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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

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Studies of the roles 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 (~2 yr later), respectively. Femoral BMD measurements were performed using dual energy x-ray absorptiometry. In women, baseline BMD levels were, on the average, 0.02 g/cm^2 [95% confidence interval (CI) for difference, −0.03, −0.00 g/cm^2] lower in individuals 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 noncarriers, 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. (*J Clin Endocrinol Metab* 88: 3878–3884, 2003)

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 and 80% (2, 3). The contribution of genetic factors to the regulation of bone loss has been much less well studied, and data are conflicting (4). Although 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 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. To date, several genes have been investigated, including the vitamin D receptor, collagen type Iα1 (COL1α1), IL-6, and TGFβ 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 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 in both males and females. Reduced plasma levels have been associated with low BMD (12–14), osteoporosis (15), and fractures (16), although it is not known whether 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 found that birth weight (28, 29), body height, and serum levels of IGF-I after age 55 yr (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).

The results of genetic studies regarding the relation of this polymorphism to osteoporosis and BMD have been contro-

Abbreviations: BMD, Bone mineral density; BMI, body mass index; CI, confidence interval; DEXA, dual energy x-ray absorptiometry.

versial. 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 sibling pairs and 363 premenopausal 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 BMD 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 (aged 55 yr 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 and Methods

The analysis was performed in two phases. In the first phase (baseline), all individuals with complete BMD and anthropometric measurements (n = 5648) were used for a cross-sectional analysis of femoral BMD levels. In the second phase (follow-up), subjects who 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-yr strata were defined, starting at age 55 yr. Height (centimeters) and weight (kilograms) were measured in a standing position wearing indoor clothes without shoes. Body mass index (BMI; kilograms per meter squared), was calculated as weight divided by the square of height. BMD measurements (grams per square centimeter) of the proximal femur were performed by dual energy x-ray absorptiometry (DEXA) using a DPX-L densitometer (Lunar Radiation Corp., Madison, WI). Methods, quality assurance, accuracy, and precision issues of the DEXA measurements have been described previously (36).

Approximately 2 yr (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 (milligrams per square centimeter 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

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 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 deoxy-NTP, 2.2 mM $MgCl_2$, 0.01% W1 (Invitrogen, San Diego, CA), and 0.4 U *Taq* DNA polymerase (Invitrogen). PCR was performed in 384-well plates (94 C for 5 min; 35 PCR cycles of 30 sec at 94 C, 30 sec at 55 C, and 30 sec at 72 C; 72 C for 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 version 3.1, PE Applied Biosystems, Foster City, CA; 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 sizes of the PCR products were determined in comparison with the internal ROX 500-size standard (PerkinElmer, Norwalk, CT). The two highest peaks were labeled (binned) with Genotyper software version 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 noncarriers 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 SDs were computed for all measurements and compared with those of the same gender in the reference population using *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 performed 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 SPSS package version 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 in the two analytical phases with all women and men of the Rotterdam study (reference population n = 7983). Overall, women showed a 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 in the group used for the follow-up analysis. The use of concurrent estrogen replacement therapy or bone modulators in women is very low.

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

TABLE 1. Allele and *192-bp* genotype frequency distributions of the study populations

Polymorphism		Reference	Baseline analysis (%)			Follow-up analysis (%)		
Length PCR product (bp)	$(CA)_n$	Total (%); (n = 7012)[a]	Total (n = 5648)	Females (n = 3265)	Males (n = 2383)	Total (n = 4134)	Females (n = 2341)	Males (n = 1793)
198	22	1.6	1.5	1.4	1.6	1.5	1.4	1.5
196	21	6.8	6.8	6.9	6.8	6.8	6.7	7.0
194	20	19.1	19.4	19.4	19.4	19.5	19.8	19.1
192	19	65.9	65.7	65.5	66.1	65.7	65.3	66.3
190	18	4.4	4.4	4.5	4.2	4.3	4.4	4.1
188	17	1.7	1.7	1.7	1.7	1.7	1.8	1.6
Other rare alleles[b]		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

[a] All Rotterdam Study participants genotyped for the IGF-I promoter polymorphism.
[b] 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			Baseline analysis		Follow-up analysis	
	Total (n = 7983)	Females (n = 4878)	Males (n = 3105)	Females (n = 3265)	Males (n = 2383)	Females (n = 2341)	Males (n = 1793)
Age (yr)	70.6 (9.8)	71.7 (10.3)	69.0 (8.7)	68.3[a] (8.2)	67.5[a] (7.6)	69.2[a] (7.7)	68.6 (7.3)
Height (cm)[b]	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 (kg)[b]	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)[b]	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 (yr)		21.2 (11.0)		18.7[a] (9.5)		19.4[a] (9.1)	
HRT[c] or bone modulators (%)		2.0		2.7		2.8	
BMD (g/cm^2)							
Femoral neck[d]	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)
Word triangle[d]	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[d]	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[e]							
Absolute (g/cm^2-yr)	−0.004 (0.03)	−0.004 (0.03)	−0.003 (0.03)			−0.004 (0.04)	−0.003 (0.03)
Relative (% of baseline-yr)	−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 (%).
[a] $P < 0.01$.
[b] Hormone replacement therapy.
[c] Based on 6917 individuals with present antropometric measurements.
[d] Based on 5823 individuals with present BMD measurements.
[e] Based on 4331 individuals with present BMD measurements.

BMD adjusted for age and BMI (Fig. 1) increased with the number of *192-bp* alleles in the genotypes (P for trend = 0.02). BMD was, on the average, 0.02 g/cm^2 [95% confidence interval (CI) for difference, −0.03, −0.00 g/cm^2] lower in women without the *192-bp* allele than in homozygotes for the allele ($P = 0.03$). This effect was also consistent at the other femoral sites (P for trend = 0.01 and $P < 0.01$ for the trochanter and Ward's triangle, respectively). When analyzing in age strata (Fig. 2), this effect was only observed in women older than 65 yr; the differences between homozygous women and noncarriers of the *192-bp* allele were −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 and 75 yr and older age categories, respectively. 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 (Fig. 3) or within age groups (data not shown).

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 and −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 (Fig. 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).

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, because 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).

The 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). As individuals were selected on the basis of having complete BMD measurements 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 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, as our allele frequencies are similar to those reported in other Caucasian populations (27, 34).

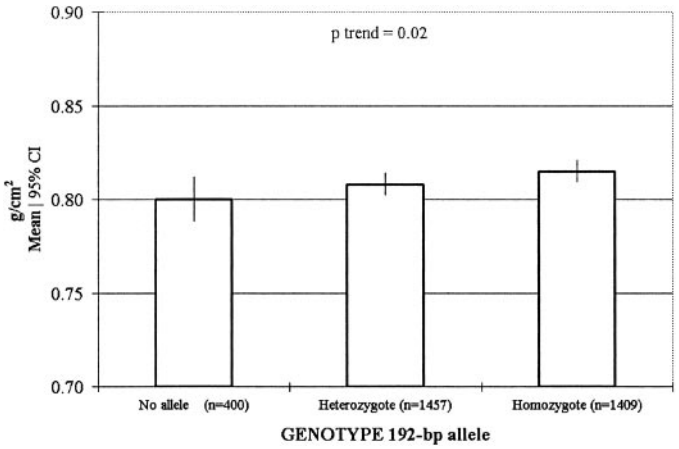

FIG. 1. Baseline mean BMD of the femoral neck within 192-bp genotype groups in women. Data are adjusted for age and BMI.

The short follow-up time (~2 yr) 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, as 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). As differences in estrogen levels influence the IGF-I regulatory axis (42–45), the hypoestrogenic state inflicted by the menopausal process in women may be (in the absence of concurrent osteoporotic treatment, *i.e.* hormone replacement therapy) directly modulating 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, as IGF-I levels begin to fall in both genders long before the age of menopause (14). Alternatively, genetically 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 yr, the genotype effect on BMD levels is only evident after age 65 yr. This may reflect the time it takes for these genotype effects

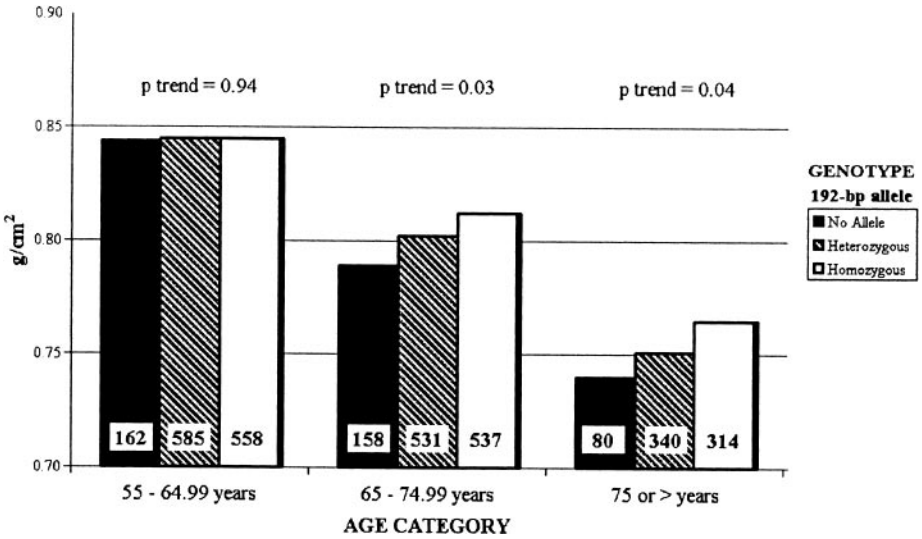

FIG. 2. Baseline mean BMD of the femoral neck within 192-bp genotype groups and age categories in women. Data are adjusted for age and BMI.

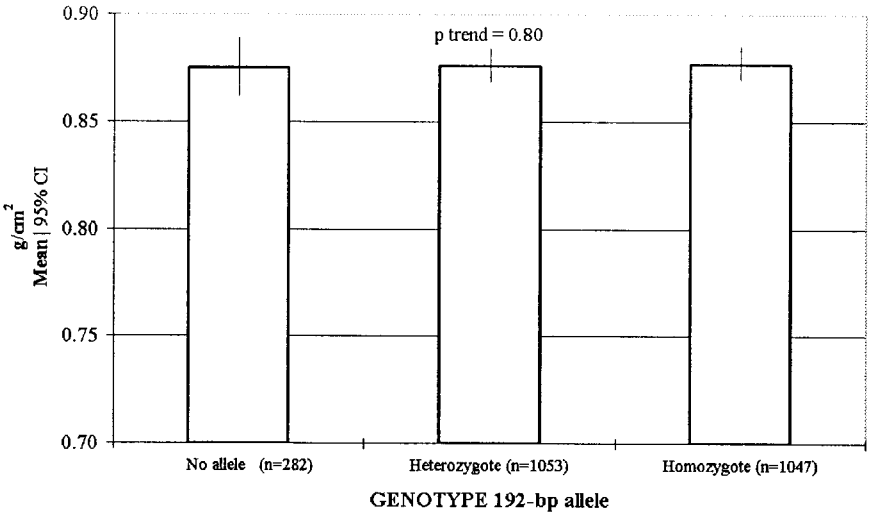

FIG. 3. Baseline mean BMD of the femoral neck within 192-bp genotype groups in men. Data are adjusted for age and BMI.

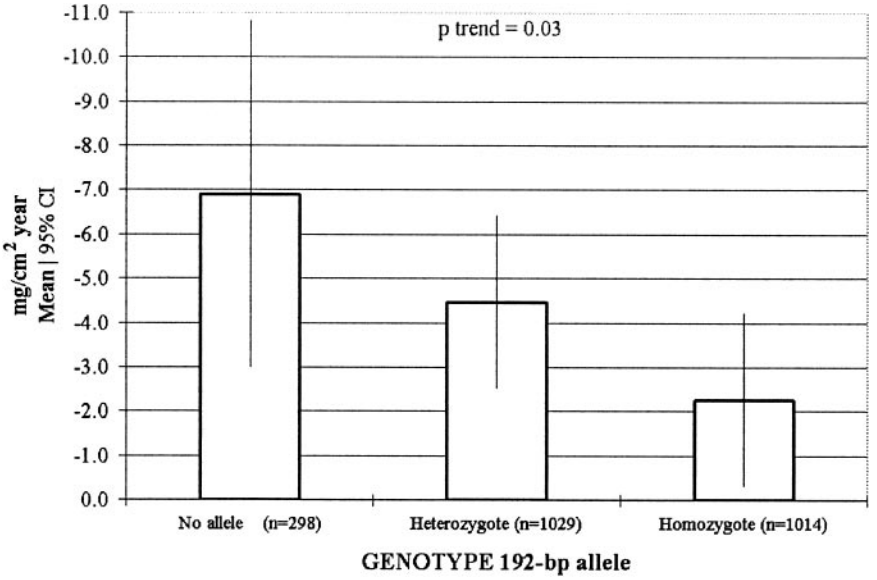

FIG. 4. Mean BMD change in the femoral neck within 192-bp genotype groups in women. Data are adjusted for age, BMI, and baseline BMD.

(during postmenopausal estrogen deficiency) to become detectable on BMD differences. Evaluation of possible biological 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 compared with 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, as DEXA 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.* (48) postulated previously that IGF-I could be related to the gender differences evidenced in bone geometry with aging.

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 premenopausal women, compared with the postmenopausal population studied by us. Furthermore, the failure to identify linkage of this polymorphism to BMD in their sibling 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 with theirs. Differences exist between the Dutch and both Japanese and Korean populations in allele and genotype frequencies, in linkage disequilibrium and in racial phenotypes.

In contrast to our findings, Rosen *et al.* (31) associated the presence of the *192-bp* allele with lower total IGF-I serum levels and male osteoporosis. Male patients with idiopathic osteoporosis represent a very distinct phenotypic trait compared with 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 whether 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), because linkage disequilibrium 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 bone architecture. This population-based study provides substantial evidence to link genetically determined levels of IGF-I to BMD in Caucasian postmenopausal women.

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