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Androgen receptor CAG and GGN repeat polymorphisms and bone mass in boys and girls

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Abstract

Introduction: **the human androgen receptor (AR) gene possesses two trinucleotide polymorphic repeats, (CAG and GGN) that affect the amount of AR protein translated. In this study, we genotyped these polymorphic tracts in a representative sample of Caucasian children (Tanner ≤5), 152 boys (11.5±2.6 yrs) and 116 girls (10.1±3.2 yrs) from Spain and investigated their association with bone mass.**

Methods: **the length of CAG and GGN repeats was determined by PCR and fragment analysis. Body composition was assessed by dual energy x-ray absorptiometry (DXA). Individuals were grouped as CAG short (CAG_S) if harboring repeat lengths of ≤21 and CAG long (CAG_L) if CAG >21. Moreover, subjects were grouped as GGN short (GGN_S) if harboring repeat lengths of ≤23 and GGN long (GGN_L) if GGN >23.**

Results: **in boys, significant differences in height, body mass, whole body bone mineral density (BMD) and content (BMC), upper extremities BMC, lower extremities BMC, femoral neck BMD, Ward's triangle BMC and BMD and lumbar spine BMD were observed between CAG_S and CAG_L groups (P<0.05). Thus, upper extremities BMD differed between GGN_S and GGN_L groups. After adjusting for confounding variables, only upper extremities BMD between GGN_S and GGN_L groups remained significant (P<0.05). No differences were observed in girls in any measured site in relation to either CAG or GGN polymorphisms length.**

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POLIMORFISMOS CAG Y GGN DEL GEN DEL RECEPTOR DE ANDRÓGENOS Y MASA ÓSEA EN NIÑOS Y NIÑAS

Resumen

Introducción: **el gen humano del receptor de andrógenos (AR) posee dos repeticiones polimórficas de trinucleótidos (CAG y GGN) que afectan a la cantidad de proteína AR traducida. En este estudio, genotipamos esos tractos polimórficos en una muestra representativa de niños caucásicos españoles (Tanner ≤5), compuesta por 152 niños (11.5±2.6 años) y 116 niñas (10.1±3.2 años) e investigamos su asociación con la masa ósea.**

Métodos: **la longitud de las repeticiones CAG y GGN se determinó mediante PCR y análisis de fragmentos. La composición corporal se midió mediante absorciometría dual de rayos X (DXA). Los participantes fueron agrupados como CAG cortos (CAG_S) si poseían una longitud de repeticiones ≤21 y CAG largos si esta era >21. Además, los participantes se agruparon como GGN cortos (GGN_S) si poseían una longitud de repeticiones ≤23 y GGN largos (GGN_L) si esta era >23.**

Resultados: **en los niños se encontraron diferencias en talla, peso corporal, densidad mineral ósea (BMD) y contenido mineral óseo (BMC) del cuerpo entero, BMC de las extremidades superiores e inferiores, BMD del cuello del fémur, BMC y BMD del triángulo de Ward's y BMD de la espina lumbar entre los grupos CAG_S y CAG_L (P<0,05). Además, el BMD de las extremidades superiores fue significativamente diferente entre los grupos GGN_S y GGN_L. Tras ajustar por variables confusoras, la única diferencia que se mantuvo significativa fue la del BMD en las extremidades superiores entre los grupos GGN_S y GGN_L (P<0,05). No se observaron diferencias entre los grupos CAG y GGN y la masa ósea en las niñas.**

Conclusions: **our results support the hypothesis that longer alleles of the AR CAG and GGN polymorphisms are associated with increased bone mass in prepubertal boys.**

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Key words: *Bone mass. Gene. Androgen receptor polymorphisms. Prepubertal growth.*

Conclusiones: **nuestros resultados apoyan la hipótesis de que los alelos largos de los polimorfismos CAG y GGN del AR están asociados con una mayor masa ósea en niños prepúberes.**

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Palabras clave: *Masa ósea. Gen. Polimorfismos del receptor de andrógenos. Crecimiento prepuberal.*

Introduction

About 60-80% of peak bone mass variability is determined by hereditary factors; although there are other important elements like diet and hormonal status that could also affect on the optimal bone mass acquisition during childhood and adolescence[1]. Bone strain elicited by physical activity promotes bone mineralization and facilitates bone mass accrual during growth[2]. The osteogenic effect of physical activity is particularly effective when exercise is started before puberty in both girls and boys[3-5]. Longitudinal studies evaluating changes in bone mass during growth, have shown that the greatest increases in bone mass occurs at ages of 12-15 years in girls compared to 14-17 years in boys[6], i.e., during the transition from adolescence to adulthood. Bone mass acquisition before or at the first stages of the pubertal phase likely depends on the ability to transcribe anabolic hormonal signals into discrete bone formation, particularly because systemic anabolic hormones (i.e. free and total testosterone or estradiol) are almost inexistent[7,8]. Although estradiol is required for the attainment of maximal peak bone mass in both sexes, testosterone has an additional action through the stimulation periosteal apposition promoting periostal expansion[7]. Therefore, those children able to generate a greater androgenic response would, in theory, benefit from a greater bone formation.

Androgenic activity in bone is mediated by the androgen receptors (ARs), a type of nuclear receptor present in osteoblasts and activated by transmembrane binding to androgenic hormones such as testosterone or dihydrotestosterone[9]. The AR gene has two polymorphic motives due to polyglutamine (CAG, encoded), polyglycine (GGN) tracts, whose repetition length is negatively associated to its transcriptional activity[10,11]. Consequently, a greater androgenic effect is expected for AR with lower CAG and GGN repeat number. In agreement, a lower number of CAG repetitions have been linked to greater fat-free mass in healthy elders[12] as well as benign prostatic hypertrophy, prostate cancer, male infertility[13-15] and higher systemic concentrations of total and free testosterone[16]. The effect of ARs polymorphisms on bone mass has also been studied by our laboratory in a group of 282 healthy men, where we found a higher femoral neck BMD in those subjects carrying a $CAG_S + GGN_S$ haplotypes[17].

Much less investigated is the effect of harboring a short or long CAG or GGN repeat polymorphism on bone mass formation during growth. In this line, Voorhoeve *et al*. have shown that height-standard deviation scores were inversely associated with AR CAG repeat length in boys at young prepubertal and early pubertal age[18].

Therefore, the aim of this study was to determine whether the CAG and GGN androgen receptor repeat polymorphisms are associated with bone mass in prepubertal boys and girls. Our hypothesis is that a short CAG or GGN repeat polymorphisms of the gene encoding the androgen receptor is associated with greater bone mass in children.

Methods

Caucasians physically active boys (152) and girls (116), aged between 7 and 13 years (Tanner stage <5), were recruited from sports clubs in Gran Canaria (Spain). Children having any chronic illness or taking medications were excluded. The study was performed in accordance with the 1975 Helsinki Declaration and was approved by the Ethical Committee of the University of Las Palmas de Gran Canaria. Parents and children provided written consent after receiving a full explanation on the benefits and risk before the start of the studies. Tanner pubertal stage[19] was self-assessed by the children[20], a procedure of recognized reproducibility ($r=0.97$)[21].

Body composition

Whole body composition was determined by dual-energy X-ray absorptiometry (DXA; QDR-1500, Hologic Corp., Software version 7.10, Waltham, Massachusetts, USA) as reported in Perez-Gomez *et al*.[22]. Calculations related to upper and lower limb lean mass was conducted by regional analysis of the whole body scan[23,24].

CAG and GGN repeat polymorphisms

DNA was extracted from saliva samples (200 ml) using High Pure PCR Template Preparation Kits (Ro-

che Applied Science). To determine the length of the CAG and GGN repeats the corresponding regions located on the exon 1 of the AR gene (Genbank accession no. M27423) were amplified using two pairs of primers whose sequences have been previously reported[25]. One primer from each pair was marked with fluorescent dye (FAM or VIC). Amplification was performed in a 25 μl reaction volume, containing 50 ng of genomic DNA, 200 μM of each deoxynucleotide triphosphate, 1x Fast Start Taq DNA polymerase Buffer (Roche Applied Science, Mannheim, Germany), 1x GC-rich solution buffer (Roche Applied Science) and 1U of Fast Start Taq DNA polymerase (Roche Applied Science). The concentration of each pair of primers was 1.2 and 1.5 μM for the amplification of the CAG and GGN repeats, respectively. PCR conditions were: 30 cycles of 95ºC for 45 sec, 56ºC for 30 sec and 72ºC for 30 sec for CAG amplification; 30 cycles of 95ºC for 1 min, 55ºC for 2 min and 72ºC for 2 min for GGN amplification. Each PCR was initiated with a denaturation step at 95ºC for 5 min and terminated with an extension step at 72ºC for 5 min. The PCR product was diluted 1:100 in distilled water and 1 μl of the dilution was mixed with 10 μl of formamide and 0.3 μl of GeneScan 500 LIZ Size Standard (Applied Biosystems, Warrington, UK), denatured at 98ºC for 5 min and cooled on ice. Fragment separation was performed by automated capillary electrophoresis, using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and the length was determined with Gene Scan Analysis Software (version 3.7) (Applied Biosystems). Internal standards supplied by the manufacturer were used for quality control. We blindly repeated the genotype analysis in 54 of the samples, and the results were completely coincident. The fragments size was confirmed by sequencing 48 DNA samples harboring different size alleles for both repeats by using the Big Dye Terminator Sequencing Kit (Applied Biosystem) at University of Las Palmas Sequencing Facility with excellent agreement between both procedures. Genotyping was performed specifically for research purposes based on the hypothesis that the aforementioned polymorphisms may influence VO_2 max, lean mass and muscle strength. The researchers in charge of genotyping were totally blinded to the subjects' identities, that is, blood samples were tracked solely with code numbers, and personal identities were only made available to the main study researcher who was not involved in actual genotyping.

Table I
Body composition, anthropometrics, maturational stage, total and regional bone mass in boys and girls (mean ± standard deviation).

	boys	*n*	*girls*	*n*
Age	11.5 ± 2.6	152	10.1 ± 3.2	116
Height (cm)	147.9 ± 14.8	152	138.4 ± 15.9	116
Body mass (kg)	41.7 ± 13.1	152	36.4 ± 12.6	116
Percentage of body fat (%)	21.1 ± 8.7	152	27.2 ± 8.5	116
Tanner G	2.5 ± 1.2	152	2.0 ± 1.2	116
Tanner H	2.6 ± 1.2	152	2.2 ± 1.4	116
Body composition				
Whole body BMC (g)	1395.9 ± 516.4	152	1150.4 ± 514.0	116
Whole body BMD (g/cm^2)	2.8 ± 0.6	140	2.6 ± 0.9	115
Upper extremities BMC (g)	71.8 ± 35.1	152	59.2 ± 33.1	116
Upper extremities BMD (g/cm^2)	0.6 ± 0.1	152	0.6 ± 0.1	116
Lower extremities BMC (g)	281.2 ± 127.9	152	217.5 ± 114.2	116
Lower extremities BMD (g/cm^2)	1.0 ± 0.2	152	0.9 ± 0.2	116
Femoral neck BMC (g)	3.3 ± 1.2	150	2.9 ± 0.9	116
Femoral neck BMD (g/cm^2)	0.8 ± 0.1	150	0.7 ± 0.1	116
Ward's triangle BMC (g)	0.9 ± 0.2	150	0.8 ± 0.2	116
Ward's triangle BMD (g/cm^2)	0.8 ± 0.1	150	0.7 ± 0.2	116
Ls BMC (g)	18.6 ± 7.8	151	17.0 ± 9.2	115
Ls BMD (g/cm^2)	1.7 ± 0.3	151	1.7 ± 0.4	115

BMC: bone mineral content; BMD: bone mineral density; Ls: mean lumbar spine (from L2, L3 and L4). *P<0.05 vs boys.

Statistical analysis

Descriptive statistics are presented as the mean values and standard deviation (SD). The homogeneity of variances was established by the Levene test. A logarithmic transformation was performed for variables that were not normally distributed, as determined by the Shapiro-Wilk test. Since both CAG and GGN repeats were not normally distributed, the Spearman correlation coefficient (rho) was used to test the strength of the association between the number of these repeats and diverse continuous variables. Differences between data subsets were analyzed using either analysis of variance or analysis of covariance, both with the Bonferroni post hoc test.

The influence of the length polymorphism repeats CAG_n and GGN_n on body composition and fitness variables was determined by taking the polymorphisms as continuous variables or dichotomous by allelic limits. The mean value showed the best balance between groups of children was used as the cutoff, therefore, subjects who had a number of repetitions ≤21 were classified as short CAG (CAG_S) and those subjects who showed a number of repeats >21 were classified as long CAG (CAG_L). In the case of GGN, subjects carrying a number of repetitions ≤23 were classified as short GGN (GGN_S), and those showing a number of repetitions >23 were considered as long GGN (GGN_L). Statistical analysis was performed with SPSS (SPSS Inc., Chicago, IL, USA). Significant differences were assumed at $P<0.05$.

Results

There were 16 different CAG alleles ranging from 13 to 33 repeats, and 12 GGN alleles ranging from 14 to 30 repeats in boys. In the girls there were 11 different CAG alleles ranging from 14 to 25, and 9 GGN alleles, ranging from 13 to 24 repeats. Boys and girls body composition, anthropometrics, total and regional bone mass are reported in table I. In general, boys were taller, heavier and had higher whole body BMD and BMC than girls ($P<0.05$, table I).

CAG repeat polymorphism

Anthropometrics, maturational stage, total and regional bone mass in the CAG_S and CAG_L groups are repor-

Table II
Body composition, anthropometrics, maturational stage, total and regional bone mass in boys and girls with CAG_S and CAG_L androgen receptor polymorphisms (mean ± standarddeviation)

	CAG_S boys	n	CAG_L boys	n	CAG_S girls	n	CAG_L girls	n
Age	11.3±2.4	90	11.7±2.8	62	10.1±3.4	51	10.2±3.0	65
Height (cm)	146.1±14.0	90	150.5±15.6*	62	138.2±15.8	51	138.6±16.0	65
Body mass (kg)	39.9±12.0	90	44.2±14.4*	62	36.3±13.3	51	36.5±12.2	65
Percentage of body fat (%)	20.9±8.1	90	21.3±9.5	62	27.0±8.7	51	27.3±8.4	65
Tanner G	2.5±1.1	90	2.5±1.2	62	2.0±1.2	51	2.0±1.3	65
Tanner H	2.6±1.2	90	2.6±1.2	62	2.2±1.3	51	2.2±1.4	65
Body composition								
Whole body BMC (g)	1340.9±505.9	90	1475.8±525.0*	62	1140.1±520.6	51	1158.5±512.7	65
Whole body BMD (g/cm^2)	2.7±0.6	90	2.8±0.6*	62	2.7±0.9	51	2.6±1.0	65
Upper extremities BMC (g)	68.3±34.2	90	76.8±36.1*	62	57.9±33.1	51	60.3±33.4	65
Upper extremities BMD (g/cm^2)	0.6±0.1	90	0.6±0.1	62	0.6±0.1	51	0.6±0.1	65
Lower extremities BMC (g)	266.9±123.9	90	302.0±131.8*	62	217.1±117.6	51	217.8±112.4	65
Lower extremities BMD (g/cm^2)	1.0±0.2	90	1.0±0.2	62	0.9±0.2	51	0.9±0.2	65
Femoral neck BMC (g)	3.2±1.2	90	3.3±1.1	60	2.8±0.9	51	2.9±0.9	65
Femoral neck BMD (g/cm^2)	0.8±0.1	90	0.8±0.1	60	0.7±0.1	51	0.7±0.1	65
Ward's triangle BMC (g)	0.9±0.2	90	0.9±0.2	60	0.9±1.1	51	0.8±0.2	65
Ward's triangle BMD (g/cm^2)	0.8±0.1	90	0.8±0.1	60	0.7±0.2	51	0.7±0.2	65
Ls BMC (g)	17.9±7.8	90	19.5±7.7	61	16.6±9.0	51	17.3±9.4	64
Ls BMD (g/cm^2)	1.6±0.3	90	1.7±0.3*	61	1.7±0.4	51	1.7±0.4	64

BMC: bone mineral content; BMD: bone mineral density; Ls: mean lumbar spine (from L2, L3 and L4). Subjects were grouped as CAG short (CAGSs) if harboring repeat lengths of ≤21 and CAG long (CAG_L) if harboring repeat lengths of >21. *$P<0.05$ vs CAG_L.

ted in table II for boys and girls respectively. CAG_L boys showed greater height, body mass, whole body bone mineral density (BMD) and content (BMC), upper extremities BMC, lower extremities BMC, femoral neck BMD, Ward's triangle BMC, Ward's triangle BMD and lumbar spine BMD than CAG_S boys ($P<0.05$, table II). No intergroup (CAG_S vs CAG_L) differences were observed in girls (Table II). After adjusting for confounding variables (age, height, weight, fat percentage, Tanner H, Tanner G), no differences were observed in any measured site in relation to CAG_S and CAG_L classification.

GGN repeat polymorphism

Anthropometrics, total and regional bone mass in the GGN_S and GGN_L groups are reported in table III for boys and girls, respectively. The boys with the polymorphism GGN_L had higher BMD in the upper extremities than those with a GGN_S ($P<0.05$, table III). This difference remained significant after adjusting for confounding variables (age, height, weight, fat percentage, Tanner H, Tanner G) ($P<0.05$). Girls classified in either GGN_S or GGN_L groups did not show any difference in any of the measured sites.

Correlations

Adjusted Spearman correlations showed a positive association between CAG repeat number and lower extremities BMD and Ward's triangle BMC in boys ($r=0.16$ and 0.14 respectively, $P<0.05$), and a negative association between Ward's triangle BMC, BMD and GGN repeat length in boys ($r=-0.18$ and 0.17 respectively, $P<0.05$).

Discussion

In contrast to our hypothesis, the main result of this study shows that boys classified as GGN long, after adjusting for potential confounders as height, weight and pubertal status, show higher bone mass in the upper extremities than those harboring the shorter GGN polymorphism. Moreover, neither CAG nor GGN polymorphism length seem to have an influence on bone mass and density in prepubertal girls.

Long CAG length has been associated with increased BMC and BMD[3,8,26]. In this study, children with long CAG have, on average, higher values in the following variables: size, weight, bone mineral content

Table III
Body composition, anthropometrics, maturational stage, total and regional bone mass in boys and girls with GGNs and GGNL androgen receptor polymorphisms (mean ± standard deviation)

	GGN_S boys	n	GGN_L boys	n	GGN_S girls	n	GGN_L girls	n
Age	11.3±2.4	93	11.8±2.8	59	10.7±3.3	50	9.7±3.0	66
Height (cm)	147.6±14.9	93	148.4±14.7	59	142.1±16.6	50	135.7±14.9	66
Body mass (kg)	41.1±12.5	93	42.6±14.2	59	39.5±13.7	50	34.0±11.3	66
Percentage of body fat (%)	21.0±8.6	93	21.1±8.9	59	28.1±8.8	50	26.5±8.4	66
Tanner G	2.5±1.2	93	2.6±1.2	59	2.3±1.3	50	1.8±1.2	66
Tanner H	2.5±1.2	93	2.7±1.2	59	2.5±1.4	50	1.9±1.3	66
Body composition								
Whole body BMC (g)	1376.3±499.6	93	1426.8±544.7	59	1268.1±547.8	50	1061.2±471.8	66
Whole body BMD (g/cm^2)	0.9±0.1	93	0.9±0.1	59	0.9±0.1	50	0.8±0.1	66
Upper extremities BMC (g)	69.3±33.3	93	75.7±37.8	59	67.0±35.8	50	53.3±29.9	66
Upper extremities BMD (g/cm^2)	0.6±0.1	93	0.6±0.1*	59	0.6±0.1	50	0.6±0.1	66
Lower extremities BMC (g)	277.6±125.9	93	287.0±131.9	59	244.0±121.8	50	197.4±104.6	66
Lower extremities BMD (g/cm^2)	1.0±0.2	93	1.0±0.2	59	0.9±0.2	50	0.9±0.2	66
Femoral neck BMC (g)	3.3±1.2	92	3.3±1.1	58	3.0±0.8	50	2.8±0.9	66
Femoral neck BMD (g/cm^2)	0.8±0.1	92	0.8±0.1	58	0.7±0.1	50	0.7±0.1	66
Ward´s triangle BMC (g)	0.9±0.2	92	0.9±0.1	58	0.8±0.2	50	0.7±0.2	66
Ward´s triangle BMD (g/cm^2)	0.8±0.1	92	0.8±0.1	58	0.7±0.2	50	0.6±0.2	66
Lumbar BMC (g)	18.4±7.5	93	18.8±8.3	58	19.0±9.9	49	15.5±8.3	66
Lumbar BMD (g/cm^2)	1.7±0.3	93	1.7±0.3	58	1.8±0.4	49	1.6±0.4	66

BMC: bone mineral content; BMD: bone mineral density; Ls: mean lumbar spine (from L2, L3 and L4). Subjects were grouped as GGN short (GGN_s) if harboring repeat lengths of ≤23 and CAG long (GGN_L) if harboring repeat lengths of >23. *$P<0.05$ vs GGN_L.

of whole body, upper and lower extremitiesm, and Ward's triangle, as well as bone mineral density of total body, lower extremities, femoral neck, Ward's triangle and lumbar spine. In agreement, Lappalainen and collaborators reported a positive correlation between BMI and the number of CAG repeats[27], whereas another study performed in adolescent boys did not confirm such an association between body composition and CAG repeats[18]. Previous work from our laboratory has shown that there is no relationship between physical fitness, muscle mass, levels of free testosterone, osteocalcin and the length of the CAG repeat number in a group of 282 healthy young men[17,26]. However, another study that investigated the relationship between the AR CAG repeat polymorphism and longitudinal growth from prepuberty until young adult age found that height-standard deviation scores were inversely associated with AR CAG repeat length in boys at young, prepubertal and early pubertal age. This association diminishes in the following years and completely disappears after the age of 16 years[18]. In agreement, we did not find differences in height between CAG_S and CAG_L in our older children. Other studies indicate that age, physical activity, body composition, sex steroid levels and anthropometrics are determinants of muscle mass and function in young men, and although the number of CAG repeats of the AR are related to sex steroid levels and anthropometrics, there is no evidence that these variations in the AR gene also affect muscle mass or function[28].

The differences observed in the present investigation with previously published data could be explained by the children's degree of physical activity. Our participants were practicing physical activity at least three times a week for two or more years at schools or after-school sport clubs. Therefore, the potential added effect of harboring a short CAG polymorphism on bone mass may not be large enough to be detected because of the well-known effects of physical activity and sports participation on bone mass acquisition during growth[29]. Additional studies are required to determine whether the CAG repeat polymorphism is associated with bone mass in sedentary children and how this polymorphism may modulate physical activity osteogenic effects.

Alternatively it may be argued that in a normally functioning adult hypothalamic–pituitary–gonadal axis, a diminished testosterone feedback, in case of a long AR CAG repeat, is compensated for by increased androgen production, because of increased LH stimulation[12,16,30]. It was suggested that several phenotypic effects are probably more attributable to oestrogen action than to androgen action, because of the increased effective bioactive oestrogen/androgen ratio in increasing AR CAG length[31]. In prepubertal boys, the hypothalamic–pituitary–gonadal axis is not responding as in adulthood, because the axis is relatively silent and the gonads only produce minor amounts of androgens. Moreover, a very small amount of androgens is produced by the adrenals, which are not under control of the hypothalamic– pituitary–gonadal axis. One could argue that in these boys, the compensatory mechanism of increasing androgen production in increasing AR CAG repeat length is absent. Therefore, prepubertal boys with long AR CAG repeats are relatively androgen deficient in compared with boys with short AR CAG repeat, leading to subtle differences in prepubertal growth as shown in our study.

The influence of AR GGN repeat polymorphisms on bone mass has not been studied in children. In the present investigation, we have found a positive correlation between AR GGN repeat length, BMD at different skeletal regions and the bone formation marker osteocalcin in young men[26] and women[32]. These studies support the abovementioned opportunity window for a prolonged bone grow span in those children with a relatively lower androgenic activity. However, more studies are needed in order to confirm this statement, especially in physically active and inactive children.

In conclusion, our results support the hypothesis that longer alleles of the androgen receptor CAG and GGN polymorphisms are associated with increased bone mass in prepubertal boys, whereas no apparent association is found in prepubertal girls. The present investigation adds to the current knowledge about the genetic influence of bone mass development during growth.

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