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Original Article

Hylonome

Lack of association between vitamin D and calcitonin receptor gene polymorphisms and forearm bone values of young Greek males

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Abstract

Introduction – hypothesis: Since the genetic bases of bone mass regulation in males are still poorly understood and the role of calciotropic hormones on bone mineral metabolism is absolute, our hypothesis is based on the certainty that specific genetic polymorphism will contribute, at least, on bone mass values. Our objective was to examine the relative contribution of genetic variables to the regulation of bone values in a population of young healthy men, focusing on the BsmI polymorphism of vitamin D receptor (VDR) gene and the AluI polymorphism of calcitonin receptor (CTR) gene. **Methods:** Areal bone mineral density (aBMD), bone mineral content (BMC) and geometrical areas at specific skeletal sites of the forearm, of 301 healthy Caucasian young men, aged 18-25, were assessed by single X-ray absorptiometry (Osteometer DTX-100). VDR and CTR alleles were determined by BsmI and AluI endonuclease restriction fragment analyses. Analysis of covariance was used as a statistical model. **Results:** No significant differences in the forearm aBMD, BMC or in area values were observed between the VDR and CTR genotypes. Findings did not change after adjusting for demographic characteristics. **Conclusions:** The BsmI and AluI polymorphisms are not related to the forearm bone values either reflecting mass or geometrical variables in this male population.

Keywords: VDR Polymorphisms, CTR Polymorphisms, Forearm Bone Mass, Geometrical Variables, Male, Peak Bone Mass

Introduction

Although it is recognized that the acquisition of optimal bone mass is mainly under genetic influence[1-3], the impact of genetic polymorphism and environmental interaction on males are rare and limited to a small portion of the study population[4-7]. Despite the fact that in developed countries the incidence of male osteoporosis is relatively high, accounting for 30% of hip fracture[8].

Since the role of calciotropic hormones such as calcitonin and vitamin D on bone mineral metabolism is absolute and in combination with the genetic impact on bone mass, the association of the regulation of distinct bone characteristics with specific gene polymorphisms seems justified.

Our hypothesis is based on the above rationale and determines the aim of the present study to investigate the relative contribution of genetics to the regulation of bone mass in a population-based cohort of young healthy men and their possible modulation by specific enviromental variables, focusing on the BsmI polymorphism of vitamin D receptor (VDR) gene and the AluI polymorphism of calcitonin receptor (CTR) gene.

Material and methods

Subjects

In this cross-sectional study, 301 healthy Greek young men, aged 18-25 were selected during gradational examinations for the Greek Armed forces. It is worthy to mention that the military service in our country is compulsory and is accomplished

The authors have no conflict of interest.

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during this age interval, approximately, by young Greek men. The randomization of our sample is based on the event that the Greek Army generally represents the general young Greek male population and on the possibility that every soldier of the same age can be included in the study's sample. Men who were treated with corticosteroids, anticonvulsants, or anticoagulants or who suffered from hypogonadism, kidney, liver, thyroid and gastrointestinal disease or diabetes mellitus were excluded from the study. Finally, soldiers with forearm and 'low energy' fractures and with prolonged immobilization were also excluded.

Approval by the hospital Ethics Committee and informed consent were obtained. Height and body weight were measured with the subjects in sportswear, standing barefoot on a fixed stadiometer and on a standard clinical balance. Height was recorded in centimetres and weight in kilograms. The stadiometer and scale were routinely monitored for accuracy and precision. Body mass index (BMI) was calculated as weight/height^2.

Dietary evaluation

Current dietary factors (calcium, proteins, alcohol, coffee and tea intake), were assessed using a food frequency questionnaire validated in the MEDOS study[9,10], and completed with an interview. Calcium intake was estimated considering milk, yogurt and cheese consumption. Protein intake was estimated by taking into account the consumption of meat and fish per week. Tea and coffee consumption was also taken into account.

Non-dietary factors were investigated using a questionnaire

Physical exercise was quantified as hours of sports activities per week (defined as taking part in organized sport for at least 12 months). Smoking behaviour was coded as 'yes' (daily smoking) or 'no', and sunlight exposure when there was exposure during the previous 3 months. The duration of immobilizations was also coded as 'less' or 'more than 1 month' (in majority, due to fractures caused by high energy trauma).

Genetic analyses

Genomic DNA was extracted from peripheral blood leukocytes with a DNA extraction kit (Puregene DNA isolation kit, Gentra Systems Inc., Munich, Germany). Polymorphisms in the CTR and VDR genes were determined by polymerase chain reaction (PCR) as previously described[11,12] using Taq DNA polymerase (Takara, Tokyo, Japan), and thermal cycler (PCR Primus 96 plus-MWG AG BIOTECH). 4 μl of CTR-PCR product were digested with AluI (New England Biolabs, UK) and 2 μl of VDR-PCR product were digested with BsmI (New England Biolabs, UK) according to manufacturer's instructions. AluI restriction digest yielded DNA fragments of 120/108-bp (TT), 228/120/108-bp (TC) and 228-bp (CC) and BsmI restriction digest yielded DNA fragments of 1200/650-bp (bb), 1850/1200/650-bp (Bb) and 1850-bp (BB), which were visualized using a 3% and 1% agarose gel, respectively, stained with ethidium bromide.

	Mean ± SD
Age (year)	23.60±3.42
Height (cm)	1.79±0. 07
Weight (kg)	82.30±13.43
BMI (kg/m^2)	25.54±3.66
Calcium intake (mg/day)	830.49±474.18
dBMC (gr)	4.24±0.56
dBMD (gr/cm^2)	0.55±0.04
udBMD(gr/cm^2)	0.50±0.06
ArRad	4.54±0.40
ArUlna	3.11±0.27
Arud	5.28±1.01
Smoking	
Yes	58.6%
No	41.4%
Sun exposure	
Never-rare-sometimes	60.5%
Often-very often	39.5%
Exercise	
<2h/week	9.2%
>2h/week	60.8%

* Values are the mean±SD. dBMC, dBMD, udBMD, bone mineral content and density at the distal (d) and ultradistal (ud) forearm, whereas ArRad, ArUlna, Arud are the geometrical areas at corresponding skeletal sites of forearm, all assessed with SXA.

Table 1. Baseline characteristics of the study group.

BMD measurements

Distal BMC (dBMC), distal BMD (dBMD) and ultradistal BMD (UdBMD) at the forearm were measured by single X-ray absorptiometry (Osteometer DTX-100, Denmark). Additionally, there were assessments of the corresponding geometrical areas, such as radial area (ArRad), ulnar area (ArUlna) and ultradistal area (ArUd). BMD is expressed as grams/cm^2; BMC is expressed in grams and Area as cm^2. The *in vivo* precision for the BMD and BMC measurements in our laboratory was 1-5%.

Statistical analysis

Descriptive statistics were determined for all variables. All variables are sufficiently represented using the mean value (mean) and standard deviation (SD).

	BB		Bb		bb	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Age (year)	23.59	±3.66	23.72	±3.38	23.24	±3.37
Height (cm)	1.80	±0.07	1.79	±0.07	1.78	±0.06
Weight (kg)	84.11	±13.55	81.25	±13.77	81.61	±12.39
BMI (kg/m^2)	25.97	±3.83	25.17	±3.64	25.60	±3.42
Calcium intake (mg/day)	744.10	±423.87	907.02	±492.57	798.06	±483.90
dBMC (gr)	4.32	±0.57	4.25	±0.56	4.20	±0.54
dBMD (gr/cm^2)	0.56	±0.05	0.55	±0.05	0.55	±0.04
udBMD(gr/cm^2)	0.51	±0.06	0.49	±0.06	0.49	±0.06
ArRad	4.56	±0.34	4.55	±0.43	4.52	±0.39
ArUlna	3.11	±0.23	3.12	±0.29	3.11	±0.27
Arud	5.30	±0.74	5.28	±1.12	5.26	±1.02

* Values are the mean±SD. dBMC, dBMD, udBMD, bone mineral content and density at the distal (d) and ultradistal (ud) forearm, whereas ArRad, ArUlna, Arud are the geometrical areas at corresponding skeletal sites of forearm, all assessed with SXA.

Table 2. Baseline characteristics of the study group according to VDR genotypes.

	TT		TC		CC	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Age (year)	23.66	±3.44	23.68	±3.57	22.35	±2.67
Height (cm)	1.80	±0.07	1.78	±0.06	1.80	±0.06
Weight (kg)	83.76	±13.66	80.59	±12.15	81.39	±15.64
BMI (kg/m^2)	25.85	±3.75	25.34	±3.53	24.85	±3.51
Calcium intake (mg/day)	813.26	±457.77	813.13	±490.54	1013.71	±472.53
dBMC (gr)	4.32	±0.61	4.19	±0.50	4.22	±0.52
dBMD (gr/cm^2)	0.56	±0.04	0.55	±0.04	0.55	±0.04
udBMD(gr/cm^2)	0.50	±0.06	0.49	±0.05	0.49	±0.04
ArRad	4.58	±0.45	4.52	±0.36	4.48	±0.26
ArUlna	3.15	±0.28	3.10	±0.27	3.04	±0.25
Arud	5.20	±1.11	5.34	±0.95	5.56	±0.77

* Values are the mean±SD. dBMC, dBMD, udBMD, bone mineral content and density at the distal (d) and ultradistal (ud) forearm, whereas ArRad, ArUlna, Arud are the geometrical areas at corresponding skeletal sites of forearm, all assessed with SXA.

Table 3. Baseline characteristics of the study group according to CTR genotypes.

Univariate analysis was performed using the two-sample Student's test or Welch-test (in a case of unequal SDs) and the model of one-way analysis of variance with no repeated measurements (pairwise multiple comparisons were analysed using the Tukey test). Analysis of covariance (ANCOVA) used the bone values (BMC, BMD, Area) as dependent variables, the VDR and CTR polymorphisms as factors and age, weight, height and calcium intake as covariates. Furthermore, multiple stepwise regression analysis was used to determine significant predictors of BMD. The impact of possible interaction of CTR and VDR genotypes on densitometric and geometrical variables of the forearm was assessed by using the 2-way ANOVA model. All tests are two sided; $P<0.05$ was defined as significant. All data analysis was performed using the Statistical Package for Social Sciences (version 10.0) software (SPSS Inc., Chicago, IL).

Results

The demographic characteristics of our sample and their diet and lifestyle habits at baseline are listed in Table 1. Moreover, Tables 2 and 3 show the above baseline characteristics according to the genotypes of VDR and CTR poly-

Variable	*Genotype*	*Number (N)*	*Mean±SD Unadjusted*	P_{unad}	*Mean±SE Adjusted*	P_{Ad}
dBMC	TT	144	4.322±0.605	0.124	4.294±0.043	0.415
	TC	126	4.187±0.495		4.222±0.046	
	CC	31	4.218±0.521		4.195±0.093	
dBMD	TT	144	0.557±4.488E-02	0.191	0.556±0.003	0.399
	TC	126	0.548±4.302E-02		0.556±0.004	
	CC	31	0.547±4.027E-02		0.548±0.008	
UdBMC	TT	144	2.643±0.675	0.848	2.621±0.052	0.677
	TC	126	2.653±0.579		2.688±0.056	
	CC	31	2.725±0.417		2.673±0.124	
UdBMD	TT	144	0.504±6.115E-02	0.090	0.503±0.004	0.239
	TC	126	0.490±5.229E-02		0.492±0.005	
	CC	31	0.493±4.319E-02		0.492±0.010	
ArRad	TT	144	4.579±0.450	0.417	4.568±0.034	0.352
	TC	126	4.522±0.355		4.541±0.036	
	CC	31	4.483±0.260		4.444±0.080	
ArUlna	TT	144	3.150±0.279	0.135	3.143±0.023	0.069
	TC	126	3.099±0.266		3.116±0.024	
	CC	31	3.040±0.248		3.008±0.054	
ArUd	TT	144	5.198±1.107	0.245	5.172±0.085	0.149
	TC	126	5.339±0.954		5.394±0.091	
	CC	31	5.564±0.771		5.455±0.203	

* Values are the mean±SD (SE). dBMC, dBMD, udBMD, bone mineral content and density at the distal (d) and ultradistal (ud) forearm, whereas ArRad, ArUlna, Arud are the geometrical areas at corresponding skeletal sites, all assessed with SXA.

Table 4. Relation of CTR genotypes and densitometric and geometrical variables of forearm unadjusted and adjusted for age, weight, height, calcium intake, sun exposure and physical activity.

morphisms. The anthropometric variables such as age, weight, height and BMI were similar among the six genotype groups of CTR and VDR polymorphisms (P=n.s.).

The genotype frequencies of the AluI CTR polymorphism were 48% for TT, 42% for CT and 10% for CC, while those of BsmI VDR polymorphism were 21% for BB, 48% for Bb and 31% for bb. The genotype distribution of each gene and the corresponding values of the examined variables are described in Tables 4 and 5. They show values of areal BMC, areal BMD and cross-sectional areas of specific skeletal sites adjacent to the wrist measured with SXA (Osteometer DTX-100). In the six groups of the specific polymorphisms, there were no significant differences in the densitometric values and in geometrical areas at the distal or ultradistal forearm. The BB and TT genotypes presented the higher values in almost all examined variables (Tables 4 and 5).

Futhermore, there was no association between the genotype groups of both gene polymorphisms and the densitometric and cross-sectional variables when adjusting for anthropometric and lifestyle factors (Tables 4, 5).

Attempting to detect any additive effect of the different alleles of the above polymorphisms, no statistically significant difference concerning the aforementioned densitometric and cross-sectional parameters was observed among the various genotype group combinations (Table 6). It is noteworthy, the TTBB polymorphism showed higher values in all the densitometric and geometrical variables examined, whereas the CCBB polymorphism revealed the lower ones.

Discussion

In the present study we found no association between forearm bone mass variables and the six VDR and CTR genotype groups defined by the specific restriction enzymes. Moreover, there was no significant statistical difference among the above genotypes concerning geometrical areas at distinct skeletal sites of the forearm, adjacent to the wrist joint. In addition, when examining the above densitometric and geometrical variables in relation to the possible gene-by-gene interaction between both polymorphisms, no significant association was found. These findings indicate that both VDR, and CTR gene polymorphisms do not contribute to

Variable	*Genotype*	*Number (N)*	*Mean±SD Unadjusted*	P_{unad}	*Mean±SD Adjusted*	P_{Ad}
dBMC	BB	64	4.319±0.567	0.404	4.282±0.066	0.754
	Bb	144	4.249±0.560		4.248±0.043	
	bb	93	4.195±0.543		4.218±0.054	
dBMD	BB	64	0.557±4.611E-02	0.554	0.556±0.005	0.694
	Bb	144	0.551±4.607E-02		0.550±0.005	
	bb	93	0.550±3.907E-02		0.552±0.004	
UdBMC	BB	64	2.692±0.508	0.852	2.630±0.081	0.936
	Bb	144	2.636±0.652		2.644±0.054	
	bb	93	2.638±0.633		2.666±0.065	
UdBMD	BB	64	0.506±5.956E-02	0.344	0.504±0.007	0.541
	Bb	144	0.494±5.627E-02		0.495±0.005	
	bb	93	0.493±5.534E-02		0.495±0.006	
ArRad	BB	64	4.557±0.337	0.791	4.521±0.053	0.845
	Bb	144	4.550±0.433		4.555±0.035	
	bb	93	4.515±0.393		4.532±0.042	
ArUlna	BB	64	3.110±0.232	0.956	3.086±0.035	0.654
	Bb	144	3.120±0.288		3.128±0.023	
	bb	93	3.109±0.266		3.118±0.028	
ArUd	BB	64	5.301±0.742	0.973	5.211±0.133	0.834
	Bb	144	5.283±1.124		5.290±0.089	
	bb	93	5.259±1.018		5.311±0.107	

*Values are the mean±SD (SE). dBMC, dBMD, udBMD, bone mineral content and density at the distal (d) and ultradistal (ud) forearm, whereas ArRad, ArUlna, Arud are the geometrical areas at corresponding skeletal sites, all assessed with SXA.

Table 5. Relation of VDR genotypes and densitometric and geometrical variables of forearm unadjusted and adjusted for age, weight, height, calcium intake, sun exposure and physical activity.

bone mass acquisition in this young healthy male population.

These data confirm the results of previous similar studies[7,13] that concluded that the AluI CTR and BsmI VDR polymorphisms are not independent predictors of early adulthood bone values either reflecting mass or geometrical characteristics. Our study is the first to show that in young male adults, AluI CTR polymorphism is not associated with forearm bone mass, since a previous study examined the same gene polymorphism in an older male population and evaluated other skeletal sites, such as spine and femoral neck instead of forearm bone values[7]. In particular, they concluded that genetic variations at the CTR were significantly associated with BMD in the hip and spine and may be related to the pathophysiology of age-related bone loss in men. Since 1994 when Morrison et al.[14] reported the relationship between VDR polymorphisms and BMD, many subsequent studies have been carried out on the association between VDR polymorphisms and diverse outcomes of osteoporosis, including BMD and fracture. In the male population there was a failure to demonstrate an effect of VDR polymorphism on bone density[7,15-17], while studies documenting the contrary showed an association with axial (lumbar spine) and not with appendicular skeleton peak BMD[18] and with several frame size characteristics such as height and lumbar vertebral body width[19,20]. Furthermore, in longitudinal studies an association of VDR genotypes with bone gain in the formative periods of life was also not found[13,19].

Current evidence suggests that genetic and enviromental factors contribute significantly to the regulation of bone mass in healthy individuals[6].The impact of the lifestyle factors is stressed in many studies, estimating their contribution to the 20-30% of the variation in peak bone values by modulating bone gain during childhood and adolescence[21-23]. Interest has focused also on the possibility that enviromental variables can modulate the apparent strength and even the direction of the different genetic effects on bone phenotypes[24]. Although it was revealed that a mean calcium daily intake less than 400 mg, physical activity and sun exposure are independent predictors of the densitometric variables dBMC and udBMD of the forearm but not of the corresponding cross-sectional areas[25], such an impact was not able to alter the findings of the present analysis of covariance (ANCOVA) for VDR and CTR polymorphisms. It seems that the modified factors (calcium intake, sun exposure, physical activity) do not mask any statistically significant difference among the above genotypes on bone values of the forearm in this young healthy male population.

Our attempt to assess bone values as a polygenic trait is

	CTR	VDR	Mean	SD	Interaction two-way ANOVA P-value
dBMC	TT	BB	4.436	0.542	0.204
		Bb	4.305	0.626	
		bb	4.238	0.618	
	TC	BB	4.300	0.621	
		Bb	4.150	0.448	
		bb	4.147	0.496	
	CC	BB	3.907	0.320	
		Bb	4.384	0.608	
		bb	4.238	0.428	
dBMD	TT	BB	0.561	0.041	0.189
		Bb	0.558	0.050	
		bb	0.552	0.041	
	TC	BB	0.564	0.055	
		Bb	0.542	0.040	
		bb	0.548	0.040	
	CC	BB	0.526	0.030	
		Bb	0.554	0.048	
		bb	0.555	0.030	
UdBMC	TT	BB	2.679	0.484	0.670
		Bb	2.638	0.740	
		bb	2.621	0.719	
	TC	BB	2.788	0.611	
		Bb	2.610	0.568	
		bb	2.610	0.592	
	CC	BB	2.485	0.213	
		Bb	2.769	0.495	
		bb	2.877	0.398	
UdBMD	TT	BB	0.518	0.059	
		Bb	0.502	0.064	
		bb	0.498	0.059	
	TC	BB	0.496	0.063	0.826
		Bb	0.487	0.049	
		bb	0.490	0.054	
	CC	BB	0.485	0.046	
		Bb	0.496	0.041	
		bb	0.495	0.049	
ArRad	TT	BB	4.657	0.351	0.449
		Bb	4.558	0.510	
		bb	4.544	0.429	
	TC	BB	4.489	0.304	
		Bb	4.529	0.355	
		bb	4.498	0.390	
	CC	BB	4.290	0.148	
		Bb	4.618	0.273	
		bb	4.476	0.234	
Ar Ulna	TT	BB	3.185	0.200	0.672
		Bb	3.136	0.320	
		bb	3.142	0.258	
	TC	BB	3.047	0.237	
		Bb	3.108	0.264	
		bb	3.090	0.271	
	CC	BB	2.942	0.256	
		Bb	3.088	0.205	
		bb	3.064	0.303	
ArUd	TT	BB	5.159	0.660	0.663
		Bb	5.215	1.229	
		bb	5.196	1.225	
	TC	BB	5.545	0.893	
		Bb	5.321	1.065	
		bb	5.203	0.791	
	CC	BB	5.273	0.546	
		Bb	5.522	0.707	
		bb	5.867	0.988	

*Values are the mean±SD. dBMC, dBMD, udBMD, bone mineral content and density at the distal (d) and ultradistal (ud) forearm, whereas ArRad, ArUlna, Arud are the geometrical areas at corresponding skeletal sites, all assessed with SXA.

Table 6. Interaction of CTR and VDR genotypes on densitometric and geometrical variables of forearm.

justified by the unsuccesful efforts in the international literature to determine the specific genes responsible for regulation of bone mass and by the assumptions of a segregation analysis that bone mineral density is under polygenic control[26]. It is likely that the genetic risk of developing low bone mineral density consists of several common gene polymorphisms that have weak effects individually but major effects when acting in concert, rather than one or only a few genes that have large individual effects. Notably, the present study, according to our knowledge, is the first that evaluates the possible additive interaction between genotype effects of two calciotropic hormones like vitamin D and calcitonin on male bone mass acquisition in early adulthood. Albeit, there was a trend towards higher distal BMC, aBMD and cross-sectional area values in the TTBB combined genotype, a significantly statistical difference among the various gene-by-gene interaction genotypes was not documented (Table 6). A similar trend was also observed for each TT and BB genotype separately (Tables 4 and 5), evidence pointing out the weak impact of these two genetic polymorphisms on bone phenotypes of the forearm, in young males.

At this point, any further assessment of possible interac-

tions among the genetic and enviromental factors on forearm phenotypes was considered unnecessary. The inability to reveal any similar interaction among the different genotypes and the magnitude of the population rule out such expectations.

This study has several strengths. First of all, the random selection of our male population and the exclusion of those with diseases and treament known to affect bone metabolism eliminate the existence of confounding factors. Only Caucasian young adults were analyzed, so our sample was an ethnically homogenous with normal hormonal and health profiles. Genotype frequencies both of CTR and VDR were similar to those previously reported for other Caucasian male populations[5,6,25]. Additionally, the groups in the three genotypes for each gene examined (CTR, VDR) were well matched for relevant anthropometric and lifestyle variables. Adjusting for these parameters did not alter our findings. The sample size is quite larger than that of previous similar studies[5,13]. Finally, the fact that early adulthood bone mineral mass is expected to be an even stronger determinant for osteoporosis in men than in women, as midlife rapid bone loss does not occur in the former as it does after menopause, makes our population ideal for association assumptions.

Some limitations of the present study should be noted. First of all, it is its cross-sectional design which makes it vulnerable to secular changes. However, cross-sectional studies like this one can provide the basis for prospective studies. Moreover, the evaluation of the gene-by-gene interaction effects between the various CTR and VDR genotypes needs a quite bigger population , since the number of subjects with CC-BB, CC-Bb, CC-bb combination genotypes were too small (6, 8, 7, respectively) for valid analysis. Furthermore, another drawback is the assessment of only one single nucleotide polymorphism (SNP) for each of two candidate genes, but the smaller sample size of similar studies[5,13], and the input of bone geometrical variables in our genotype associations were considered advantages for confirming our initial hypothesis.

In conclusion, the results of this study point out that the AluI CTR and BsmI VDR polymorphisms are not related to forearm bone values, either reflecting mass or geometrical characteristics, in this young healthy male population. In addition to that, additive effects of both polymorphisms and interactions with dietary and lifestyle factors were not detected on bone variables examined. Therefore, other unidentified genes must play an important role in BMD attained at adulthood in males. The identification of those genes and understanding the mechanisms underlying bone mass acquisition during the genetic and enviromental interplay will have a major impact on the diagnosis, prediction, and prognosis of osteoporosis.

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