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Association between androgen receptor gene polymorphism and bone density in older women using hormone replacement therapy

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Abstract

Objective: The objective of this study was to investigate the relationship between bone mineral density (BMD) and both CAG repeat polymorphism of the androgen receptor (AR) gene and skewed X chromosome inactivation (SI) in postmenopausal women.
Methods: BMD was measured by DEXA. Both the number and the X-weighted biallelic mean of the CAG repeats of AR were analysed by PCR, before and after DNA digestion with methylation-sensitive *Hpa*II in 192 healthy Caucasian postmenopausal women.
Results: The number of CAG repeats ranged from 10 to 34, with a median value of 22. $CAG)_{n\leq 22}$ and $CAG)_{n\geq 23}$ alleles were designated as short and long alleles, respectively. In women using hormone replacement therapy (HRT) ($n=81$), lumbar spine BMD was significantly lower, and femoral neck and total body BMD marginally lower in those with long-long alleles when compared with those with other genotypes. SI ($\geq$80%) was observed in 24% of the women and was not associated with BMD. In women using HRT, femoral neck BMD was significantly lower, and lumbar spine and total body BMD marginally lower in those whose X-weighted CAG repeat biallelic was greater than 22.59 (median value) when compared to other genotypes. These results were not found in women not using HRT.
Conclusion: In conclusion, our results suggest that BMD may be associated with AR gene polymorphism in postmenopausal women using HRT but not with SI. Further studies are needed to investigate the mechanisms of the interaction between HRT, BMD and AR found in the present study.

Keywords: Androgen receptor polymorphism; X-chromosome inactivation; Bone mineral density; Postmenopausal women; Osteoporosis; CAG repeat

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1. Introduction

Osteoporosis is a major public health problem in postmenopausal women because of the association between low bone mineral density (BMD) and vertebral and hip fractures [1]. BMD is under strong genetic control, with as much as 40–60% of the variance of BMD attributed to genetic factors [2–4].

Hormonal factors, and particularly estrogens and androgens, are known to play a role in bone physiology [5]. Both total and free testosterone concentrations are correlated with BMD in pre-, peri- and postmenopausal women [6–8]. Moreover, the addition of long-term testosterone replacement confers more benefits than estrogen replacement alone on the BMD of postmenopausal women [9–11]. Androgens may act on bone indirectly *via* the estrogen receptors after their aromatization into estrogens [5,12]. The recent detection and functional characterization of the androgen receptor (AR) in osteoblasts, osteoclasts, and osteocytes suggests that bone is also a possible direct androgen target tissue [12–14]. Indeed, in the androgen insensitivity syndrome, which is caused by mutations in the AR [15], the affected patients are deficient in the action of androgens and have abnormal bone mass accretion [16], even after estrogen replacement therapy [16,17].

The AR contains a polyglutamine tract of variable size in the N-terminal transactivation domain that can modulate the ability of the receptor to enhance transcriptional events *in vitro* [18]. This tract is encoded by a highly polymorphic CAG repeat microsatellite in exon 1 of the AR gene, located on human chromosome Xq12-13. AR proteins with shorter polyglutamine tracts that possess theoretically greater transactivation activity have been shown to be associated with a higher risk of prostate cancer [19], endometrial, ovarian and breast cancers [20–23], and hirsutism [24,25].

The somatic cells of females contain two X chromosomes and theoretically, one is inactive in half the cells, while the other is active in the other half. This process of X chromosome inactivation, that occurs early in the development and is random, results therefore normally in the generation of tissues with approximately equal numbers of cells in which the active X chromosome is of maternal or paternal origin [26]. A deviation from such an equal distribution of the two cell types can however occur, this phenomenon being possibly acquired with aging [27–29]. Since a skewed pattern of X chromosome inactivation affecting the CAG repeat polymorphism of AR has been associated with hormone-related diseases [26,30,31], this phenomenon may be crucial when studying AR polymorphism in relation with diseases in older women.

Few studies have assessed the relationship between the CAG repeat polymorphism in the AR gene and BMD in women [32–38]. Moreover, their results are conflicting. The bone site considered [34,35,38], age [38], estrogenic status [33,36] and racial characteristics [38] of studied women may explain some of the discrepancies. Since no information is available on the relative extents of inactivation of the S and L alleles of AR in older women from these previous studies, whether a skewed pattern of X chromosome inactivation may influence the relationship between CAG repeat polymorphism of AR and BMD in postmenopausal women remains to be elucidated.

The aim of the present study was to investigate the relationship between the AR CAG repeat polymorphism of AR and total body, lumbar spine, and femoral neck BMD in late postmenopausal Caucasian women, while taking into account the patterns of X-chromosome inactivation and use of hormone replacement therapy (HRT) and estradiol levels.

2. Subjects and methods

A total of 192 postmenopausal women aged 47–90 years were consecutively recruited from various urban community organisations. Inclusion criteria were Caucasian race, apparent good health, and no personal or family history of osteoporosis or fracture. Exclusion criteria were any diseases, especially endocrinologic or rheumatologic pathologies, or treatment that could influence BMD (e.g. fluoride, calcium, corticosteroids, bisphosphonates, Vitamin D, calcitonin, anti-Vitamin K, diuretics). Informed written consent was obtained from all participants and the study design was approved by our ethics committee. For all women, we recorded the age at menopause, the time since menopause, and the use of HRT.

2.1. Genotyping

Genomic DNA was extracted and purified from EDTA blood samples using the QI amp Blood Kit (Qiagen S.A., Courtaboeuf, France). DNA from all subjects was used to amplify the polymorphic $(CAG)_n$ repeat of the human AR gene. PCR reactions were performed using the Taq PCR Master Mix Kit (Qiagen S.A., Courtaboeuf, France). The primers for amplification were 5′-TCCAGAATCTGTTCCAGAGCGTGC-3′ labelled with FAM fluorescent dye and the antisense primer 5′-GCTGTGAAGGTTGCTGTTCCTCAT-3′. The quality of the PCR products was assessed by 1.5% agarose gel electrophoresis. Each PCR product was diluted in water (10×) for analysis, and 2 μl were mixed with deionized formamide and a fluorescent molecular weight marker [Genescan 400HD (ROX), Size Standard, Applied Biosystems, Foster City, CA]. After denaturation for 1 min at 95 °C, each sample was submitted to capillary electrophoresis on an ABI 310 automated sequencer and analysed with the GeneScan software (Applied Biosystems, Foster City, CA). The number of CAG repeats was calculated from the size of the PCR products, in relation to a series of standards obtained by direct sequencing of PCR products from XY individuals [39].

2.2. X-chromosome inactivation analysis

The X-inactivation assay, which is based on the AR gene methylation patterns using lymphocyte DNA, has been described elsewhere [24,40]. Briefly, the heterozygous samples were analysed for the X-inactivation ratio by assessment of methylation status using the methylation-sensitive restriction enzyme, *Hpa*II (New England BioLabs, Beverly, MA). Non-methylated (active X) DNA segments were enzyme-digested and were thereby unavailable for PCR amplification. Methylated (inactive X) *Hpa*II sites are not enzyme-digested and remain intact for amplification. Postdigestion PCR products therefore represent only methylated (inactive X) DNA sequences.

Equivalent 500-ng DNA aliquots were digested with *Hpa*II (10 U) overnight at 37 °C in a 20-μl reaction volume, with a final enzyme denaturation step of 65 °C for 20 min. Aliquots of digested and undigested DNA were amplified by PCR and analysed on Genescan as described above. To quantify the relative inactivation of the AR alleles, the peak areas of both alleles using digested and undigested DNA were obtained using the Genescan software. For each patient, the ratio between the areas of the two alleles using undigested PCR product was calculated and served as a correction factor. This correction factor was applied to compensate for unequal amplification of the alleles, using digested PCR product. The degree of X inactivation was then calculated by normalizing the sum of both alleles to 100%, and each allele was then calculated and expressed as a percentage. The degree of skewing (DS) was defined as the percentage of activity of each allele in the chromosome. Skewing was considered to be present when the activity of one allele in the X chromosome was more than 80% [41].

2.3. Allele distribution profile

Distribution of the number of CAG repeats in AR (range, mean ± S.D.; median) was determined in 192 women (384 alleles). All alleles were divided into two groups. Those with fewer CAG repeats than the median value were defined as short (S), and those with more than the median were defined as long (L). The subjects could thus be divided into three groups: short-short (SS), short-long (SL), and long-long (LL). For each woman, the biallelic mean of CAG repeats was then calculated. We also used the method described by Hickey et al. to calculate the X-weighted biallelic means, whereby the results of X-inactivation analysis are used to create a mean value that represents differences in the expression of constituent alleles. This was achieved by multiplying each allele in a genomic pair by its percentage of total expression (100 − % inactivity) and adding the two adjusted repeat values [24].

2.4. Measurements

All women were weighed (kg) and their height was measured (cm). The body mass index (BMI) was expressed as weight (kg) per squared height (m^2). Dietary calcium intake was estimated with a self-administered food-frequency questionnaire assessing intake of 20 common foods rich in calcium over the past week [42].

Total body BMD (g/cm^2), BMD at the lumbar spine (L2–L4) and the femoral neck, and lean mass (kg) were determined using dual energy X-ray absorptiometry

(DEXA, Lunar, WI; software Version 2.5.2). The coefficient of variation for our machine during measurement on a standard phantom is less than 1%. In our centre, the coefficient of variation of BMD measurement in human subjects is less than 3%. Serum concentration of Estradiol (E2) was determined by immunoassy (Immunotech S.A.; intrassay variation 6.3%, interassay variation of 3.4%).

2.5. Statistical analysis

Descriptive statistical analyses of continuous variables were performed using means, medians, and standard deviations. Comparisons of BMD according to the three groups [short-short (SS), short-long (SL), and long-long (LL)] were performed using an ANOVA when the distribution was normal and the Kruskal–Wallis non-parametric test otherwise. Correlations between continuous variables were performed using the Pearson correlation coefficient when the distribution was normal and the Spearman correlation coefficient otherwise. The relationships between categorical variables were tested using the Chi-Square test or, when Chi-Square was not a valid test, Fisher's exact test. A p value of less than 0.05 was considered to indicate statistical significance. Statistical analysis was performed using SAS software V8.2 (SAS Institute, Cary, NC).

3. Results

The characteristics of the study population are shown in Table 1. Irrespective of the bone site, BMD was inversely correlated with age ($p < 0.001$) and time since menopause ($p < 0.001$) and positively correlated with lean mass ($p < 0.05$), body mass index ($p < 0.001$), calcium intake ($p < 0.05$), E2 ($p < 0.001$) and the use of HRT ($p < 0.001$).

Genotype was obtained in all subjects ($n = 192$) but the X-chromosome inactivation analysis could not been obtained for technical reasons (unsuccessful DNA digestion) in 41 subjects. AR CAG repeats ranged from 10 to 34 CAGs, with a median value of 22 (Fig. 1). We designated $CAG)_{n \leq 22}$ and $CAG)_{n \geq 23}$ alleles as short (S) and long (L) alleles, respectively. The number of AR CAG repeats was not correlated with age, time since menopause, or anthropometric parameters ($p > 0.1$).

Table 1
Characteristics of the study population ($n = 192$)

	Mean ± S.D.
Age (years)	65.4 ± 8.4
CAG repeat	22.4 ± 2.6
HRT (year) ($n = 81$)	5.6 ± 4.5
Age at menopause (years)	50.67 ± 4.68
Time since menopause (years)	14.7 ± 9.0
Dietary calcium intake (mg/day)	981.0 ± 388.1
Height (cm)	159.5 ± 5.7
Weight (kg)	62.3 ± 10.2
BMI (kg/m^2)	24.4 ± 3.77
Lean mass (kg)	36.3 ± 5.1
Percent of lean mass	56.3 ± 6.4
Serum E2 (pg/ml)($\times 0.003$ nmol/l)	32.61 ± 54.8
Total body BMD (g/cm^2)	0.88 ± 0.1
Femoral neck BMD (g/cm^2)	0.72 ± 0.1
Lumbar spine BMD (g/cm^2)	0.89 ± 0.1
X-weighted CAG repeat biallelic means ($n = 151$)	22.7 ± 2.0

HRT, hormone replacement therapy; E2, estradiol; BMI, body mass index; BMD, bone mineral density.

In the overall sample of women and among women not using HRT, BMD was not significantly different in those with short-short, short-long, and long-long AR alleles. In women using HRT however, lumbar spine BMD was significantly lower, and femoral neck and total body BMD marginally lower in those with long-long alleles when compared with those with short-short and short-long alleles (Table 2). Although women with HRT ($n = 81$) had significantly higher serum E2 levels that women without HRT ($n = 109$) (57.3 ± 76.4 pg/ml versus 13.8 ± 8.4 pg/ml; $P < 0.01$), no interaction was found between E2 categorized by quartiles and the association between AR gene polymorphism and the BMD.

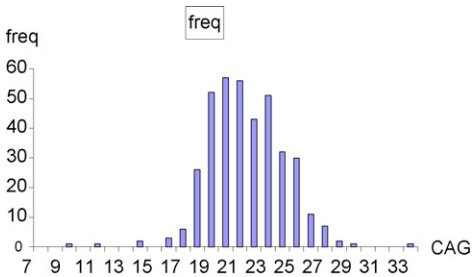

Fig. 1. The frequency distribution of the androgen receptor gene trinucleotide (CAG) repeat polymorphism in 192 postmenopausal women (384 alleles).

Table 2
Bone mineral density (mean ± S.D.) according to androgen receptor genotype and treatment group

BMD (g/cm^2)	Short-short	Short-long	Long-long	*P* value
Whole sample	*n* = 57	*n* = 92	*n* = 43	
Total body	0.88 ± 0.13	0.89 ± 0.13	0.85 ± 0.11	0.21
Femoral neck	0.74 ± 0.14	0.73 ± 0.12	0.70 ± 0.11	0.32
Lumbar spine	0.89 ± 0.17	0.91 ± 0.17	0.86 ± 0.15	0.28
Not HRT women	*n* = 36	*n* = 47	*n* = 26	
Total body	0.85 ± 0.11	0.85 ± 0.11	0.84 ± 0.10	0.93
Femoral neck	0.72 ± 0.15	0.68 ± 0.10	0.69 ± 0.11	0.77
Lumbar spine	0.84 ± 0.17	0.85 ± 0.15	0.85 ± 0.17	0.94
HRT women	*n* = 21	*n* = 43	*n* = 17	
Total body	0.93 ± 0.14	0.93 ± 0.13	0.85 ± 0.12	0.08
Femoral neck	0.78 ± 0.14	0.78 ± 0.12	0.71 ± 0.12	0.14
Lumbar spine	0.97 ± 0.13	0.98 ± 0.17	0.87 ± 0.12	0.04

Alleles with fewer CAG repeats than the median value (22) are defined as short (S), and those with more CAG repeats than median are defined as long (L). BMD, bone mineral density; HRT, hormone replacement therapy.

The distribution of X-weighted CAG biallelic means calculated with the method described by Hickey et al. [24] ranged from 17.66 to 27.40, with a median value of 22.59. Among women using HRT, those whose X-weighted CAG biallelic mean was greater than 22.59 had significantly lower femoral neck BMD and marginally lower total body and lumbar spine BMD than women with other genotypes. In the overall sample of women and among those not using HRT, BMD was not significantly different in women whose X-weighted CAG biallelic mean was lower or greater than the median value (Table 3). Twenty-four percent of the women displayed a skewed X-chromosome inactivation as defined by Naumova et al. (≥80%) [41]. BMD was not significantly different between women with or without skewed X-chromosome inactivation (Table 4).

4. Discussion

The clinical and biochemical effects of androgens on bone metabolism have been extensively studied [5]. However, the extent to which the effect of androgens on bone is mediated by AR gene polymorphism is not clear. The AR genotype and BMD have been

Table 3
Comparison of the bone mineral density (mean ± S.D.) between women whose X-weighted biallelic means of CAG repeats of androgen receptor is greater vs. lower than the median value (22.59) according to the treatment group

BMD (g/cm^2)	X-weighted biallelic means of CAG repeats < 22.59	X-weighted biallelic means of CAG repeats > 22.59	*P* value
All women	*n* = 75	*n* = 76	
Total body	0.91 ± 0.13	0.87 ± 0.12	0.16
Femoral neck	0.75 ± 0.13	0.71 ± 0.24	0.07
Lumbar spine	0.90 ± 0.17	0.89 ± 0.17	0. 88
Not HRT women	*n* = 43	*n* = 47	
Total body	0.86 ± 0.11	0.86 ± 0.10	0.89
Femoral neck	0.70 ± 0.11	0.69 ± 0.11	0.75
Lumbar spine	0.83 ± 0.16	0.86 ± 0.17	0.25
HRT women	*n* = 32	*n* = 29	
Total body	0.96 ± 0.13	0.90 ± 0.15	0.14
Femoral neck	0.81 ± 0.13	0.74 ± 0.14	0.04
Lumbar spine	0.98 ± 0.15	0.92 ± 0.17	0.15

X-weighted biallelic means of CAG repeats has been calculated using the method described by Hickey et al. [24]. BMD, bone mineral density; HRT, hormone replacement therapy.

Table 4
Bone mineral density (mean ± S.D.) according to skewed X-chromosome inactivation

BMD (g/cm^2)	Short allele, $n = 16$	Long allele, $n = 34$	No skewed inactivation, $n = 100$	P-value
Total body	0.89 ± 0.16	0.85 ± 0.12	0.90 ± 0.12	0.16
Femoral neck	0.71 ± 0.15	0.70 ± 0.12	0.74 ± 0.13	0.25
Lumbar spine	0.90 ± 0.16	0.89 ± 0.18	0.89 ± 0.17	0. 97

$CAG)_{n\leq 22}$ and $CAG)_{n\geq 23}$ alleles are designated as short and long alleles, respectively. BMD: bone mineral density.

found to be associated in pre- and perimenopausal American women, with an over-representation of certain AR genotypes in those with the lowest femoral neck BMD (12/12, 12/13, 12/14, 12/15, 13/13, 13,14, and 13/15) [32]. Yamada et al. showed additionally that total body BMD was significantly lower in premenopausal Japanese women with long-long genotype than in those in the combined group of short-short and short-long genotypes [38]. Taken together, these studies may suggest that AR polymorphism may affect the achievement of peak bone mass and perhaps the bone loss that occurs in the first half of the life in women. The influence of AR genotype on bone loss that occurs after menopause is more controversial.

BMD was not associated with AR genotype in Japanese postmenopausal women [38] whereas a study conducted in postmenopausal women from Taiwan found that those with genotype 20+ had lower BMD values and a significantly greater risk for osteoporosis at the femoral neck than those with genotype 20− [35]. Conflicting results have also been found in Scandinavian postmenopausal women. AR gene polymorphism was not found to be associated either with BMD, 5-year BMD change, or fracture in early post-menopausal Finnish women, nor with BMD in late postmenopausal Danish and Swedish women [33,34,37]. In contrast, the short-short genotype (short alleles defined as CAG repeat ≤ 20) was associated with a higher lumbar spine BMD and a lower osteoporotic fracture risk in Danish postmenopausal women [34].

Differences in estrogenic status among postmenopausal women may explain a part of these apparent discrepancies. In the Finnish population studied by Salmen et al., the relationship between AR genotype and BMD was found significant in postmenopausal women using HRT. Of these, women with the short-short alleles of AR had higher BMD and lower five-year annual change of BMD at the lumbar spine ($p = 0.028$ and 0.043, respectively) and at the femoral neck ($p = 0.086$ and 0.049, respectively) than those with other genotypes [33]. However, because no differences were found in the overall sample, the authors considered these results to be due to type I error. In the study of Tofteng et al., and although the direction of the association was somewhat puzzling, a modifying effect of sex hormone-binding globulin (SHBG) was present, AR genotype being associated with femoral neck BMD in women whose SHBG was in the highest quartile [36].

Present data support the hypothesis of an interaction between the use of HRT and the association of AR gene polymorphism and BMD. Indeed, the number of AR CAG repeats was found significantly associated with lumbar spine BMD and marginally associated with total body BMD and femoral neck BMD in women who used HRT but not in the overall sample or in women not using HRT. This interaction appears unrelated to body mass or body mass index since present results are after adjustment for anthropometric parameters [42]. Although women with HRT were found to have significantly higher serum E2 levels that women not using HRT, we failed to find a significant interaction between E2 levels and the association of the AR gene polymorphism and BMD.

Although our results must be considered within the context of a relatively modest sample size, several hypotheses may be proposed to explain a possible interaction independent of serum estradiol levels between the use of HRT and the association of the AR gene polymorphism and the BMD. First, since the effect of estradiol on bone is not linear and bio E2 levels have been shown to be associated with volumetric BMD and some structural bone parameters at low but not high bio E2 levels [43], it can be postulated that the association between AR polymorphism and BMD may be observed over a threshold for estrogen insufficiency that cannot be determined here, due to a lack of power. Other factors that have not been investigated in the present study, such as testosterone levels, or not accounted for a single measure of serum estradiol, such as the duration of HRT and the type of HRT, may also be involved. Another

explanation relies on the fact that HRT is known to be associated with a reduction of some diseases and symptoms, and with a better functional, psychological, and health status in older women, due to a direct effect of estradiol but also to better lifestyle habits (physical activity and smoking habits) and to a better socio-economic and cultural level [44,45]. Women using HRT could therefore be considered as an elite that is less prone to be exposed to environmental and lifestyle factors involved in age-related bone loss and their characteristics could be closer to premenopausal women than other postmenopausal women. The association found in present and previous studies between bone mass and AR polymorphism in premenopausal women and in postmenopausal women using HRT only, could support the concept that the influence on bone of genetic factors may decrease with aging [46], particularly in older women not using HRT, whose bone loss is also dependent on the effects of lifestyle and environment that may be cumulative and become dominant in later life [47].

Missing information on the phenomenon of X-chromosome inactivation may also explain apparent divergent results in previous studies. AR is situated on the X-chromosome and is therefore affected by X-chromosome inactivation in women [48]. Although this phenomenon is normally random, some studies have reported skewed inactivation related to AR polymorphism in women, especially in older ones [27–29], and have suggested a pathogenic influence [26,30,31]. Skewed inactivation related to AR (>80%) [41] was observed in 24% of postmenopausal women recruited in the present study (versus 16% and 37.9% in women aged 60 years and older in two previous studies) [27,28]. This confirms that skewed inactivation related to AR is a relatively common phenomenon in Caucasian postmenopausal women. However, present data suggest that the influence of skewed X inactivation on BMD is rather minor. Indeed, BMD of women with skewed inactivation was not found significantly different from that measured in other women. Moreover, present data using X-weighted biallelic means, that represent differences in the expression of constituent alleles, were found quite similar to those obtained without adjustment for skewing of X-chromosome inactivation.

Present results must be considered within the context of several possible limitations. First, the relatively modest sample size may explain in part some of the marginal associations between AR gene polymorphism and BMD found in women using HRT. The present study, therefore, only allows the drawing of hypotheses on the association of the AR gene polymorphism and the BMD and the interaction of the use of HRT on this association, hypotheses that must be tested in further studies with larger samples of postmenopausal women using and non using HRT. Second, some factors such as testosterone levels, the type and duration of the HRT, and other factors associated with HRT, including, lifestyle and environment, that have not been taken into account in the present study will have to be included in further studies, to better understand the mechanisms involved in the possible interaction of HRT use and the association of the AR gene polymorphism and the BMD in postmenopausal women. Although present results suggest that skewed X inactivation in peripheral lymphocytes is not associated with low BMD, further studies are needed to investigate this phenomenon in bone cells [28]. The inclusion of only Caucasian volunteers may limit the generalizability of our findings as racial differences in the number of CAG repeats have been demonstrated elsewhere [49,50]. Nevertheless, the mean number of CAG repeats in the present study (22.4 ± 2.6) was found close to those found in Japanese (22.8 ± 2.6) [38], and Danish (21.9 ± 3.0) [36] postmenopausal women.

In conclusion, present results suggest that AR CAG repeat polymorphism may be associated with BMD in postmenopausal Caucasian women using HRT, as it has been observed in premenopausal women. Moreover, skewed X chromosome inactivation does not appear to be associated with BMD in postmenopausal women regardless of the use of HRT. Further studies are needed to confirm these results in a greater number of postmenopausal women using and non-using HRT and to investigate the mechanisms of the possible interaction between HRT, BMD, and AR.

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