3	0.60
Weight (kg) [a]	69.0 ± 0.3	69.5 ± 0.3	69.6 ± 0.4	0.41	78.1 ± 0.4	78.4 ± 0.3	79.0 ± 0.5	0.32
BMI (kg/m^2) [a]	26.5 ± 0.1	26.7 ± 0.1	26.9 ± 0.2	0.21	25.5 ± 0.1	25.7 ± 0.1	25.8 ± 0.1	0.24
Age at menopause (years) [a]	48.6 ± 0.2	48.9 ± 0.1	48.7 ± 0.2	0.47	-	-	-	-
Age at menarche (years) [a]	13.7 ± 0.06	13.7 ± 0.04	13.6 ± 0.07	0.31	-	-	-	-
BMD lumbar spine (g/cm^2) [b]	1.04 ± 0.01	1.03 ± 0.00	1.03 ± 0.01	0.33	1.16 ± 0.01	1.17 ± 0.01	1.16 ± 0.01	0.33
Lumbar spine area (g/cm^2) [c]	43.1 ± 0.1	42.8 ± 0.1	42.6 ± 0.2	0.08	51.7 ± 0.2	51.6 ± 0.1	51.9 ± 0.2	0.68
BMD femoral neck (g/cm^2) [b]	0.83 ± 0.00	0.83 ± 0.00	0.82 ± 0.01	0.26	0.91 ± 0.01	0.92 ± 0.00	0.92 ± 0.01	0.25
Neck Width (cm) [b]	3.01 ± 0.01	3.00 ± 0.01	3.04 ± 0.01	0.02	3.45 ± 0.01	3.46 ± 0.01	3.44 ± 0.01	0.60
Section Modulus (cm^3) [b]	0.95 ± 0.01	0.97 ± 0.01	0.96 ± 0.01	0.21	1.37 ± 0.01	1.40 ± 0.01	1.38 ± 0.01	0.15
Cortical Thickness (mm) [b]	0.128 ± 0.00	0.129 ± 0.00	0.125 ± 0.00	0.01	0.139 ± 0.00	0.141 ± 0.00	0.141 ± 0.01	0.28
Buckling Ratio [b]	13.60 ± 0.12	13.49 ± 0.09	14.12 ± 0.14	0.001	14.08 ± 0.13	13.85 ± 0.10	13.76 ± 0.15	0.21

Data are mean ± SE [a] adjusted for age [b] adjusted for age, height and weight [c] adjusted for age, weight

TABLE 4. PARAMETERS OF CHANGE IN BMD AND BONE GEOMETRY WITH FOLLOW-UP BY *H APLOTYPE 4* CARRIER STATUS

	WOMEN				**MEN**			
	ESR2 HAPLOTYPE 4				***ESR2 HAPLOTYPE 4***			
Measurements	**Non-carriers**	**Heterozygotes**	**Homozygotes**	*p*	**Non-carriers**	**Heterozygotes**	**Homozygotes**	*p*
BMD change [a]								
Absolute (g/cm² -yr)	-0.008 ± 0.00	-0.007 ± 0.00	-0.006 ± 0.00	0.04 [c]	-0.003 ± 0.00	-0.004 ± 0.00	-0.004 ± 0.00	0.8
Relative (% of baseline -yr)	-0.9 ± 0.00	-0.8 ± 0.00	-0.6 ± 0.00	0.02 [c]	-0.4 ± 0.00	-0.4 ± 0.00	-0.4 ± 0.00	0.68
Width change [b]								
Absolute (cm-yr)	0.005± 0.03	0.001 ± 0.03	0.004 ± 0.04	0.52	-0.003 ± 0.03	-0.001 ± 0.03	0.006 ± 0.04	0.16
Relative (% of 1st follow-up -yr)	1.3 ± 0.5	0.3 ± 0.4	0.9 ± 0.7	0.33	-0.2 ± 0.5	0.09 ± 0.4	1.5 ± 0.5	0.05 [c]
Buckling Ratio change [b]								
Absolute (units-yr)	0.19 ± 0.04	0.12 ± 0.03	0.12 ± 0.05	0.29	0.05 ± 0.04	0.02 ± 0.03	0.11 ± 0.04	0.23
Relative (% of 1st follow-up-yr)	9.0 ± 1.4	6.6 ± 1.1	6.1 ± 1.9	0.31	3.0 ± 1.3	2.5 ± 1.0	6.2 ± 1.6	0.14
Cortical Thickness change [b]								
Absolute (mm-yr)	-0.001 ± 0.01	-0.001 ± 0.00	-0.001 ± 0.00	0.75	-0.0006 ± 0.00	-0.0001 ± 0.00	-0.0005 ± 0.00	0.27
Relative (% of 1st follow-up -yr)	-2.7 ± 0.9	-2.5 ± 0.7	-1.5 ± 1.2	0.71	-1.0 ± 0.8	0.05 ± 0.6	-1.2 ± 1.0	0.43
Section modulus change [b]								
Absolute (cm³-yr)	-0.006 ± 0.003	-0.007 ± 0.002	-0.003 ±0.004	0.71	-0.01 ± 0.003	-0.001 ± 0.002	-0.004 ± 0.004	0.07
Relative (% of 1st follow-up -yr)	-0.4 ± 1.4	-1.6 ± 1.1	0.5 ± 1.8	0.59	-1.8 ± 1.0	0.3 ± 0.8	-0.8 ± 1.2	0.22

Data are mean ± SE [a] Between 2nd follow-up and baseline [b] Between 2nd follow-up and 1st follow-up [c] p-trend < 0.05

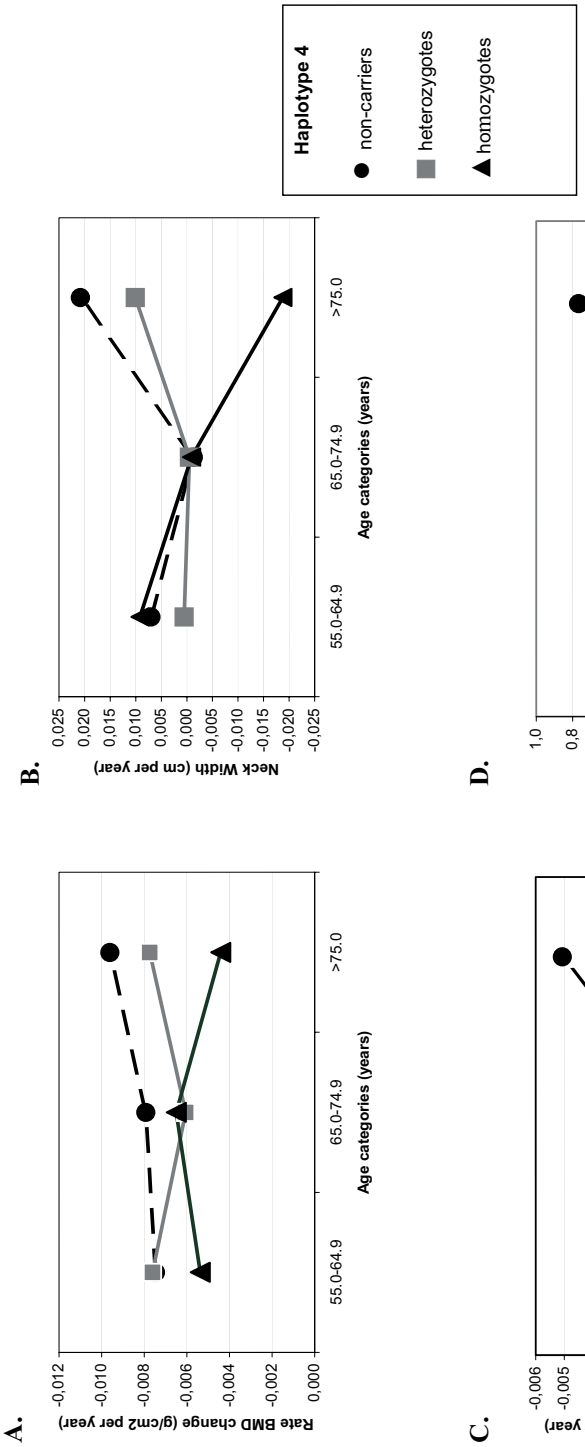


Figure 2. Rate of change by *haplotype 4* carrier status, between age categories in **A.** BMD **B.** Neck width **C.** Cortical thickness **D.** Buckling ratio

In Figure 2, patterns of change in BMD, cortical thickness, neck width and buckling ratio with time are graphed for women (n = 949) across age strata and *haplotype 4* carrier status. A similar pattern is seen in all bone geometry parameters, where differences are accentuated in the oldest age category (>75 yrs). Through age, for non-carriers, there is a higher decrease in BMD, higher increase in neck width, higher decrease in cortical thickness and higher increase in buckling ratio. A significant trend was seen in the oldest age category.

Adjustment for baseline BMD (or first follow-up BMD) did not essentially modify the results on changes in BMD or geometry (data not shown). In males, no (major or) significant effects were observed in the follow-up analysis.

ESR2 haplotype 4 and mortality risk

In men, a small effect of *haplotype 4* was seen on survival (Figure 3). Male homozygous carriers had 1.2 (95%CI, 1.0-1.4) increased mortality risk as compared to non-carriers of *haplotype 4*, effect which is consistent through age. No such effect was seen in women.

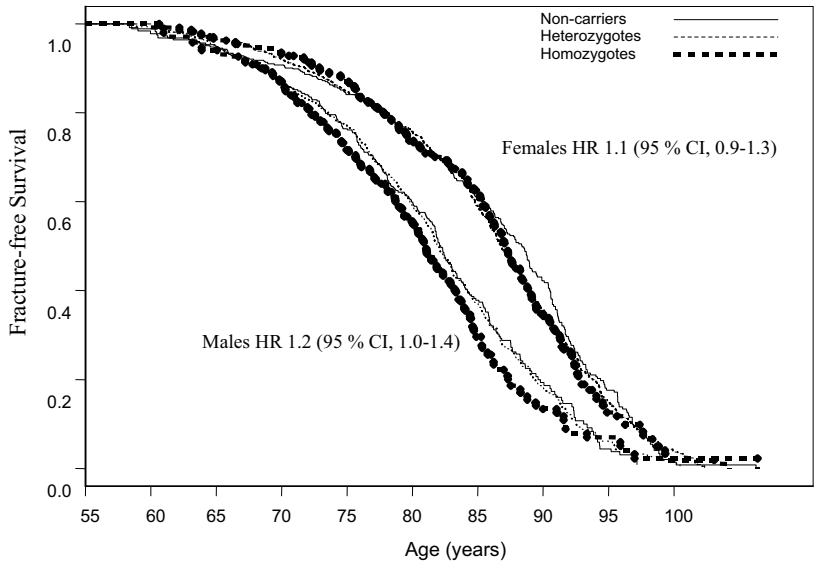


Figure 3. Overall mortality risk in males and females according to *ESR2 haplotype 4* carrier status

ESR2 haplotype 4 and non-vertebral fracture risk

Tables 5 show the fracture incidence rates and fracture-free survival analysis by genotype in both genders. In general, males have lower incidence rates as compared to women. No increase in fracture risk was seen in men homozygous carriers of *haplotype 4* (Table 5A). Instead, this group of men have 0.5 (95% CI, 0.3-1.0) decreased risk for "other type" (not hip, pelvis, proximal humerus or wrist) of fractures as compared to non-carriers of *haplotype 4*. Figure 4 shows that this *protective* effect for suffering "other type" of fractures is driven by the abscense of fracture events after the age of 80 years, while the other two groups (heterozygotes and non-carriers of *haplotype 4*) undergo an increase in the number of event with a signifi cant allele-

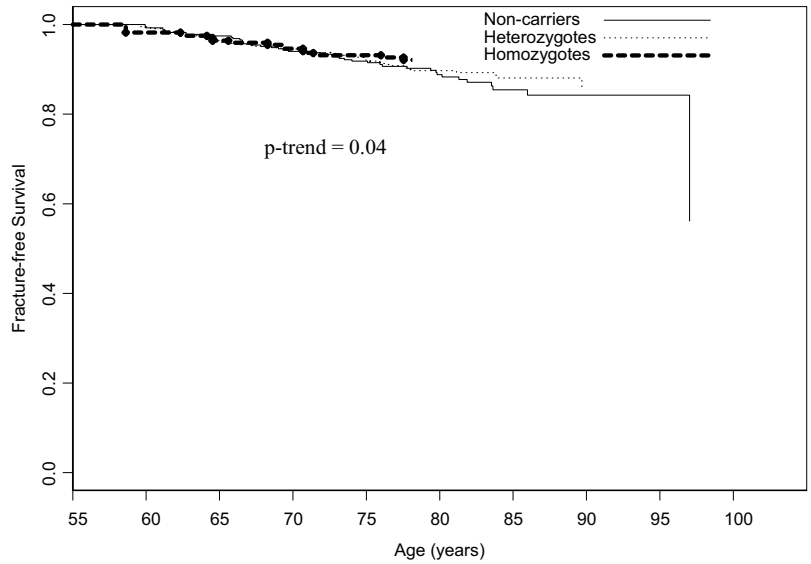


Figure 4. Proportion of other types (not hip, pelvis, proximal humerus or wrist) of non-vertebral fractures with age according to *ESR2 haplotype 4* carrier status in men.

dose effect (p = 0.04).

Female homozygous carriers of *haplotype 4* have 1.4 (95% CI, 1.0-1.8) increased risk for fragility fractures (hip, pelvis and proximal humerus) as compared to non-carriers Table 5B). As shown in Figure 5, the higher rate of fragility fracture in women homozygous for *haplotype 4* is consistent throughout all ages (p-trend = 0.08). Adjustment for BMD, height and weight did not essentially change these risk estimates.

TABLE 5A. OVERALL AND NON-VERTEBRAL FRACTURE FREE SURVIVAL BY *HAPLOTYPE 4* CARRIER STATUS IN MEN

	ESR2 Haplotype 4 carrier status in males						**Cox analysis of *fracture-free* survival***				
	Non-carriers		**Heterozygotes**		**Homozygotes**		**Heterozygotes vs Non-carriers**		**Homozygotes vs Heterozygotes**		**Trend**
	Subjects	**Person-years**	**Subjects**	**Person-years**	**Subjects**	**Person-years**					
	1398	9707	2392	16343	939	6400					
Type of fracture	**Cases**	**Rate**	**Cases**	**Rate**	**Cases**	**Rate**	**HR**	**95%CI**	**HR**	**95%CI**	***p***
All types	84	0.013	111	0.011	47	0.011	0.9	[0.6-1.2]	0.8	[0.6-1.1]	0.19
Osteoporotic	65	0.010	78	0.008	34	0.008	0.8	[0.6-1.1]	0.7	[0.5-1.1]	0.12
Fragility	32	0.005	38	0.004	20	0.004	0.8	[0.5-1.3]	0.9	[0.5-1.5]	0.55
Hip	24	0.004	26	0.003	11	0.002	0.7	[0.4-1.2]	0.6	[0.3-1.2]	0.13
Pelvis	3	0.000	4	0.000	2	0.000	0.9	[0.2-4.1]	1.0	[0.2-6.3]	0.99
Proximal Humerus	5	0.001	8	0.001	7	0.002	1.2	[0.3-4.0]	2.4	[0.7-8.3]	0.14
Wrist	10	0.001	14	0.001	4	0.001	1.0	[0.4-2.2]	0.6	[0.2-2.0]	0.45
Other	38	0.006	46	0.005	14	0.003	0.8	[0.5-1.2]	**0.5**	**[0.3-1.0]**	**0.04**

ESR2 haplotype 4 carrier hazard ratios (HR) have 0 copies as reference group. Adjustment for age, height and weight.

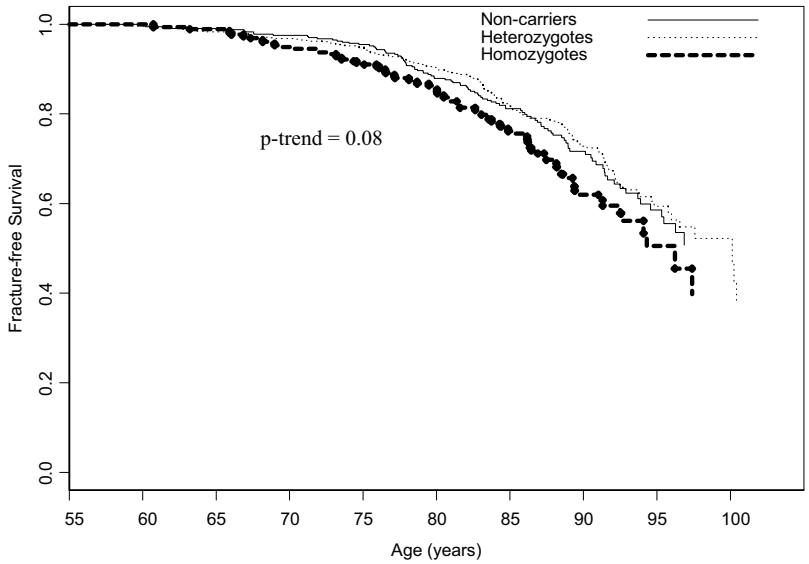


Figure 5. Proportion of fragility (hip, pelvis, proximal humerus) fractures with age according to *ESR2 haplotype 4* carrier status in women.

ESR2 haplotype 4 and vertebral fracture risk

Table 6 shows the risk of vertebral fracture by carrier status. Women homozygous carriers of *haplotype 4* have 2.4 (95%CI 1.5-3.8) increased risk for prevalent vertebral fractures at baseline and 1.8 (95% CI 1.2-2.6) increased risk for having any type of vertebral fractureas compared to non-carriers of the haplotype. Both showed evidence for an allele dose effect (p-trend < 0.01 and p-trend 0.01 for prevalent and *all type* of vertebral fractures, respectively). Further adjustment for the presence or absence of baseline prevalent vertebral fracture or for baseline lumbar spine BMD did not essentially change these results (data not shown). In men no such effect on vertebral fractures was seen.

TABLE 5B. OVERALL AND NON-VERTEBRAL FRACTURE FREE SURVIVAL BY *HAPLOTYPE 4* CARRIER STATUS IN WOMEN

	ESR2 Haplotype 4 carrier status in females						**Cox analysis of *fracture-free* survival***				
	Non-carriers		**Heterozygotes**		**Homozygotes**		**Heterozygotes vs Non-carriers**		**Homozygotes vs Heterozygotes**		**Trend**
	Subjects	**Person-years**	**Subjects**	**Person-years**	**Subjects**	**Person-years**					
	1398	9707	2392	16343	939	6400					
Type of fracture	**Cases**	**Rate**	**Cases**	**Rate**	**Cases**	**Rate**	**HR**	**95%CI**	**HR**	**95%CI**	***p***
All types	271	0.0279	459	0.0281	193	0.0302	1.0	[0.9-1.2]	1.1	[0.9-1.4]	0.27
Osteoporotic	202	0.0208	346	0.0212	147	0.0230	1.0	[0.9-1.2]	1.2	[0.9-1.4]	0.22
Fragility	100	0.0103	157	0.0096	82	0.0128	0.9	[0.7-1.2]	**1.4**	**[1.0-1.8]**	**0.08**
Hip	61	0.0063	95	0.0058	51	0.0080	1.0	[0.7-1.4]	1.4	[0.9-2.0]	0.15
Pelvis	12	0.0012	20	0.0012	8	0.0013	0.9	[0.4-2.0]	1.1	[0.5-2.9]	0.81
Proximal Humerus	27	0.0028	44	0.0027	24	0.0038	1.0	[0.6-1.6]	1.4	[0.8-2.5]	0.28
Wrist	78	0.008	115	0.0070	42	0.0066	0.9	[0.6-1.2]	0.8	[0.6-1.2]	0.27
Other	73	0.0075	142	0.0087	56	0.0088	1.1	[0.8-1.5]	1.2	[0.8-1.6]	0.18

ESR2 haplotype 4 carrier hazard ratios (HR) have 0 copies as reference group. Adjustment for age, height and weight.

TABLE 6. VERTEBRAL FRACTURE RISK ACCORDING TO *ESR2 HAPLOTYPE 4* CARRIER STATUS

	ESR2 Haplotype 4						**Heterozygotes vs Non-carriers**		**Homozygotes vs Non-carriers**		
	Non-carriers		**Heterozygotes**		**Homozygotes**						
Females	Cases	Percentage	Cases	Percentage	Cases	Percentage	*OR*	95%CI	*OR*	95%CI	*p-trend*
Prevalent at baseline	34	6.1	61	6.9	41	12.1	1.1	[0.7-1.7]	**2.4**	**[1.5-3.8]**	**0.001**
Incident at follow-up	36	6.4	48	5.4	28	8.3	0.8	[0.5-1.2]	1.4	[0.8-2.4]	0.31
All types	61	10.9	95	10.7	56	16.5	0.9	[0.7-1.3]	**1.8**	**[1.2-2.6]**	**0.01**
Males	Cases	Percentage	Cases	Percentage	Cases	Percentage	*OR*	95%CI	*OR*	95%CI	*p-trend*
Prevalent at baseline	26	5.9	55	8.2	21	7.4	1.4	[0.9-2.3]	1.2	[0.7-2.3]	0.40
Incident at follow-up	18	4.1	20	3	7	2.5	0.7	[0.4-1.4]	0.6	[0.2-1.4]	0.21
All types	39	8.9	72	10.7	27	9.5	1.2	[0.8-1.9]	1.1	[0.6-1.8]	0.73

ESR2 Haplotype 4 carrier odds ratios (*OR*) have non-carriers as reference group. Adjustment for age, height and weight

DISCUSSION

This population-based study showed that female homozygous carriers for *haplotype 4*, of the *ESR2* gene, have increased risk of fragility (HR 1.4 95%CI 1.0-1.8) and vertebral fracture (OR 1.8 95%CI 1.2-2.6). Consistently, hip structural analysis showed that, as compared to non-carriers of *haplotype 4*, homozygous women had wider femoral necks ($p = 0.02$), thinner cortices ($p = 0.01$) and higher buckling ratios ($p = 0.001$) at baseline, while BMD was not significantly different. The patterns of change in BMD and bone geometry with follow-up showed that homozygous carriers of *haplotype 4* have lower rates of BMD loss compared to non-carriers. Consistently, female non-carriers of *haplotype 4* have higher rates of cortical thinning, increase in neck width (bone expansion) and buckling ratios (bone instability), while section modulus (bone strength) is decreased more as compared to homozygous carriers of *haplotype 4*. In contrast, male homozygous carriers of *haplotype 4*, had a protective effect for suffering other types of non-vertebral fractures (not hip, pelvis, proximal humerus or wrist). Further, a small effect on overall mortality risk was seen in male homozygous carriers of *haplotype 4* (OR 1.2 95 % CI 1.0-1.4). No other effects were observed in males for vertebral fracture, BMD or bone geometry.

To our knowledge, this is the first study that investigated the relationship between variations in the *ESR2* gene and the risk of fracture. Our results on the risk for non-vertebral fracture are in line with our findings on bone geometry at baseline. Women who were homozygous carriers of *haplotype 4* had thinner cortices in wider femoral necks, consequently presenting higher buckling ratios (bone instability). This is consistent with the observation that homozygous female carriers were more likely to suffer fragility fractures (26,33). Adjustment for BMD or bone geometry parameters at baseline did not modify the genotype effect on risk of fracture, suggesting that the haplotype dependent fracture risk does not seem to be entirely mediated by differences in BMD or hip bone geometry at baseline. When we looked at the patterns of change in bone geometry with follow-up, we observed that non-carriers of *haplotype 4* had higher rates of BMD decline than homozygous carriers of *haplotype 4*. On first sight this may appear contradictory, however this may be explained by the structural changes shown in the analysis of bone geometry with time. We know that expansion of the femoral neck is a compensatory mechanism in bone which preserves bending strength in the presence of thinning cortices. Both cortical thinning and bone expansion result in decreased bone mineral density and consequently in a relative higher rate of decline in BMD over time. Considering that homozygous carriers of *haplotype 4* had thinner cortices and wider necks than non-carriers at baseline, it is plausible that the processes of cortical thinning and compensatory bone expansion may occur earlier in postmenopausal life in carriers of *haplotype 4* than in non-carriers. Similarly, it has been proposed that women with hip fracture have wider necks than controls, not only due to the occurrence of

periosteal apposition (bone expansion) after menopause but also for differences in bone growth during early life (34). Although we did not observe genotype–mediated differences in body height, the latter can be still an explanation for our findings considering that there is an effect on vertebral area.

Consistent with the finding on fragility fractures, women homozygous for *haplotype 4* also had increased risk of suffering vertebral fractures. It has been previously shown that women with vertebral fractures have less vertebral bone mass and bone size than women without fractures (35). From a biomechanical perspective, less vertebral size provides less resistance to axial compressive forces, which are the main determinant of vertebral (crush) fracture. In line with this, our results show that women who are homozygous carriers of *haplotype 4* have lower vertebral area than non-carrier women, with no observed differences in lumbar spine BMD. This way, it is likely that variations in the *ESR2* gene in women are influencing the structural adaptation with aging of the femoral neck and of the vertebral bodies in a similar way. Considering that previously we have also found variations in the *ESR1* gene associated with vertebral fracture in women (8), possible genetic interaction between both estrogen receptors should be evaluated.

Not observing these genotype effects in men probably suggests that there are gender differences on how the *ESR2* influences fracture risk. It should be considered that circulating estrogen levels in elderly men are higher than in postmenopausal women (5), which could be an explanation for the gender differences if bioavailability of estrogen plays a role. An effect of the polymorphism was present for the occurrence of "other type" of fractures (not hip, pelvis, proximal humerus or wrist) with opposing direction to that observed in women, that is, carrying *haplotype 4* exerted a protective effect over the occurrence of this type of fractures. Nevertheless, the fracture results in men should be interpreted with caution, considering that genotype frequencies in males showed deviancy from Hardy-Weinberg equilibrium (HWE). Both the deviation in HWE and the protective effect seen over the risk of "other type" of non-vertebral fractures appear to be explained by differential survival across *ESR2* genotypes. Further research is also warranted to elucidate the etiology of the early death observed in men.

Our findings with BMD and bone geometry differ from those observed by Shearman et al. (17), the only other population-based study reported for the *ESR2* intron 2 polymorphism that we have used in our study. Their cross-sectional study, which included relatively younger men and women, displayed a significant association with femoral neck BMD in men. The highest BMD was observed in men with the TT homozygous (equivalent to non-carriers of *haplotype 4* in our study). In addition, the heterozygous had the lowest BMD compared to both homozygous (suggesting negative heterosis). In women they found no significant association. These

conflicting findings could be due to the fact that individuals in the Rotterdam Study are much older than the subjects included in the Framingham Study. Similarly, in contrast to the exclusively postmenopausal population of the Rotterdam Study, Shearman et al. (17) included premenopausal women (~16,7%) in their analyses. Replication of our findings on the risk of fracture in the Framingham population should be sought to help elucidate these differences between studies.

Our study has some limitations. Within the follow-up analyses, individuals were selected on the basis of having complete BMD and geometry measurements at the first and second follow-up. This might have caused selection bias, due to the fact that healthier individuals are most likely to have both BMD-measurements performed. As we presented in table 1, individuals in the follow-up analyses were younger and had higher BMD at baseline. Individuals without follow-up measurements are probably older, with higher morbidity and probably with lower BMD than individuals remaining in the study. Fracture rates in the complete Rotterdam Study were not essentially different in the genotyped cohort, making unlikely the effect of selection bias in the fracture results. Nevertheless, selection bias may play a role in our analyses of vertebral fractures, considering that the screening of vertebral fractures was performed on a survival cohort of our population.

As described earlier, software updates, which altered scan image acquisition, made follow-up data from the HSA not comparable to baseline. This did not allow us to use bone geometry changes in the longest follow-up period of 6.5 years. Measurement error could also be an issue as seen in the age-stratified analyses of neck width in time (Figure 2B) where it is suggested that women after age 65 years are "decreasing" neck width. Since there is no current evidence that normally loaded bones contract, this is probably reflecting no change in neck width in this age group. In addition, the age-specific effects on bone geometry should be interpreted carefully because this analysis could be susceptible to age cohort effects due to its cross-sectional nature. Longer follow-up is needed to determine accurate changes in bone geometry with aging.

Considering the population-based approach of our study, and the high LD observed across the *ESR2* gene, we cannot distinguish whether the genetic variants we have considered in this study are responsible for the observed associations or if it is in LD with another variant within or in the vicinity if the *ESR2* gene. Additional research is needed to evaluate the possible functionality of these polymorphisms.

In conclusion, this population-based study showed a significant association, between the *ESR2* gene and the risk of both fragility and vertebral fractures in post-menopausal women. Differences in vertebral size at the lumbar spine and in bone geometry at the femoral neck were in line with these findings; homozygous

carriers of the risk *haplotype 4* had lower vertebral area, increased femoral neck width, decreased cortical thickness and increased buckling ratio (bone instability) as compared to non-carriers. In contrast, male homozygous carriers of *haplotype 4* had a protective effect for all type of non-vertebral fractures had 0.5 (95% CI 0.3-1.0). In line with these findings male homozygous carriers tended to have, thicker cortices, less neck width and less bone instability (lower buckling ratios), while in female homozygous carriers had thinner cortices, greater neck width and higher instability (higher buckling ratios). In men, in homozygous carriers a small but significant effect on survival 1.2 (95%CI 1.0-1.4) was seen. The association with fractures in males is probably explained by selection on survival. These findings implicate the *ESR2* gene in the pathogenesis of osteoporotic fracture in post-menopausal women.

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Gene Interaction between *ESR1*, *ESR2* and *IGF-I* Influences the Risk of Osteoporotic Fracture

6

ABSTRACT

Introduction: *ESR1*, *ESR2* and *IGF-I* gene polymorphisms have been associated previously with the risk of osteoporotic fracture. The aim of our study was to examine the joint influence of variants in these genes on the risk of osteoporotic fracture.

Methods: Participants are part of the Rotterdam Study, a prospective population-based cohort of Caucasian men and women age 55 and over. Genotypes for *ESR1* (XbaI, PvuII haplotypes), *ESR2* (-10550 intron and +38 3'UTR haplotypes) and *IGF-I* (CA_n repeat in promoter) were available in 6343 individuals. Gene interaction was studied for vertebral fracture (screened in X-ray films of 3469 individuals), incident non-vertebral fracture (average follow-up time of 8.6 years), BMD and bone geometry measured by hip structural analysis (HSA).

Results: Female homozygote carriers of the risk haplotypes in the *ESR2* gene and non-carriers of the risk haplotype in the *ESR1* gene have 3.5 (*95%CI* 1.4 - 8.4) and 1.8 (*95%CI* 1.0 - 3.4) increased risk of vertebral and fragility (hip, pelvis and proximal humerus) fracture, respectively, as compared to non-carriers of any of the risk haplotypes in the estrogen receptor genes (*p interaction* = 0.06 and 0.10 for the risk of vertebral and fragility fracture, respectively). This interaction was stronger in women homozygous for the *192-bp* allele of the *IGF-I* gene promoter polymorphism (*p interaction* = 0.002 and 0.01 for the risk of vertebral and fragility fracture, respectively). Similar interactions were observed for BMD, bone geometry and body height. In men, no evidence of interaction was observed.

Conclusion: Interlocus interaction between *ESR1*, *ESR2* and *IGF-I* gene polymorphisms influences the risk of osteoporotic fracture in postmenopausal women.

INTRODUCTION

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and micro architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. From the genetic perspective osteoporosis is a multifactorial disease resulting from multiple independent gene effects, and gene-gene and gene-environment interactions (1-3). Using the so-called candidate gene approach, several gene variants have been shown to influence independently an individual's genetic susceptibility to fracture (4) with modest, but real effects (5). However, only few studies have addressed the issue of gene interaction which probably underlies most of the complexity of multifactorial disorders including osteoporosis (1).

Estradiol is a pleitropic sex hormone which regulates many physiological processes, including normal cell growth, development, and tissue-specific gene regulation in the reproductive tract, the central nervous and skeletal systems (6). The estrogen receptors *ESR1* (*6q25.1)* and *ESR2* (*14q22-24*) are obvious candidates to study in complex diseases (7). We (8) and others (9-14) have found previously, that variants in the *ESR1* gene (XbaI, PvuII) lead to increased susceptibility for osteoporotic fracture in women, while a large meta-analysis as part of the GENOMOS project is currently under way (Ioannidis et al.; unpublished results). In addition, we have previously found that variants in this gene may interact with variants in the vitamin D receptor in influencing the risk of vertebral fracture (15). The estrogen receptor beta (*ESR2*) has been less often studied in osteoporosis, with only two studies performed on its relation with BMD (16,17). Recently, we have found in women that variants in this *ESR2* gene (-10550 intron and +38 3'UTR) are related to increased risk of both vertebral and fragility fracture, BMD and bone geometry (18). It has been established that there is a complex molecular interplay between both estrogen receptors resulting from estrogen-mediated gene regulation which affects several biologic processes (19). When both estrogen receptors are co-expressed, *ESR2* exhibits an inhibitory action on ERS1-mediated gene regulation (20-22).

Similarly, insulin-like growth factor I (IGF-I) is a ubiquitous polypeptide that stimulates osteoblast activity, subsequently leading to bone matrix formation and inhibition of bone collagen degradation (23). In the *IGF-I* gene (*12q22-24*) we have studied a $(CA)_n$ microsatellite repeat polymorphism (24) located in the 5'-flanking promoter region (25-30), and observed association of the *192-bp* (wilt type) allele with increased IGF-I serum levels and body height (31), higher BMD levels (32), favorable bone geometry (less bone instability) and decreased risk of fragility fracture in women (33).

Considering that *ESR2* modulates *ESR1* transcriptional activity (34), and that *IGF-I* and *ESR1* have common activation pathways and receptor cross-talk (35), biological interaction between variants in these three genes can be expected. We therefore explored possible interactions between these genes in relation to the occurrence of osteoporotic fracture. Therefore, we have examined the possible interaction between genotypes of both estrogen receptors, by themselves, and also by genotypes of the IGF-gene, in relation to the risk of osteoporotic fracture and bone parameters in the Rotterdam study, a large population-based cohort study in elderly men and women.

SUBJECTS AND METHODS

Subjects

The Rotterdam Study is a large prospective population-based cohort study of Caucasian men and women aged 55 and over, living in the Ommoord district of the city of Rotterdam in The Netherlands. The objective is to study the determinants of disease and disability in the elderly, with special focus on neurological, cardiovascular, ophthalmologic and locomotor diseases. The Medical Ethics Committee of the Erasmus Medical Centre has approved the Rotterdam Study. Both the rationale and the study design have been described previously (36). All 10,275 inhabitants of Ommoord, a district in Rotterdam, The Netherlands, were invited to participate. Of these, 7983 (4878 women) participated (resulting in a response rate of 78%). This study is based on 6343 individuals (3787 women) who had genotypes for the three genes available.

Fracture assessment

Radiographs of the spine (extending from the fourth thoracic to the fifth lumbar vertebrae) were available in 3469 individuals (1971 women) and assessed for the presence of vertebral fractures at baseline by the McCloskey-Kanis method as described previously (37). Information on incident non-vertebral fractures was collected from baseline (1990-1993) until December 31st 2001, comprising an average follow-up period of 8.6 (SD 3.4) years. Fracture events were retrieved from computerized records of the general practitioners (GP) in the research area (covering 80% of the cohort). Research physicians regularly followed participant information in GP's records outside the research area and made an independent review, encoding of all reported events. Subsequently, a medical expert in the field reviewed all coded events for final classification. Site-specific incidence rates of fracture have been reported previously (33,38). In line with the associations observed previously with *IGF-I* (33) and *ESR2* (18), we have focused on the occurrence of "fragility" fractures occurring at older age including hip, pelvic and proximal humerus fractures.

BMD and Bone Geometry Measurements

Bone mineral density measurements (g/cm^2) of the proximal femur were performed by dual energy X-ray absorptiometry (DXA) using a Lunar DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA) and analyzed with DPX-IQ v.4.7d software. Methods, quality assurance, accuracy and precision issues of the DXA measurements have been described previously (39). We have used the hip structural analysis (HSA) software developed by Thomas J. Beck (40) to measure hip bone geometry from the DXA scans of the narrow-neck (NN) region across the narrowest point of the femoral neck. BMD, bone width (outer diameter) and cross-sectional moment of inertia (CSMI) were measured directly from mineral mass distributions using algorithms described previously (41). In addition, estimates of cortical thickness and endocortical diameter are obtained modeling the NN region as a circular annulus which assumes a proportion of cortical/trabecular bone of 60/40. For the current work, calculations of section moduli (Z), an index of bending strength, and buckling ratios (BR), index of bone instability, were slightly modified from those reported previously (40) to account for shifts in the center-of-mass. Z was calculated as CSMI /ds, where ds is the maximum distance from the center of mass to the medial or lateral surface. Buckling ratios were computed as ds divided by estimated mean cortical thickness. In some earlier work (40), ½ the outer diameter was used instead of ds in calculations of Z and BR.

Clinical examination

Height (cm) and weight (kg) were measured in standing position wearing indoor clothes and without shoes. Age at and reason of menopause was assessed as defined previously (42).

Genotyping

For *ESR1* we determined haplotypes of the *PvuII* (rs2234693 *T>C*) and *XbaI* (rs9340799 *A>G*) restriction fragment length polymorphisms (RFLP) located in intron 1 of the *ESR1* gene, 397 bp and 351 bp upstream of exon 2 respectively, using direct molecular haplotyping methods as described previously (43). Specific genotyping methods and quality control procedures have been described earlier (8). The alleles were defined as haplotypes such as "Px", capitals denoting absence and lower cases letters denoting presence of the restriction site for the PvuII (P/p) and XbaI (X/x) enzymes on each of the alleles. The haplotype alleles were coded as haplotype numbers 1 through 4 in order of decreasing frequency in the population (1 = px, 2 = PX, 3 = Px, and 4 = pX). Based on our previous associations we focused our analysis on the presence of the risk *haplotype 1* (8), and classified individuals in three genotype groups: non-carriers (with frequency of 22%), het-

erozygotes carriers (with frequency 49%) and homozygote carriers (with frequency of 29%) of *haplotype 1* .

For *ESR2* we genotyped initially six SNP's scattered across the gene. Taqman allelic discrimination assay was used, while primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands). Specific genotyping methods and quality control procedures have been described earlier (18). Using PHASE (v.2.0) software (University of Washington, Seattle, WA) we determined linkage disequilibrium (LD) among these six SNP's, and selected two SNP's located on intron 2 (rs1256031 *T>C*) and in the 3'UTR regions (rs4986938 *C>T*) for the final analysis. Using PHASE haplotype alleles were constructed from the two SNP's and coded as numbers 1 through 4 in order of increasing frequency in the population (1=$C_{int}C_{utr}$ 2=$T_{int}T_{utr}$, 3=$T_{int}C_{utr}$, 4=$C_{int}T_{utr}$). Based on our previous associations (18) we focused our analysis on the presence of the risk *haplotype 4*, and classified individuals in three genotype groups: non-carriers (with frequency of 31%), heterozygotes carriers (with frequency of 49%) and homozygote carriers (with frequency of 20%) of *haplotype 4*.

The polymorphic number of $(CA)_n$ repeats in the *IGF-I* gene promoter region was determined as described earlier (32). From sequence analysis it is known that the *192-bp* allele (based on its frequency in our population referred to as *"wild-type"*) corresponds to 19 CA-repeats ($CA_{(19)}$) (44). Based on the relationship between the polymorphism and serum IGF-I levels, genotypes were assembled from two allele categories as previously described (31): the *192-bp* allele and *all-other* alleles pooled as "*non-192-bp*" alleles. Individuals were classified in three genotypes groups: homozygotes for the *192-bp* allele (44% of the population), heterozygotes for the *192-bp* allele (44%) and non-carriers of the *192-bp* allele (12%).

Statistical analysis

We stratified all analyses by gender, and by *IGF-I* genotypes when we studied interaction between the estrogen receptor genotypes in relation to *IGF-I* genotypes. In all analyses we defined as reference the group of non-carriers of either risk *haplotype 1* or *4* in the *ESR1* and *ESR2* genes, respectively. Nine genotype groups resulted from the pair wise combination of estrogen receptor loci with (/nc denoting noncarriers of risk haplotype), (/ht denoting heterozygous carriers of risk haplotype) and (/r denoting homozygous carriers of the risk haplotype): 1) *ESR1*/nc *ESR2*/nc (reference); 2) *ESR1*/ht *ESR2*/nc; 3) *ESR1*/r *ESR2*/nc; 4) *ESR1*/nc *ESR2*/ht ; 5) *ESR1*/ht *ESR2*/ht; 6) *ESR1*/r *ESR2*/ht; 7) *ESR1*/nc *ESR2*/r; 8) *ESR1*/ht *ESR2*/r ; 9) *ESR1*/r *ESR2*/r. Relative risks were estimated for the analysis of vertebral fracture as Odds Ratios (OR) with 95% confidence intervals (CI) using multiple

logistic regression, while risk for incident fragility fractureswas estimated from Cox proportional hazards models as hazard ratios (HR) with 95% confidence intervals (CI). Differences in continuous measurements were compared across genotype groups using multiple linear regression and analyses of covariance (ANCOVA). All estimates were adjusted for age, height and weight (with exception of body height and age-at-menopause). Genetic interaction between *ESR1* and *ESR2* was modeled assuming additive genetic effects (45), with an interaction term obtained from the product of main (genotype) effects. Finally, model assumptions were verified and model residuals were checked for goodness-of-fit. All analyses were performed using the SPSS-package V.11 (SPSS Inc. Chicago, IL).

RESULTS

Table 1 shows the independent effects estimated for each of the studied genes in the Rotterdam Study population, of which most have already been previously reported. As shown earlier for *ESR1*, women who are homozygote carriers of *haplotype 1* have increased risk of vertebral fracture, lower body height and earlier age at menopause. Similarly for *ESR2*, women who are homozygote carriers of *haplotype 4* have increased risk of both vertebral fracture and fragility fracture. In line with the latter finding, HSA showed that these women have greater periosteal expansion and cortical instability of the femoral neck. In contrast for *IGF-I*, women who do not carry the 192-bp or "wild type" allele have increased risk of fragility fracture, lower BMD levels at baseline, greater bone instability of the femoral neck and lower body height. No associations of these genotypes with osteoporosis genotypes were observed in men.

In Table 2 the genotype frequencies of different genotype combinations are presented. In general, the absolute and relative number of women in each genotype is higher than the number of men. For ease of interpretation, in Table 2 values are highlighted for the groups with combinations of homozygous genotypes. These specific coloring is used in the figures below.

Figure 1 illustrates the risk of both vertebral and fragility fracture (hip, pelvis and proximal humerus) in women with the above mentioned combination of estrogen receptor genotypes, overall and across IGF- genotypes. In Figure 1A, it is seen overall that, homozygous carriers of the risk haplotype in *ESR1* but who did not carry the risk haplotypes in *ESR2* (*ESR1*/r *ESR2*/nc) had 2.6 (*95%CI* 1.1 - 5.8) increased risk of vertebral fracture. This risk was higher for homozygous carriers of the risk haplotype in *ESR2* but who did not carry the risk haplotypes in *ESR1* (*ESR1*/nc *ESR2*/r) 3.5 (*95%CI* 1.4 - 8.4). The risk of vertebral fracture was 3.1 (*95%CI* 1.3 - 7.4) in homozygous carriers of both risk haplotypes in *ESR1*

TABLE 1. ASSOCIATIONS OF *ESR1*, *ESR2* AND *IGF-1* POLYMORPHISMS WITH FRACTURE RISK, BONE PARAMETERS AND ANTHROPOMETRICS IN WOMEN

Selected Endpoints	ESR1 Risk Haplotype 1			ESR2 Risk Haplotype 3			IGF-I 192-bp (wild type) allele			
	Non-carriers	*Homozygotes*	*p*	*Non-carriers*	*Homozygotes*	*p*	*Homozygotes*	*Non-carriers*	*p*	
Vertebral fracture risk*[a]	1.0 (Reference)	**1.6 (1.1 - 2.5)**	0.03 [8]	1.0 (Reference)	**1.8 (1.2 - 2.6)**	0.005 [18]	1.0 (Reference)	1.1 (0.7 - 1.7)	0.64	
Fragility fracture risk[a]**	1.0 (Reference)	1.0 (0.7 - 1.3)	0.89	1.0 (Reference)	**1.4 (1.0 - 1.9)**	0.06 [18]	1.0 (Reference)	**1.4 (1.0 - 1.9)**	0.07 [33]	
Femoral neck BMD (g/cm^2)¤[a]	0.83 (Reference)	-0.003 (0.01)	0.65 [8]	0.83 (Reference)	-0.012 (0.007)	0.21 [18]	0.83 (Reference)	**-0.013 (0.01)**	0.06 [32]	
Narrow-neck width (cm)¤[a]	3.004 (Reference)	0.012 (0.01)	0.32	3.009 (Reference)	**0.026 (0.01)**	0.03 [18]	3.007 (Reference)	0.008 (0.01)	0.53 [33]	
Cortical buckling ratio (cm)¤[a]	13.6 (Reference)	0.1 (0.18)	0.52	13.6 (Reference)	**0.5 (0.18)**	0.005 [18]	13.6 (Reference)	**0.3 (0.20)**	0.10 [33]	
Lumbar spine BMD (g/cm^2)¤[a]	1.03 (Reference)	-0.001 (0.001)	0.92 [8]	1.04 (Reference)	-0.01 (0.009)	0.24 [18]	1.04 (Reference)	-0.003 (0.01)	0.70 #	
Vertebral area (cm)¤[a]	42.9 (Reference)	-0.2 (0.19)	0.31 [8]	43.1 (Reference)	-0.3 (0.20)	0.19 [18]	42.9 (Reference)	-0.3 (0.21)	0.12	
Body height (cm)¤[b]	161.6 (Reference)	**-0.6 (0.29)**	0.03 [47]	161.2 (Reference)	-0.2 (0.30)	0.58 [18]	161.4 (Reference)	**-0.7 (0.32)**	0.04 [31]	
Age at menopause (years)¤[b]	48.7 (Reference)	**0.5 (0.25)**	0.08 [48]	48.8 (Reference)	0.1 (0.26)	0.68 [18]	49.0 (Reference)	0.0 (0.28)	0.99	

Estimates are *Odds Ratios (95%CI) **Hazard Ratios (95%CI) ¤Mean and Δ (SEM)

[a]Adjusted age, height and weight [b]Adjusted age

TABLE 2. COUNT AND PERCENT (%) OF INDIVIDUALS IN EACH COMBINATION OF ESTROGEN RECEPTOR GENOTYPES

			Overall		**IGF-I 192-bp allele**					
					Homozygotes		**Heterozygotes**		**Non-carriers**	
ESR1	**ESR2**		Males	Females	Males	Females	Males	Females	Males	Females
/nc	**/nc**		**179 (7.0)**	**259 (6.8)**	**83 (7.5)**	**126 (7.7)**	70 (6.2)	106 (6.4)	**23 (8.1)**	**26 (5.8)**
/ht	**/nc**		397 (15.5)	543 (14.3)	161 (14.6)	215 (13.2)	192 (16.9)	233 (14.1)	41 (14.4)	84 (18.6)
/r	**/nc**		**224 (8.8)**	**321 (8.5)**	**90 (8.2)**	**132 (8.1)**	104 (9.1)	147 (8.9)	**29 (10.2)**	**37 (8.2)**
/nc	**/ht**		248 (9.7)	407 (10.7)	110 (10.0)	177 (10.9)	100 (8.8)	174 (10.6)	34 (12.0)	53 (11.7)
/ht	**/ht**		613 (24.0)	961 (25.4)	271 (24.6)	424 (26.0)	277 (24.4)	409 (24.8)	55 (19.4)	116 (25.7)
/r	**/ht**		343 (13.4)	554 (14.6)	143 (13.0)	251 (15.4)	154 (13.5)	244 (14.8)	41 (14.4)	47 (10.4)
/nc	**/r**		**128 (5.0)**	**158 (4.2)**	**67 (6.1)**	**59 (3.6)**	49 (4.3)	79 (4.8)	**11 (3.9)**	**19 (4.2)**
/ht	**/r**		282 (11.0)	351 (9.3)	119 (10.8)	149 (9.1)	130 (11.4)	153 (9.3)	30 (10.6)	40 (8.8)
/r	**/r**		**142 (5.6)**	**233 (6.2)**	**58 (5.3)**	**96 (5.9)**	61 (5.4)	104 (6.3)	**20 (7.0)**	**30 (6.6)**

Risk haplotype genotypes -> **/nc** : non-carrier of risk haplotype **/ht** : heterozygous carrier of risk haplotype **/r** : homozygous carrier of risk haplotype

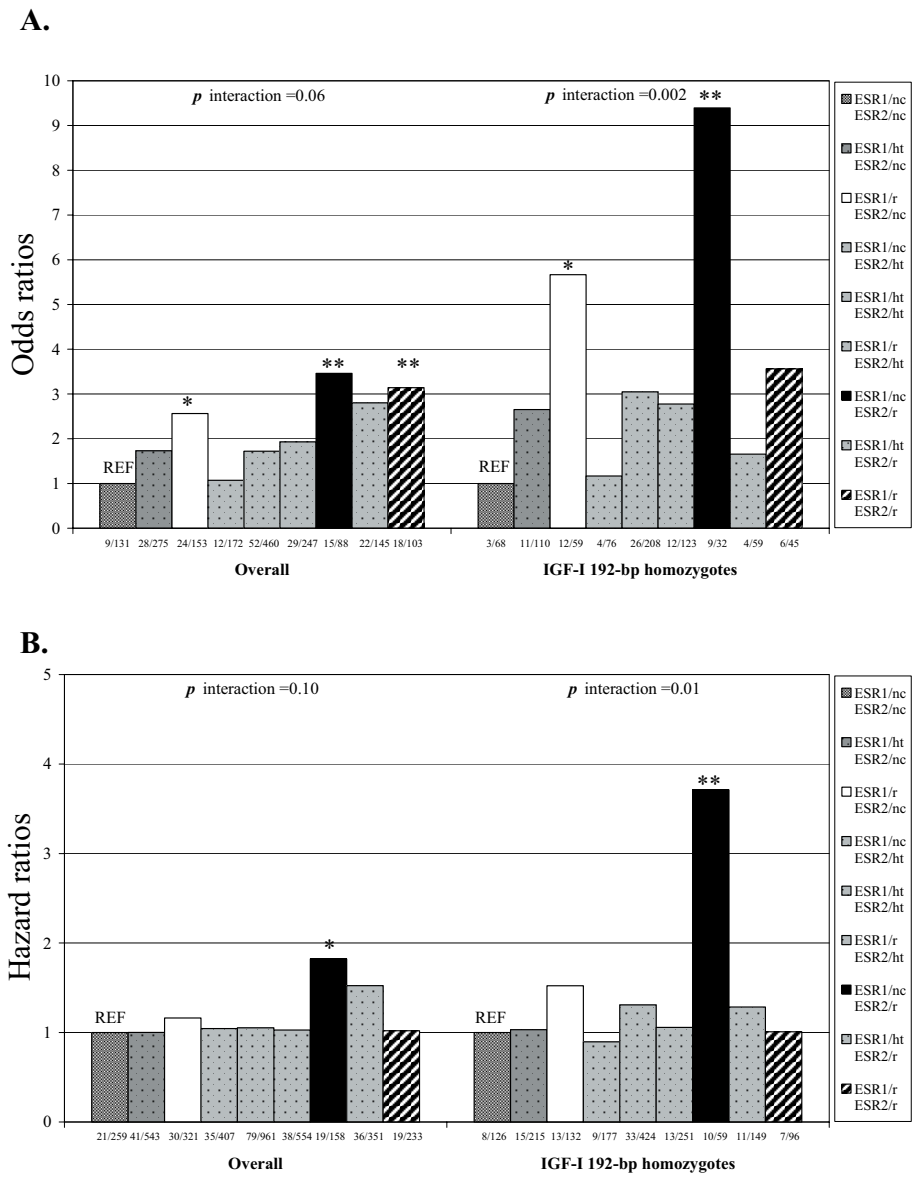


Figure 1. Interaction between ESR1and *ESR2* genotypes, overall and in *IGF-I* 192-bp homozygotes group for **A.** the risk of vertebral fracture and **B.** the risk of fragility fracture.

ESR1/**nc**: non carriers haplotype 1, *ESR2*/**nc**: non-carriers *haplotype 4*
ESR1/**ht**: heterozygous haplotype 1 *ESR2*/**ht**: heterozygous *haplotype 4*
ESR1/**r**: homozygous haplotype 1 *ESR2*/**r**: homozygotous *haplotype 4*
Data are adjusted for age, height and weight Significance: * $p<0.05$ ** $p<0.01$

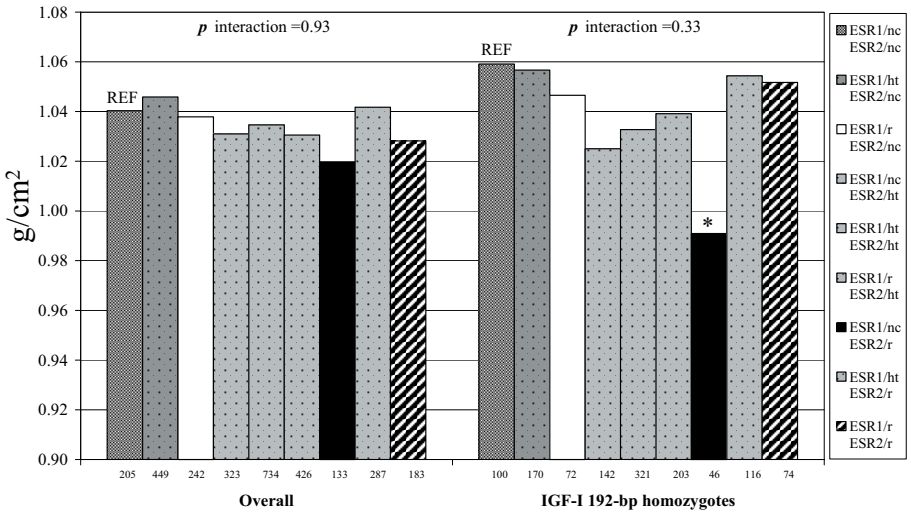
and *ESR2* (*ESR1*/r *ESR2*/r) with *p* interaction =0.06. In the group of individuals homozygous for the *IGF-I 192-bp* allele, a highly significant interaction (p=0.002) is seen where the risk of vertebral fracture in individuals *ESR1*/nc *ESR2*/r has a prominent increase to 9.4 (*95%CI* 2.3 - 38.2) while it is unexpectedly lower in carriers of both risk haplotypes (*ESR1*/r *ESR2*/r). In Figure 1B it is shown that individuals with the *ESR1*/nc *ESR2*/r combination have 1.8 (*95%CI* 1.0 - 3.4) increased risk of fragility fracture, while no other combination of genotypes showed increased risk. A significant gene interaction (p=0.01) with a similar pattern to that observed in vertebral fractures, is seen for the risk of fragility fracture, where homozygous women for the *IGF-I 192-bp* allele and with the *ESR1*/nc *ESR2*/r combination, the risk increases to 3.7 (*95%CI* 1.4 - 9.8), while no risk is seen for homozygous carriers of both risk genotypes (*ESR1*/r *ESR2*/r). No relationship between the combination of genotypes and the risk of vertebral fracture was seen in males.

As shown in Figure 2, this pattern of interaction, although not significant, is also seen in women for lumbar spine and femoral neck BMD in the overall group, with individuals with the *ESR1*/nc *ESR2*/r combination showing the lowest BMD levels. In women who are homozygous for the *IGF-I 192-bp* allele and carry the *ESR1*/nc *ESR2*/r combination we observed significantly lower BMD than all the other genotype groups, with a significant interaction seen only at the femoral neck (p=0.03). No such findings were seen in men.

In Figure 3 the hip structural analysis revealed no significant differences or evidence of interaction in neck width, although carriers of any of the risk haplotypes tend to have wider femoral necks than non-carriers of the risk haplotypes (Figure 3A). This pattern is also observed for the endocortical diameter (Figure 3B). However, in the group of women with the *ESR1*/nc *ESR2*/r combination these differences reach significance, both overall and in the group of women homozygous for the *IGF-I 192-bp* allele. The indexes of bending strength (section modulus) and bone instability (buckling ratio) (Figure 3C and 3D respectively) show concordantly that, overall, women with the *ESR1*/nc *ESR2*/r combination have significantly lower bone strength and higher bone instability (interpreted as susceptibility to fracture), while this parameters did not differ significantly from the reference group in women homozygous for both risk haplotypes (*ESR1*/r *ESR2*/r). When we analyzed the *ESR1* and *ESR2* combinations by *IGF-I* genotypes, evidence of interaction is suggested for bone strength and instability (p=0.10 and p=0.06, respectively). Again, no such relations were observed in males.

Finally, a similar interaction (p=0.14) is observed for body height (Figure 4A), where women with the *ESR1*/nc *ESR2*/r combination and who are homozygote carriers of the *192-bp* allele are approximately 2 cm taller than individuals who don't carry risk haplotypes in any of the two estrogen receptors *ESR1*/nc *ESR2*/nc)..

A.



B.

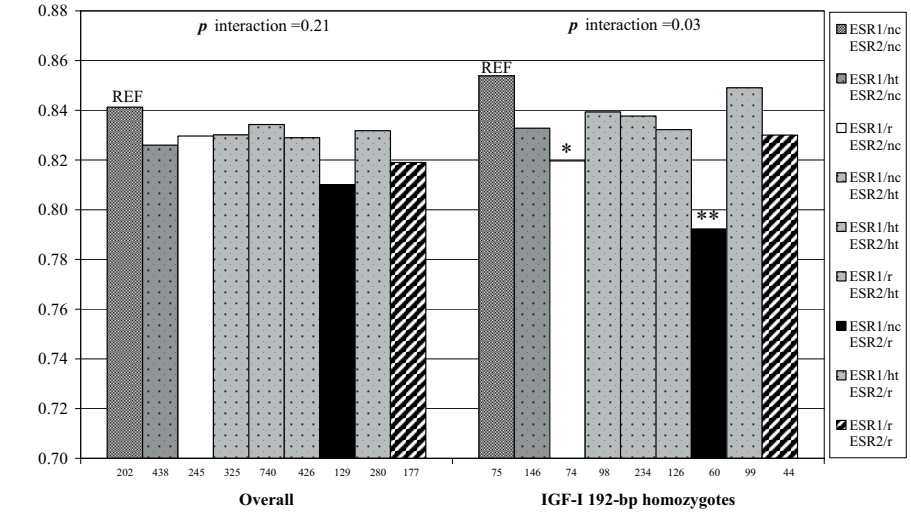


Figure 2. Interaction between ESR1and *ESR2* genotypes, overall and in *IGF-I* 192-bp homozygotes group for **A.** lumbar spine BMD **B.** Femoral neck BMD.

ESR1/**nc**: non carriers haplotype 1, *ESR2*/**nc**: non-carriers *haplotype 4*
ESR1/**ht**: heterozygous haplotype 1 *ESR2*/**ht**: heterozygous *haplotype 4*
ESR1/**r**: homozygous haplotype 1 *ESR2*/**r**: homozygotous *haplotype 4*
Data are adjusted for age, height and weight Significance: * $p<0.05$ ** $p<0.01$

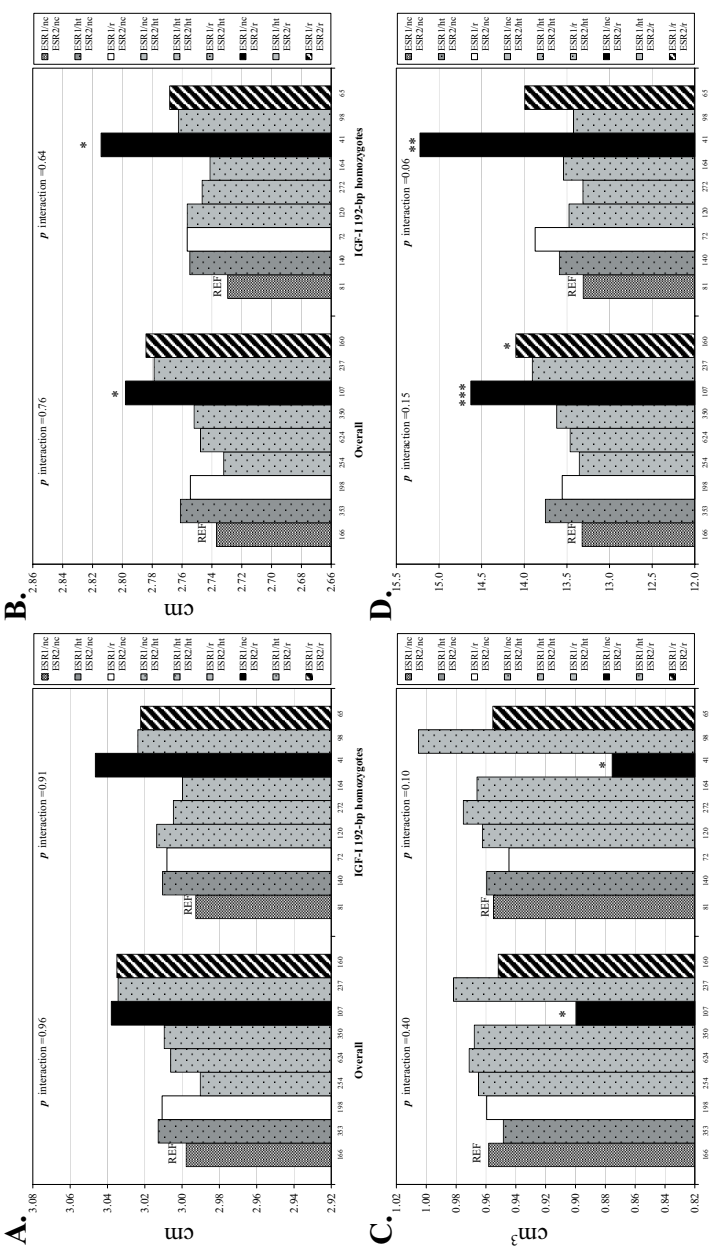


Figure 3. Interaction between ESR1and *ESR2* genotypes, overall and in *IGF-I* 192-bp homozygotes group for:
A. Femoral neck width **B.** Endocortical diameter **C.** Section modulus and **D.** Buckling ratio.
ESR1/**nc**: non-carriers haplotype 1 *ESR1*/**ht**: heterozygous haplotype 1 *ESR1*/**r**: homozygous haplotype 1
ESR2/**nc**: non-carriers *haplotype 4ESR1*/**ht**: heterozygous *haplotype 4* *ESR2*/**r**: homozygous *haplotype 4*
Data are adjusted for age, height and weight Significance: * $p<0.05$ ** $p<0.01$

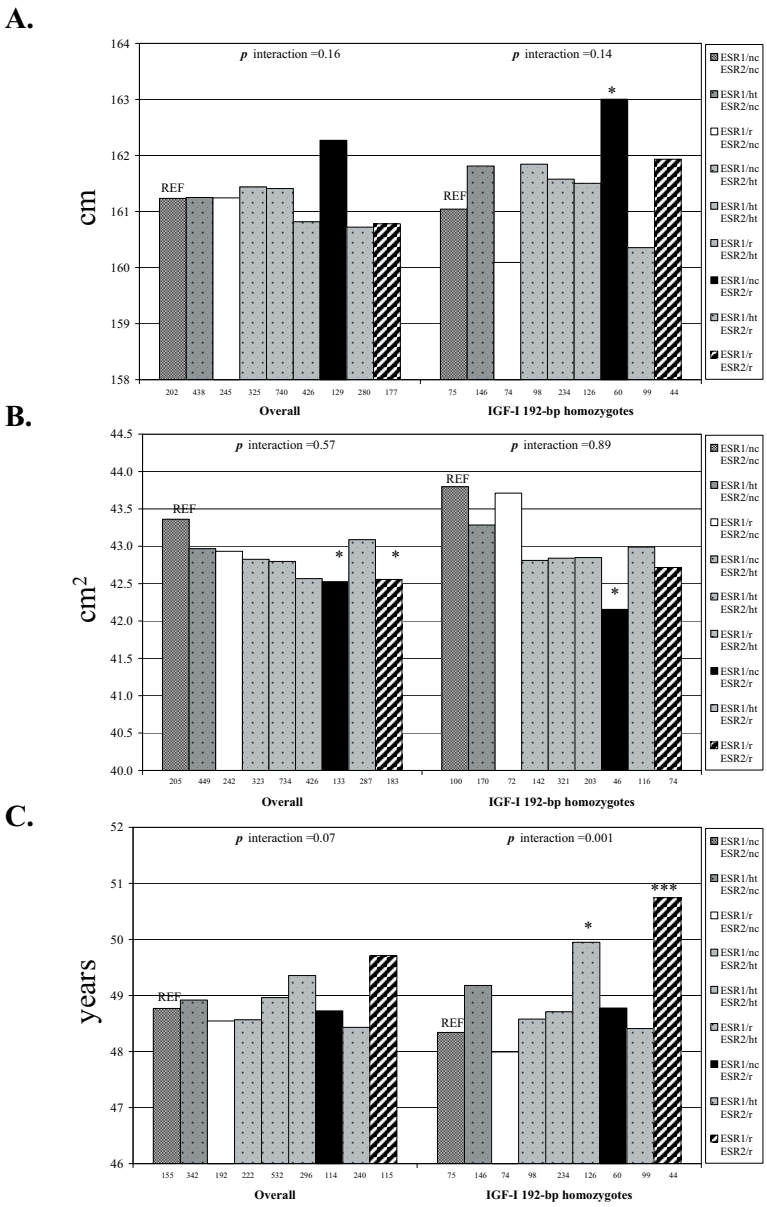


Figure 4. Interaction between ESR1and *ESR2* genotypes, overall and in *IGF-I* 192-bp homozygotes group for **A.** Body height **B.** Vertebral area and **C.** Age at menopause

ESR1/**nc**: non carriers haplotype 1, *ESR2*/**nc**: non-carriers *haplotype 4*
ESR1/**ht**: heterozygous haplotype 1 *ESR2*/**ht**: heterozygous *haplotype 4*
ESR1/**r**: homozygous haplotype 1 *ESR2*/**r**: homozygotous *haplotype 4*
Data are adjusted for age, height and weight Significance: * $p<0.05$ ** $p<0.01$

Even though there is no evidence of interaction for vertebral area (Figure 4B), women with the *ESR1*/nc *ESR2*/r combination have significantly lower vertebral area, just as seen for women with the *ESR1*/r *ESR2*/r combination. These differences became greater in the group of women who were also homozygous carriers of the 192-bp allele of the *IGF-I* promoter polymorphism. No relation with body height or vertebral area was observed in males.

When we examined age at onset of menopause in relation to this genes (Figure 4C), we also observed evidence of interaction between *ESR1* and *ESR2* (p=0.07), and which was more prominent in women homozygous for the *192-bp* allele in the *IGF-I* gene (p=0.01). Nevertheless, it was women who carried both of the risk haplotypes in the two estrogen receptor genes (*ESR1*/r *ESR2*/r) who had significantly delayed onset of menopause as compared to the other genotype groups.

DISCUSSION

This population-based study suggests for the first time interaction between *ESR1*, *ESR2* and *IGF-I* genes in relation to risk of vertebral and fragility fracture in Caucasian postmenopausal women. We found that women who are homozygote carriers of the risk *haplotype 4* in the *ESR2* gene and non-carriers of the risk *haplotype 1* in the *ESR1* gene (*ESR1*/nc *ESR2*/r combination) have 3.5 (*95%CI* 1.4 - 8.4) and 1.8 (*95%CI* 1.0 - 3.4) increased risk of vertebral and fragility fracture, respectively, as compared to non-carriers of any of the risk haplotypes in the estrogen receptor genes (*ESR1*/nc *ESR2*/nc combination). When looking at the interaction between estrogen receptor genes across *IGF-I* genotypes, we found evidence of augmented interaction between the same genotypic combination (*ESR1*/nc *ESR2*/r) in women who were also homozygote for the *192-bp* allele of the *IGF-I* gene (*p interaction* = 0.002 and 0.01 for the risk of vertebral and fragility fracture, respectively). This gene interaction was also observed for other bone parameters including BMD, bone geometry and body height.

Most association studies in humans focus on effects of single genes and very few address the issue of interaction. One of the main reasons for this is that examining (pair wise) gene interactions usually requires large sample sizes to preserve sufficient power to analyze usually infrequent combination of genotypes and/or to control for the occurrence of chance findings. This problem is exacerbated if a third or higher number of loci is considered. This has made the mouse model the preferred tool to examine multiple genetic interactions (46), though extrapolation to humans remains an obstacle. One alternative to address this problem is to examine the interaction between candidate genes in large population-based studies in humans.

Previously, we have proposed that carriers of the risk *haplotype 1* in *ESR1* appear to be less sensitive to estrogen probably due to differential expression of the receptor. This has been supported by our previous findings, where body height in pre- and postmenopausal women (47) and estradiol levels in premenopausal women decreased, while age at menopause increased (48) with the number of copies of *haplotype 1* in their genotype. Similarly, we have shown previously that the presence of the *192-bp* allele in the *IGF-I* promoter genotype is associated with higher IGF-I levels (31) and body height in postmenopausal women (31,33) with evidence for an allele-dose effect. Additionally, we have recently shown that IGF-I levels and peak body height appear to be related to an "optimum" in the length of repeats in this polymorphism, where as, IGF-I levels and body height in individuals decreases considerably as alleles in their genotype deviate from the length of the *192-bp* (wild-type) allele (49). The interaction between *ESR1* and *IGF-I* genes is plausible because both share common activation pathways and because *ESR1* is needed for some *IGF-I* induced responses (35). In addition, it has been demonstrated that *ESR1* can interact in an indirect manner with some promoters by physically associating with prebound AP1 or SP1 protein complexes, mechanisms that appears to explain how estrogens regulate the cyclin D, c-Myc, and *IGF-I* promoters (50). Few studies in humans have examined the relation between variations in the *ESR2* gene and estrogen levels. Nevertheless, several studies have demonstrated the regulatory action that *ESR2* exerts over *ESR1* transcriptional activity. The ability of *ESR2* to heterodimerize with and inactivate *ESR1*, positions *ESR2* as the dominant regulator of the estrogen signalling (34,51). So, in this perspective, the *ESR1*/nc *ESR2*/r combination of genotypes appears to reflect women with higher (*ESR1*-mediated) sensitivity to estrogen (*ESR1*/nc) while regulatory capacity of *ESR2* is compromised (*ESR2*/r). In addition, individuals with the *192/192* genotype in the *IGF-I* promoter polymorphism, appear to have the optimum configuration for *IGF-I* transcription, which is expected to be compromised in homozygote non-carriers of the *192-bp* allele.

Results from our current study are in line with this biological framework and may explain why the *ESR1*/nc *ESR2*/r genotypic combination could be detrimental to the bone health of individuals who carry it, and why the possible deleterious effect arising from this combination is exacerbated if they are in addition homozygous for the 192-allele of the *IGF-I* promoter polymorphism.

We have looked at BMD and bone geometry trying to elucidate further the underlying mechanisms that could increase the susceptibility to fracture. It has been postulated that in long bones, estrogen (via both of its receptors) favours endocortical over periosteal apposition (52). The presence of estrogen before menopause causes bone formation in the inner border but not on the external borders of the cortices (limiting the occurrence of bone expansion). This phenomenon

has important biomechanical implications and is an important sexual dimorphism observed in bone (53). It has been suggested that the rapid phase of BMD decline observed after menopause is both the result of bone loss (in the form of cortical thinning consequence of endocortical expansion) and also, of the increase in bone volume (at expense of periosteal apposition) (54). As bone diameter increases the same bending strength (section modulus) can be achieved with progressively less material (40). Nevertheless, if the process is carried too far, the section modulus no longer governs bending strength, because the cortex and the trabeculae that internally support it become unstable and prone to fracture (55). Considering this biomechanical relationship with estrogen and also because IGF-I has been implicated in periosteal bone formation (56), we propose a model which integrates this findings of our study.

Our results show that women with the *ESR1*/nc *ESR2*/r combination have lower femoral neck BMD, which is in line with the finding that they also tend to have greater increase in endocortical diameter (cortical thinning) and neck width (bone volume). This could be the result of enhanced *ESR1* mediated bone formation (*ESR1*/**nc**) which is not properly opposed by *ESR2* (*ESR2*/**r**). If, in addition, these women are homozygous for the *192-bp* allele in the *IGF-I* promoter genotype (optimized IGF-I response), there would be two driving elements enhancing bone formation with inadequate modulation of the response. In line with this, bending strength (section modulus) is decreased and cortical instability increased (buckling ratios) in women with this combinations. Similarly, this higher cortical instability would explain the higher risk of fragility fracture observed in these women. In addition, our results also show that homozygous women with both risk haplotypes (*ESR1*/r *ESR2*/r), instead of having a more deleterious phenotype as would be expected, appear to benefit from this combination and have decreased risk of osteoporotic fractures. This could also be explained within the framework of our hypothesis. If there is *ESR1* mediated bone formation, which is compromised in carriers of the risk haplotype (*ESR1*/**r**), the improperly modulated opposition on bone formation exerted by a compromised *ESR2* (*ESR2*/**r**) will not result in the excessive and consequently harmful response on bone formation. No major differences are observed in women homozygous for the *192-bp* allele in the *IGF-I* promoter genotype, possibly because *ESR1* integrity is needed for optimum IGF-I action on bone formation.

Recently, it has been proposed that such a structural basis of bone fragility has some features that could have originated during growth and not only during aging (57,58). This concept is supported by the fact that both estrogen receptors are highly expressed in the epiphysial plate during different stages of human growth (59) and by the observation that estrogens in combination with IGF-I initiate the pubertal growth spurt (58). Our finding of increased body height in women with

this specific genotypic combination (even though they suffered more vertebral fractures) is also suggestive of an effect driven by the interaction between *ESR1*, ESR2 and *IGF-I* during growth.

No difference in age at menopause was observed for women with the *ESR1*/nc *ESR2*/r combination. In contrast, women who carried both risk haplotypes of the estrogen receptors (*ESR1*/r *ESR2*/r combination) appeared to have later age at menopause, also in interaction with the *192-bp* homozygosis in the *IGF-I* promoter polymorphism. These findings with age at menopause merits further investigation that is beyond the scope of this study.

In concordance with the increased risk of vertebral fractures women with the *ESR1*/nc *ESR2*/r combination had relatively lower lumbar spine BMD levels, although no interaction was observed. In addition, women with this genotypic combination had significantly lower vertebral area which is consistent with biomechanical theory (60-62), but contrasts with our finding of greater height and does not fit our proposed hypothesis. Nevertheless, it should be considered that vertebral bodies are mostly trabecular bone where the action of *ESR2* is less clear (63). This could suggest that *ESR2* may, by itself or in combination with *ESR1*, exert a distinct type of regulation over the maintenance of bone mass at the vertebral bodies and merits further investigation.

An aspect that should be realized is that the polymorphisms which constitute the haplotypes of both estrogen receptors genes are most likely anonymous. Similarly, the (microsatellite) CA-repeat promoter polymorphism of the IGF1 gene has yet to be proved functional. This way, specific functional studies are needed to determine how these variants (or others in linkage disequilibrium) may interact at the molecular level, yet there is some epidemiological evidence to suspect that the polymorphisms we studied are functional because of a number of biologically consistent observations.

The absence of interactions in males could be explained by lack of power, considering the fact that the number of fractures which occur in men is considerably lower than in women. On the other hand we can not exclude that gender-differences could reflect truly different biological mechanisms, which should be the target of future studies.

Given the high number of statistical interactions that have been tested across the multiple genotypic combinations, multiple testing could play a role in our results. Nevertheless, the consistency in the occurrence of significant findings (among independent parameters) makes it unlikely that our results could be explained by

chance alone. Considering this, replication of these genetic interactions in other population-based (multicenter) studies is warranted.

In summary, this population-based study in elderly men and women age 55 and over, has shown interlocus interaction between the *ESR1*, *ESR2* and *IGF-I* genes in relation to the occurrence of bone fracture in postmenopausal women. A deleterious genetic combination for bone health may arise from having a risk genotype in *ESR2*, in the presence of higher (*ESR1*-mediated) estrogen sensitivity and enhanced genetically-determined IGF-I activity. These findings provide some insight into etiology, but most importantly, they reinforce the complex and polygenic character of osteoporosis.

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General Discussion

7

The objective of this thesis was to identify genetic determinants of osteoporosis using a candidate gene approach framework. Two main aspects of genetic association studies were addressed:

- *phenotype definition:* which included a comprehensive dissection of the epidemiological and genetic properties of the traits
- *analyses of candidate genes:* which included the selection and posterior evaluation of their genetic variants in relation to the targeted traits.

In the previous chapters we have discussed the merits and shortcomings of the individual studies. This discussion evaluates the different aspects of the proposed framework and provides suggestions for future research.

7.1 The framework at a glance

Even though the occurrence of fracture is the most important clinical outcome in osteoporosis, identifying genetic determinants determining the risk of fracture has been difficult due to its multifactorial nature and occurrence late in life. Intermediate phenotypes which could help explain the risk of fracture have therefore attracted the interest of researchers in the field of osteoporosis. Measurements of BMD by dual-energy X-ray absorptiometry (DXA) are currently the most widespread method to evaluate the risk of fracture, and thus the object of genetic studies. However, BMD values are dynamic and ambiguous in nature, considering that many biomechanical properties intrinsic to the risk of fracture are not well reflected by the BMD measurement. Consequently, the first step we took in our quest for genetic determinants was a phenotypic dissection of the osteoporosis traits. Initially, we evaluated the epidemiological aspects of femoral neck BMD decline and of changes in bone geometry in the elderly population of the Rotterdam Study. Then, we quantified how much of the variability in BMD and bone geometry is explained by genetic factors. To do this, we have studied a genetic isolated population part of the Erasmus Rucphen Family (ERF) Study, for the analysis of heritability and genetic correlation across diverse osteoporosis traits including BMD and bone geometry measurements. Based on these analyses, we proposed a theory on how the risk of fracture could be heritable and related to biomechanical adaptation. After the definition of the most heritable osteoporosis traits we would focus on, we moved on to a candidate-gene approach to identify specific genetic factors. The first candidate gene we chose was the insulin-like growth factor I (*IGF-I*). IGF-I is a ubiquitous polypeptide with a recognized role in bone metabolism and an obvious candidate gene to study in osteoporosis. Nevertheless, previous studies remained conflicting regarding its possible relationship with BMD while none had yet studied its relationship with fracture(1-4). The second candidate gene we chose

was the estrogen receptor β (*ESR2*), far less well-studied in relation to osteoporosis than the other estrogen receptor α (*ESR1*) (5-12) which in fact had been subject of previous investigation in our population (5). Only few studies had examined the relationship between *ESR2* and BMD but none addressed the relationship with the occurrence of fracture (13,14). Finally, we analyzed and compared the independent gene effects with their patterns of interaction of the different traits of osteoporosis.

7.2 Osteoporosis defined

Osteoporosis has been defined as a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (15). There are many aspects within this definition which are of relevance for genetic studies. The first is that the most important clinical outcome of osteoporosis is bone fracture, even though its occurrence is not needed for the diagnosis. It has been proposed that fractures are too uncommon to allow detection of an association with genes that regulate structural determinants of bone strength (16). However, the studies presented in this thesis and other previous works prove otherwise, indicating that genetic studies of osteoporosis should always target the relationship with fracture. Furthermore, while low BMD is also a hallmark in the definition of osteoporosis, even though it has been shown that the majority of fractures occur in fact above the risk threshold set for the diagnosis of osteoporosis (17). We have therefore also focused on bone geometry, which represents the underlying structural properties that determine the way mass is distributed. In addition, bone geometry measurements allow evaluating more directly how bone fragility and susceptibility to fracture differ between individuals.

7.3 Epidemiology of osteoporosis

7.3.1 Fracture risk

The prevalence and incidence of osteoporotic fractures in the Rotterdam Study has been object of detailed research in the past (17,18). It has been shown how fractures occurring at different skeletal sites have different risk factors and specific epidemiological patterns. Considering that osteoporotic fractures are rare events and that in a complex disease like osteoporosis the contribution of individual gene variants are expected to be modest, one of the questions is if there is justification to pool together different types of fractures. Distinct epidemiological patterns suggest that diverse levels of heterogeneity exist between different types of fracture. Nevertheless, we have shown in Chapter 2 that even though the heritability of

BMD varies across the different sites of measurement, there is also a certain degree of genetic correlation between them. This allows to group different types of fractures also based on biomechanical, epidemiological and biostatistical (to gain power) aspects. Probably, the most prominent differences exist between vertebral and non-vertebral fractures. In contrast to non-vertebral fractures, vertebral fractures usually occur without falling history and are usually asymptomatic (19,20). This implies that in a prospective population-based study like the Rotterdam Study, vertebral fractures cannot be reliably ascertained from clinical records but need to be screened for in the complete population. We should, however, realize that as suggested from cross-sectional studies, the prevalence of vertebral fractures also increases dramatically (especially in women) with age (21) and they are assessed only in those subjects who survived to the second follow-up assessment. In contrast, the occurrence of the clinically evident non-vertebral fractures can be reliably monitored by accurate registry by general practitioners of the health care system. Even though both methodologies are subject to health selection bias, in contrast to vertebral fractures, the analysis of non-vertebral fracture does allow consideration of time-to-event and age-dependent relationships.

The group considered as "fragility" fractures has traditionally been suggested to share common etiological factors (i.e. to be more related to aspects of bone quality and with an increase of their prevalence with old age) (22). They include vertebral, hip, pelvic, proximal humerus and distal radius (wrist) fractures. Given the methodological differences in ascertainment described above, we have considered vertebral fractures separately. Among the rest of non-vertebral fragility fractures, we have distinguished also that the prevalence of wrist fractures undergoes a rapid increase before 75 years, to level off there after, in contrast to hip, pelvic and proximal humerus fractures which only after age 75 years show a substantial increase in their prevalence (17). Without disregarding that wrist fractures are also fragility fractures, the fact that they occur at a younger age and with specific falling patterns made us treat them as etiologically distinct phenomena. To reduce etiological heterogeneity in the genetic studies of osteoporotic fractures we have focused on the group of fragility fractures at older age (hip, pelvic and proximal humerus) rather than on all type of fractures.

7.3.2 Longitudinal changes in BMD and bone geometry

When using changes in BMD as an outcome for genetic studies some technical considerations need to be taken into account. Even within a large study as the Rotterdam Study, the use of standard protocols which guarantee quality control and adequate subject positioning, is important to diminish measurement error in serial measurements of BMD (23). In contrast to manufacturer's claims, we and others (24) have found that software upgrades do interfere with the use of longitudinal

measurements. We therefore had to reanalyze with one uniform version (DPX-IQ) all scans whose image acquisition had been done with different software versions (DPX-L), and also had to perform additional retrospective calibrations (25) to allow comparability between measurements of the same individual in time.

Another important aspect of the longitudinal evaluation of the change of BMD measurements in time are the underlying causes for those changes. It would be expected that faster rates of decrease in BMD at a moment in time are invariably reflecting increased risk of fracture. Nevertheless, BMD decreases in time due to bone loss but also due to bone expansion, with different and opposing biomechanical effects. That is why it is important to also consider changes in bone geometry in the interpretation of BMD decline. Comparatively faster or slower rates of decrease in BMD across groups should also be interpreted carefully. A group with current higher rates of BMD decline could be just going through a phase of decrease in BMD that has already happened in another group, which with a lower rate of BMD decline may still be having increased risk of fracture. This was well depicted by our findings on the patterns of BMD decline (Chapter 2) where higher levels of BMD at baseline are associated with higher rates of decline in BMD. In this respect, another possibility to explain this is the occurrence of "regression to the mean" which could be influencing the patterns of change in BMD. Though, this would only play a major role after short follow-up intervals and not in a long follow-up like we discussed in chapter 3.1.

7.3.3 Health and survival biases

Other epidemiological aspects to be considered in genetic studies is the influence of survival and health selection. As described in Chapters 1 and 4 survival plays an important role in the determination of fracture risk or patterns of BMD decline in time. In chapter 4 we observed how a relation of the *ESR2* genotype with survival can confound the relation with fracture, generating a spurious association with the occurrence of fracture. Similarly, in Chapter 1 we observed how the estimation of change in BMD was done on a relatively healthier cohort that survived until follow-up, which does not necessarily reflect the patterns occurring in the population. It should also be noticed that a genetic determinant may be related to lower BMD (and risk of fracture) through comorbidity and weight loss without influencing directly bone health. One final epidemiological aspect is the presence of secular trends that may influence the relationship between a given genotype and osteoporosis or the risk for fracture. Considering the secular trend in body height that is seen in our study population (where older generations have considerably lower height than younger ones) we have opted to adjust for age, height and weight in the vast majority of analyses between genotypes and BMD, bone geometry or the risk of fracture, instead of adjusting for age and BMI alone.

7.4 The osteoporosis phenotype

It has been proposed that progress in the genetics of bone fragility is slow because the phenotype is poorly defined (16). Low trauma fractures are the clinically relevant osteoporosis phenotype, however, they are too uncommon and the detection of an association with genes that regulate a structural determinant of bone strength is difficult. Alternatively, some genetic studies have focused on surrogate traits that assess certain characteristics of bone health. These include the determination of BMD, quantitative ultrasound and the use of biochemical markers of bone metabolism. BMD measurements are the most widely used method to assess the risk of fracture. Nevertheless, BMD is too ambiguous to allow detection of the cell- and surface- specific genetic determinants of the complex bone traits (16). We have therefore refocused the attention away from genes which determine BMD and towards the gene loci (their products, the structures formed and the genetic regulation) involved with adaptation of bone to changing loads (16). As we showed in Chapter 3, mechanosensitivity indexes of the hip were shown to be heritable and may reflect more in general the predisposition towards bone fragility with aging. And unlike BMD, they were genetically independent from size and showed high genetic correlation with bone instability, which we propose is the biomechanical property that predisposes individuals to fracture. In this perspective we propose that the head/skull BMD measurement, which has so far been neglected from studies in osteoporosis, could be of use in genetic studies of osteoporosis. Not only it is one of the most highly heritable traits in the human skeleton (probably due to the lack of influencing environmental factors especially strains), but also, we showed that it can be regarded as a possible indicator of mechanosensitivity. Rather than using isolated BMD measurements, future studies should target bone geometrical parameters and indexes of mechanosensitivity to further elucidate the underlying genetic basis of osteoporosis.

7.5 Genetics of osteoporosis

7.5.1 Candidate genes

Bone remodeling makes bone tissue one of the most metabolic active tissues in the human body. The complex underlying processes underlying bone remodeling are modulated by mechanical, endocrine, paracrine and autocrine regulation, among the most important ones. From this perspective the number of candidate genes that could be contemplated for research in osteoporosis is enormous. One of the reasons we have focused on this subset of hormones and growth factors is due

to the expertise in our group which has evolved towards the study of ligand-receptor functions. With no doubt many other candidates lie within the group of bone matrix proteins and mineral metabolism. The known biology of such genes eases the choice of them, but also facilitates interpretation of results. This explains why the candidate gene approach has been the method of choice used so far to identify most of the genes known to influence the risk of osteoporotic fracture (26).

As already described in Chapter 1, we have chosen to study the *IGF-I* and the estrogen receptor genes considering that they are ubiquitous and known to share common metabolic pathways. This provides a framework to evaluate initially their independent effects and to explore their joint action.

7.5.2 Screening for polymorphisms in candidate genes

The CA_n microsatellite polymorphism located in the 5'promoter region of the *IGF-I* gene (27) has been studied in relation to diverse outcomes, including osteoporosis. Even though no functional studies have been performed to date, its location in the 5'regulatory region of the gene made it a very interesting variant to be studied. Considering that in our population this polymorphism has been related to IGF-I plasma levels and to body height, it is likely that it or another genetic variant in LD with this polymorphism, is responsible for differential IGF-I action between individuals.

In contrast, genetic variants in the *ESR2* gene are however, much less studied. Therefore, we have opted to search for polymorphisms in the gene by consulting literature and SNP databases in order to select the most suitable ones for analysis. The rationale behind this approach is that LD seems to have a block-like structure in genes and that a judiciously chosen subset of SNP's can identify most of the genetic variation in any genomic region through haplotypes (28). A previous study has demonstrated that, within LD blocks, more than 90% of the diversity of common haplotypes (>5%) may be captured by six to eight common SNPs and that these common haplotypes explain the vast majority of genetic variation contributed by unmeasured or undiscovered SNP's (29). In this way, a good representation of the polymorphisms in the gene can be tested by choosing a number of polymorphisms similarly spaced through out the gene and analyzed in the form of haplotypes (30). *ESR2* showed a high degree of LD between most of the polymorphisms, a finding that concurs with another population-based study of *ESR2* variants in a Caucasian cohort (31). The haplotype analysis we have performed in the *ESR2* gene makes it likely that the genetic variant(s) responsible for differential effects exerted by the *ESR2* gene are being picked up by the constructed haplotype. However, more in depth analysis of *ESR2* variants will be necessary to test this.

7.5.3 Genetic effects

It has become clear that there is not one major single gene responsible for the risk of osteoporosis (32). Rather, an individual's susceptibility to develop osteoporosis is determined as in most common diseases, by several common gene variants with modest but real genetic effects (33). We have found how the *IGF-I* promoter and *ESR2* polymorphisms are related independently to the risk of osteoporotic fracture in the general population. In concordance with these findings, we have also found them associated in a consistent way to diverse structural parameters of bone, suggesting that probably, these are not spurious findings. Nevertheless, replication in other large population cohorts is obviously necessary. As expected for complex diseases their independent genotype effects are quite modest (33,34). Yet, when considered in combination, we have seen that their genetic effects may rise beyond the sum of their individual deleterious effects, postulating gene interaction as an important determinant of disease in a subset of individuals (fraction of the population with risk alleles) with a specific deleterious combination of genetic determinants. Nevertheless, an important lesson we have come upon the study of gene interactions is that a higher risk for disease will not only arise from the combination of risk genotypes, but may also arise from unexpected combinations involving non-risk genotypes.

7.5.4 Population stratification

Spurious associations can result from population stratification either because of a recent admixture of a different population or because of inappropriate matching of cases and controls (35). This is a growing concern for association studies, especially in the United States of America where much recent admixture has occurred. The occurrence of spurious associations due to population stratification is unlikely in our study population, since both cases and non-cases are sampled from the same ethnically homogeneous source population, while the population is also stable with very little immigration/emigration.

7.6 Future research

Our research has only evaluated a small set of candidate genes that could be related to the risk of osteoporosis. Nevertheless, we think we have made some progress towards elucidating the genetic basis of osteoporosis when using the framework described in this thesis. We have contributed to the understanding of how genetic variants of the *IGF-I* and estrogen receptors could influence the occurrence of osteoporosis independently or in interaction. The next step is to continue

the study of additional candidate genes, which could provide further elucidation of these metabolic systems.

With no doubt, rapid progress in the fields of genetics and the availability of DNA chips and microarray technology, will lead to the common execution of large-scale genome-wide association studies (36). Such association studies involve the evaluation (dependent on linkage disequilibrium) of all existing genes in the genome of an individual, using evenly spaced SNP markers with a very dense resolution (5-6 kb) and in relation to disease status or traits (37,38). Although high throughput genotyping is currently possible, some time will pass before it is feasible to apply such approximation on population-based studies in thousands of individuals.

In the immediate future, we will target the study of other polymorphisms within the *IGF-I* gene, but also in other genes of the IGF-I regulatory pathway (i.e. IGFBP-3 or IGF-I receptor) and other genes of relevance for estrogen production and action.

Similarly, we will have to develop new analytical strategies to evaluate gene interactions, considering the power limitations which arise from these attempts to evaluate epistatic effects. Even in a large population-based study like the Rotterdam Study (n=7000), the evaluation of a small number of gene interactions already rapidly compromises the power needed for robust assessment. It has been proposed that alternative statistical modeling may provide the tool necessary to overcome such situations (39-44). Replication and validation of such interactions will have to be pursued in large scale collaborations between studies, which increase power by pooling cohorts. The recently set up European consortium GENOMOS is an excellent example of such an effort in osteoporosis research, and has already identified the *ESR1* haplotype 1 as a susceptibility allele in osteoporosis (Ioannidis et al. JAMA, in press). We have been recently funded with a project to develop genetic risk models targeted on the identification of gene interactions that influence the risk of osteoporosis. In this project we will focus on developing analytical tools that will allow the simultaneous evaluation of multiple genetic determinants and the identification of genetic risk profiles that could be of use in clinical practice to predict the occurrence of multifactorial diseases.

Additionally, our future research will not only involve a candidate gene approach in a population-based setting like the Rotterdam Study. The Family Osteoporosis (FAMOS) sample collection and database is an example where family-based studies genome scans are pursued to identify genetic determinants of bone mineral density, fracture and other osteoporosis-related phenotypes. The FAMOS study group involves in addition to our group in Rotterdam, other leading figures in osteoporosis research based in Oxford, Cambridge, London, Southampton, Aberdeen, Glasgow

and Aarhus. Furthermore, within our group we also have the opportunity to further explore the linkage approach methodology in a genetically isolated population like the ERF study (already described in this thesis) which possesses well-defined advantages that favor the identification of genetic determinants in complex diseases (45). Together, these resources provide important research material and opportunities in our quest for genetic determinants of osteoporosis.

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Summary

8

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. It is a condition of very high prevalence in the elderly, with associated morbidity and mortality which represents enormous costs for health systems. Since the completion of the human genome project, the study of the genetic basis of complex diseases and hence osteoporosis has gained evolving importance. This etiologic genetic research may result in further understanding of the underlying pathophysiology of osteoporosis and could lead to the development of specific diagnostic, therapeutic and preventive strategies. The objective of this thesis was to identify genetic determinants of osteoporosis using a candidate gene approach framework. Two main aspects of genetic association studies were addressed: (1) a thorough definition of osteoporosis phenotypes including a comprehensive dissection of the epidemiological and genetic properties of the traits, and (2) the analyses of variations in two candidate genes: the insulin-like growth factor I (*IGF-I*) and the estrogen receptor beta (*ESR2*).

In **Chapter 2** we evaluated in the elderly population of the Rotterdam Study the epidemiological aspects of femoral neck BMD decline and of changes in bone geometry as assessed by hip structural analysis (HSA) after an average follow-up of 6.5 years. We found that increasing age (age 75 years or older), BMD and weight loss with follow-up are the most significant determinants of bone loss in both genders. HSA showed that BMD declines in the elderly due to bone loss (cortical thinning), but also due to increase in neck width (periosteal apposition), with both processes being more prominent in (postmenopausal) women than in men. Similarly, it was found that women had faster progression towards bone instability than men, which could explain the higher susceptibility for hip fracture in elderly women.

In **Chapter 3** we quantified how much of the variability in BMD and bone geometry is explained by genetic factors. To this end, we studied a part of the genetically isolated population of the Erasmus Rucphen Family (ERF) Study, for the analysis of heritability and genetic correlations across diverse DXA-based measurements including BMD and HSA. We found that the heritability of BMD is site-specific and influenced by genes determining height and lean mass. In addition we determined that HSA parameters of bone dimensions, strength and instability are also heritable. In addition, we studied the mechanosensitivity of individuals (defined as the relation between skeletal loading and bone strength) and found it to be a heritable trait genetically-correlated to bone instability. We propose that susceptibility to fragility fracture at older age is heritable partly because they reflect genetically-determined differences in mechanosensitivity.

In **Chapter 4** we applied the candidate gene approach to study a polymorphism in the promoter region of the insulin-like growth factor I (*IGF-I*) gene in relation to bone mineral density, bone geometry and the risk of non-vertebral fractures in the Rotterdam Study. In **Chapter 4.1** we evaluated the relationship with BMD and determined that this promoter polymorphism or another functional polymorphism in linkage disequilibrium may be a genetic determinant of BMD levels and rate of bone loss in postmenopausal women. In **Chapter 4.2** we examined the relationship with the risk of fracture and found that the absence of the *wild-type* allele of the promoter polymorphism is associated with increased risk of fragility fracture in women but not in men. In addition, we analyzed an approximation of hip bone geometry (from DXA scans). The results of this analysis suggested the polymorphism is associated with bone strength and stability in gender-specific ways.

In contrast to estrogen receptor alfa (*ESR1*), genetic variants in estrogen receptor beta (*ESR2*) have been much less well studied in relation to osteoporosis. In **Chapter 5** we evaluated within the Rotterdam Study variations in *ESR2* in relation to bone mineral density, and for the first time in relation to bone geometry and the risk of osteoporotic fractures. We determined that there is high linkage disequilibrium between polymorphisms scattered through the gene and constructed haplotypes for analysis. We found that female homozygous carriers of the most frequent haplotype have increased risk of vertebral and fragility fractures. In line with these findings, hip structural analysis of DXA-scans showed that this group of women have wider femoral necks and thinner cortices, and consequently higher bone instability. In males, a decreased risk for non-vertebral fracture was seen in homozygous carriers of the haplotype, which can possibly be explained by selection on male survival.

In **Chapter 6** we studied the interaction between the two estrogen receptors (*ESR1* and *ESR2*) in relation to the risk of osteoporotic fracture and associated traits within the Rotterdam Study. In addition, we evaluated this interaction in relation to the *IGF-I* promoter polymorphism genotypes. We found that as compared to non-carriers of any of the risk haplotypes in the estrogen receptor genes, women who are homozygous carriers of the risk haplotypes in the *ESR2* gene and non-carriers of the risk haplotype in the *ESR1* gene have increased risk of vertebral and fragility (hip, pelvis and proximal humerus) fracture. This effect was accentuated in the group of individuals who were homozygous for the *wild-type* allele of the *IGF-I* promoter polymorphism. Similar interactions were observed for BMD, bone geometry and body height measurements. No evidence of such interactions was observed in men. This way, we conclude that interlocus interaction between *ESR1*, *ESR2* and IGF-I gene polymorphisms influences the risk of fracture in postmenopausal women and reinforces the complex and polygenic character of osteoporosis.

In the general discussion in **Chapter 7** general principles and problems of this candidate gene approach framework are discussed. In addition the findings in this thesis are placed in perspective of future research and opportunities that arise within our quest for genetic determinants of osteoporosis.

Osteoporose (botontkalking) is een aandoening die wordt gekenmerkt door een lage botmassa en een verslechtering van de microarchitectuur van het botweefsel, wat resulteert in een verhoogde botfragiliteit (broosheid van het bot) en een verhoogde kans op botbreuken. Het is een aandoening die vooral voorkomt bij ouderen en gepaard gaat met een verhoogde kans op ziekte en sterfte, maar ook met hoge kosten voor de maatschappij.

De voltooiing van het in kaart brengen van het menselijk genoom (human genome project) heeft het mogelijk gemaakt om de genetische basis van complexe ziekten, zoals osteoporose, te bestuderen. Door genetisch onderzoek kan een groter begrip van de onderliggende oorzaken van osteoporose ontstaan, hetgeen zou kunnen leiden tot de ontwikkeling van specifieke diagnostische, therapeutische en preventieve strategieën. De doelstelling van dit proefschrift is het identificeren van genetische factoren die een rol spelen bij het ontstaan van osteoporose. Er is hier gebruik gemaakt van twee belangrijke aspecten van genetische associatiestudies: (1) een grondige definitie van de osteoporose fenotypen, met een uitgebreide ontrafeling van de relevante epidemiologische en genetische eigenschappen en (2) de analyse van genetische varianten in het insuline-achtige groeifactor I (*IGF-I*) gen en in het oestrogeenreceptor beta (*ESR2*) gen.

In **hoofdstuk 2** worden de epidemiologische aspecten van afname van de botmineraaldichtheid (BMD) en veranderingen in de botgeometrie bestudeerd in de Rotterdam Studie, over een periode van gemiddeld 6,5 jaar. Bij zowel mannen als vrouwen zijn de belangrijkste determinanten van botverlies een hogere leeftijd (75 jaar of ouder), een hogere uitgangs-BMD en gewichtsverlies. Onderzoek van de geometrie van het heupbot toonde aan dat afname van de botmassa bij ouderen zowel een gevolg is van botverlies (dunner worden van de cortices) als van een toename van de wijdte van de dijbeenhals (periosteale botvorming). Beide processen zijn meer uitgesproken bij vrouwen na de overgang dan bij mannen. In overeenstemming hiermee hadden vrouwen een snellere progressie naar bot-instabiliteit, hetgeen een verklaring zou kunnen zijn voor de grotere kans op heupfracturen bij oudere vrouwen.

In **hoofdstuk 3** onderzochten we in welke mate de variatie in BMD en botgeometrie verklaard kan worden door erfelijke factoren. In een deel van de genetisch geïsoleerde populatie uit de Erasmus Rucphen Familie (ERF) studie, bepaalden we de erfelijkheid en genetische correlaties van verschillende met DXA (dual energy X-ray absorptiometry) gemeten parameters, inclusief BMD en geometrie. Uit de analyses van de gegevens bleek dat de mate van erfelijkheid van botmineraaldichtheid afhangt van de plaats in het skelet en dat de erfelijkheid wordt beïnvloed door genen die de lichaamslengte en de vetvrije massa bepalen. Ook hebben we vastgesteld dat de parameters van botgeometrie zoals botafmetingen, botsterkte

en instabiliteit erfelijk zijn. Daarnaast hebben we de relatie tussen de belasting van het skelet en sterkte van het bot bestudeerd in de vorm van een index van mechanosensitiviteit. Deze index bleek eveneens erfelijk te zijn en genetisch gecorreleerd met botinstabiliteit. Wij zijn van mening dat de erfelijkheid van de gevoeligheid voor osteoporotische fracturen (breuken) op hogere leeftijd, de genetisch bepaalde verschillen in mechanosensitiviteit weerspiegelen.

In **hoofdstuk 4** hebben we een polymorfisme in het promotor gebied van het insuline-achtige groeifactor I (*IGF-I*) gen in relatie tot BMD, botgeometrie en de kans op fracturen bestudeerd. Dit hebben we gedaan in de Rotterdam Studie. In **hoofdstuk 4.1** beschrijven we de relatie met BMD. Het door ons bestudeerde promoter polymorfisme of een ander hieraan gekoppeld polymorfisme lijkt een genetisch bepalende factor voor de hoogte van de BMD en de snelheid van botverlies bij postmenopausale vrouwen. In **hoofdstuk 4.2** bestudeerden we de risico op fracturen en vonden we dat afwezigheid van de meest voorkomende variant van het promotor-polymorfisme (het zogenaamde wild type allel) geassocieerd is met een verhoogd risico op osteoporotische fracturen bij vrouwen, maar niet bij mannen. Daarnaast analyseerden we ook een benadering van de heupgeometrie. De resultaten van deze analyse suggereerden dat het polymorfisme voor de twee geslachten op verschillende wijzen samenhangt met botsterkte en botstabiliteit.

Vergeleken met het oestrogeenreceptor alfa (*ESR1*) gen is er relatief weinig onderzoek gedaan naar de relatie tussen genetische varianten in het oestrogeenreceptor beta (*ESR2*) gen en osteroporosis. In **hoofdstuk 5** evalueren we in de Rotterdam Studie de varianten in het ESR2 gen in relatie tot BMD en voor het eerst in relatie tot botgeometrie en de kans op osteoporotische fracturen. We typeerden verschillende polymorfismen verspreid in het *ESR2* gen. Omdat er een hoge mate van afhankelijkheid was tussen de varianten van de verschillende polymorfismen (linkage disequilibrium) construeerden we haplotypen. Deze haplotypen gebruikten wij bij de verdere analyses. Een verhoogd risico op wervel- en andere osteoporotische fracturen werd gezien bij vrouwelijke homozygote dragers van het meest frequente haplotype. Hiermee samenhangend liet de geometrie-analyse zien dat deze vrouwen een wijdere dijbeenhals, een dunnere cortex en een hogere botinstabiliteit hadden. Mannelijke homozygote dragers van dit haplotype hadden een verlaagd risico op het ontstaan van niet-vertebrale fracturen, hetgeen mogelijk te verklaren is door selectie in overleving van mannen.

In **hoofdstuk 6** bestudeerden we in de Rotterdam Studie de interactie tussen de twee oestrogeenreceptoren (*ESR1* en *ESR2*) en het risico op het ontstaan van osteoporotische fracturen en geassocieerde kenmerken. Daarnaast hebben we deze interactie bekeken in relatie tot de genotypen van het *IGF-I* promotor polymorfisme. In vergelijking met individuen die geen drager zijn van de risicohaplotypen

van beide oestrogeengenen, hebben vrouwen die homozygote dragers van het risicohaplotype van het *ESR2* gen zijn maar *geen* drager zijn het risicohaplotype van het *ESR1* gen, een verhoogd risico op wervel- en andere osteoprotische (heup, bekken en bovenarm) fracturen. Eenzelfde interactie zagen we voor BMD, botgeometrie en lichaamslengte. Deze interactie was het sterkst in homozygote dragers van het wild type variant op het *IGF-I* gen. Bij mannen vonden we geen aanwijzingen voor een dergelijke interactie. Hieruit concluderen we dat er een interactie tussen polymorfismen van het *ESR1*, *ESR2* en *IGF-I* gen bestaat in relatie tot het ontstaan van fracturen bij postmenopausale vrouwen. Bovendien benadrukken deze uitkomsten het complexe en polygenetische karakter van osteoporose.

In de algemene discussie in **hoofdstuk 7** worden algemene principes en problemen van deze genetische associatiestudie bediscussieerd. Daarnaast worden de bevindingen in dit proefschrift geplaatst in een perspectief van toekomstig onderzoek en mogelijkheden die voortkomen uit onze zoektocht naar genetische determinanten van osteoporose.

Epilogue

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Prof. Dr. Jaime E. Bernal Villegas my mentor who I owe my passion for genetics. I hope we will be launching soon many more Human Expeditions and Mega Research Projects. El doctor Ignacio M. Zarante, Compadre Nacho, with whom I've shared my passion for research, thanks for your friendship… será Ubaté el nuevo megaproyecto? En la Pontificia Universidad Javeriana quisiera agradecer al Padre Rector Gerardo Remolina Vargas S.J. y de manera especial al Doctor Francisco Henao, Decano de la Facultad de Medicina, quien ha apoyado desde un principio mi entrenamiento en Holanda. Toda la gente del Instituto de Genetica Humana, con la que aprendí muchísimo de estudiante, Martaele T., Juan Carlos P. y Clemencia D., Martha B., Juana A. y Manuel F., Gloria O., Alberto G., Ángela U., Ignacio B., Diana T., Sara P., Roberto M., Carlos G. y Fernando S. y mis superamigas Maria Claudia L. y Adriana O. De la Unidad de Epidemiología Clínica de entonces Juan Manuel L., Rodolfo D., Juan Gabriel R., Álvaro R., Carlos G., Mery B., Darío L., Martha D., Claudia G., M. Nelcy R. y Adriana P., Gabriel G., Javier A. Por supuesto, a Luis Gabriel en UK con quien seguimos en contacto.

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Fernando Rivadeneira Ramírez
Rótterdam, October 2004

BIOGRAPHY

Personal view by ANTONIO JOSE RIVADENEIRA VARGAS
English version by ALICIA RAMIREZ DE RIVADENEIRA

Fernando Rivadeneira Ramírez, was born in Bogotá, Colombia, on January 12th, 1971. He is the youngest of six siblings born to Antonio José Rivadeneira Vargas and Alicia Ramírez Díaz. He attended San Carlos School in Bogotá, which is run by Benedictine monks. There, he was known as excellent student, great classmate and sports leader. While still a boy, he travelled with his father to the neighbouring country of Venezuela. In the city of Güanare, he improvised a public greeting to the former president, Luis Herrera Campins. For this reason, a commendable chronic was written in a newspaper in Caracas under the title "Interesting People". In the same column, they also published one of his short stories titled "A Desire of Love". His visit to the Pantheon (in Caracas) grave of Simon Bolívar, the *Liberator* of many Latin-American countries, inspired him another story called "The Defeat". In it, he emphasized his faith in freedom. At the age of 11 years, a third story, "The Rose Bush and the Lover" was published in his school newspaper in April 1982.

In 1989 he graduated from high school with excellent results and started medical studies at the Pontificia Universidad Javeriana. He had to interrupt his studies to join the compulsory military service. He performed his military task with great duty and he was highly valued by his superiors. Nevertheless, he clearly stated to them that he considered himself destined by God to save lives and not to take them. As a reward to his conduct and his proficiency in the English language, in March 1990 he joined up the Multinational Force & Observers in the Sinai Peninsula, Egypt. During his stay there, he followed an intensive course of paramedic aid organized by the American Army and was awarded a medal for achieving the first place. He returned to Colombia to continue his medical training. In 1996, he received his medical degree from the Universidad Javeriana. Thanks to the support, stimuli and inspiration from his professor, doctor Jaime Eduardo Bernal Villegas, he started his research work in the Human Genetics Institute while he made his Masters in Clinical Epidemiology in the same university.

In the year 2000 he travelled to Rotterdam, The Netherlands to continue his post-graduate training. He received a Master in Science Degree in Genetic Epidemiology in the Netherlands Institute of Health Sciences (NIHES). In 2001, he was appointed by the departments of Internal Medicine and Epidemiology & Biostatistics to perform the studies described in this thesis.

Since his childhood, he showed great aptitude for study and research. He is the pride of his parents and brothers. His relatives, as well as his professors, colleagues, collaborators and friends admire his wonderful human qualities. His great loves are his wife, Maria Carolina Pardo and his daughters born in The Netherlands: Gabriëlla, who according to his grandfather and author of this biography, combines the delicate essences of the tulip and the orchid; and Paulina, who thanks to God only opened her eyes to the world on September 26th 2004, after her father had finished this thesis.

SEMBLANZA BIOGRAFICA

Perspectiva personal por ANTONIO JOSE RIVADENEIRA VARGAS

Fernando Rivadeneira Ramírez nació en Bogotá, Colombia, el día 12 de enero de 1971, es el menor de seis hermanos en el hogar formado por Antonio José Rivadeneira Vargas y Alicia Ramírez Díaz. Cursó estudios primarios en el Colegio San Carlos de Bogotá, regentado por padres Benedictinos. Allí se distinguió como estudiante sobresaliente, gran compañero y líder deportivo. Niño aún, viajo con su padre a la República de Venezuela y en la población de Güanare improvisó un saludo al entonces Presidente Luis Herrera Campins. Con este motivo se escribió sobre él una elogiosa crónica en un periódico de Caracas bajo el título "Gente Interesante". Allí se reprodujo además uno de sus cuentos titulado "Un Deseo de Amor". Otro escrito titulado "La Derrota" fue inspirado en su visita al Panteón de Caracas, donde reposan los restos del Libertador Simón Bolívar. En el destacó la fe que siempre tuvo en el triunfo de la libertad. Un tercer cuento suyo, "El Rosal y el Enamorado" fue publicado en el periódico de su colegio en abril de 1982 a la edad de 11 años.

En 1989, una vez terminado el bachillerato en forma sobresaliente, se matriculó en la Pontificia Universidad Javeriana, en la carrera de Medicina, pero debió interrumpir los estudios para prestar el servicio militar obligatorio, donde actuó con pundonor y ganó el aprecio de sus superiores a quienes de manera enfática les manifestó que se consideraba destinado por Dios para salvar vidas y no para quitarlas. Como premio a su conducta y por su dominio del idioma inglés, en marzo de 1990 fue destinado a formar parte de la Fuerza Multinacional de Observadores en la Península del Sinaí, Egipto. Allí siguió un curso intensivo de paramédico, organizado y dictado por el Ejercito Norteamericano, quienes le otorgaron una medalla tras ocupar el primer puesto. De regreso en Colombia prosiguió sus estudios de manera que en 1996 la Universidad Javeriana le otorgó el grado de Doctor en Medicina. Gracias al apoyo, estímulo e inspiración que le brindó, su profesor, el doctor Jaime Eduardo Bernal Villegas, se vinculó como investigador al Instituto de Genética Humana mientras realizaba su Maestría en Epidemiología Clínica en la misma Universidad.

En el año 2000 viajó a Rótterdam, Holanda a continuar sus estudios de postgrado. Allí recibió su grado de Maestría en Epidemiología Genética del Netherlands Institute of Health Sciences (NIHES). Desde el año 2001 forma parte de los departamentos de Medicina Interna y Epidemiología & Bioestadística del Centro Médico Erasmus donde realizó los estudios de investigación descritos en esta tesis.

Desde su infancia ha demostrado particular vocación por el estudio y la investigación, es orgullo de sus padres y hermanos y posee una estupenda calidad humana que le ha merecido el cariño de familiares, como también la admiración y el aprecio de sus profesores, colegas, colaboradores y amigos. Constituyen el centro de sus afectos su esposa, Maria Carolina Pardo y sus hijas nacidas en Holanda: Gabriëlla, de quién su abuelo y autor de esta semblanza dijo que reunía en sí las delicadas esencias del tulipán y la orquídea; y Paulina, quien gracias a Dios abrió sus ojos al mundo el 26 de septiembre del 2004, sólo después que su padre hubiese terminado esta obra.

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Young Investigator Award 2003. American Society for Bone and Mineral Research (ASBMR). *Association of an IGF-I Gene Promoter Polymorphism and Hip Bone Geometry and its Influence on the Risk of Hip Fracture: The Rotterdam Study.*

NIHES Best Article Award 2000-2001. Netherlands Institute for Health Sciences (NIHES). *Association Between an Insulin-like growth Factor I Gene Promoter Polymorphism and Bone Mineral Density in the Elderly: The Rotterdam Study.*

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