J Bone Miner Metab (2006) 24:329–336
DOI 10.1007/s00774-006-0691-2

ORIGINAL ARTICLE

Siobhan Cusack · Christian Mølgaard · Kim F. Michaelsen
Jette Jakobsen · Christel J.E. Lamberg-Allardt
Kevin D. Cashman

Vitamin D and estrogen receptor-α genotype and indices of bone mass and bone turnover in Danish girls

Received: August 31, 2005 / Accepted: January 23, 2006

Abstract Peak bone mass is a major determinant of osteoporosis risk in later life. It is under strong genetic control; however, little is known about the identity of the genes involved. In the present study, we investigated the relationship between polymorphisms in the genes encoding the vitamin D receptor (VDR) (*Fok*I, *Taq*I) and estrogen receptor-α (ERα) (*Pvu*II, *Xba*I), and bone mineral density (BMD), bone mineral content (BMC), and markers of bone turnover in 224 Danish girls aged 11–12 years. BMD and BMC were measured by dual-energy X-ray absorptiometry. Serum osteocalcin, 25(OH)D, and parathyroid hormone (PTH) were measured by ELISA assays and urinary pyridinium cross-links by HPLC. Physical activity, dietary calcium, and Tanner stage were assessed by questionnaire. In general, there were no significant differences in anthropometrical variables, physical activity, dietary calcium, serum 25(OH)D, or PTH among genotype groups. BMD or BMC of lumbar spine or whole body (adjusted for body and bone size and pubertal status) were not associated with VDR or ERα genotypes or the combination of these genotypes. This lack of association remained even after adjustment for dietary and environmental factors. VDR genotypes had no effect on bone turnover markers. *XX* and *PP* ERα genotypes were associated ($P < 0.05$) with reduced levels of urinary pyridinium cross-links, whereas serum osteocalcin was similar among genotypes. These findings suggest that the rate of bone resorption was influenced by ERα genotypes, even though these biochemical differences were not evident in bone mass indices.

Key words vitamin D receptor · estrogen receptor · genotype · bone

S. Cusack · K.D. Cashman (✉)
Department of Food and Nutritional Sciences, and Department of Medicine, University College, Cork, Ireland
Tel. +353-21-4901317; Fax +353-21-4270244
e-mail: k.cashman@ucc.ie

C. Mølgaard · K.F. Michaelsen
Research Department of Human Nutrition, Centre for Advanced Food Studies, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark

J. Jakobsen
Danish Institute for Food and Veterinary Research, Söborg, Denmark

C.J.E. Lamberg-Allardt
Calcium Research Unit, Department of Applied Chemistry and Microbiology, University of Helsinki, Finland

Introduction

The peak level of bone mass, generally attained before the third decade, is considered to be a major independent indicator of risk of osteoporotic fracture in later life. This peak bone mass (PBM) is believed to be under strong polygenic control [1]. Over the past decade, there has been considerable research emphasis on the identification of the specific genes involved. Among the major genes implicated in the determination of bone mineral density (BMD) are the genes encoding the vitamin D receptor (VDR) and estrogen receptor-α (ERα).

Morrison et al. [2] suggested that a polymorphism at the VDR gene could be responsible for up to 75% of the total genetic effect on PBM. Although the results of Morrison et al. [2] have been confirmed in some studies, other studies have failed to find a relationship between bone mass and VDR genotype [3–9]. Furthermore, although the number of studies that have investigated the relationship between polymorphisms in the ERα gene and BMD are far fewer than those for the VDR gene; these studies have also produced conflicting results [9]. Moreover, much of this research has been in adult population groups, especially pre- and postmenopausal women. Even less is known about the effects of these candidate genes on bone mass in childhood, a life stage of critical importance to the achievement of PBM [10].

Theoretically, bone gain would be a stronger candidate for an effect of candidate genes, such as the VDR and ERα,

because bone loss has a very low heritability [11]. However, of the limited number of association studies that have investigated the effect of VDR genotype on bone mass/bone turnover in children or adolescents, the results have been conflicting, with no consistent pattern emerging across studies indicating how VDR alleles might be involved [12–17]. Similarly, the limited data on the relationship between ERα genotype and bone mass/bone turnover in children and adolescents are also conflicting [10,13,16,18–20]. Many of these studies concerned either entirely or largely a group of prepubertal [12–14,18,19] or late-postpubertal children [15–17,21]. There has been less research emphasis on children during the peripubertal period. Theintz et al. [22] suggested that the rate of increment in BMD in childhood can be very pronounced over the 3-year period from 11 to 14 years of age in girls.

Therefore, the objective of the present study was to investigate the relationship between polymorphisms in the VDR (*Fok*I, *Taq*I) and ERα (*Pvu*II, *Xba*I) genes and BMD, bone mineral content (BMC), and biochemical indices of bone turnover in 224 Danish girls, aged 11–12 years. In addition, the potential impact of dietary and environmental factors, such as dietary calcium, vitamin D status, and physical activity, on the relationship between genotype and bone mass was also investigated in these girls.

Materials and methods

Subjects

A total of 225 Danish girls, aged between 10.9 and 11.9 years, were included in the study. The girls were recruited for participation in a 12-month vitamin D intervention trial on bone health [as part of the Optimal Strategy for Vitamin D Fortification (OPTIFORD) project; www.optiford.org] and the BMD, BMC, and bone turnover data reported in the present work were the baseline values from the intervention study. Subjects were recruited using information from the Danish National Central Offices of Civil Registrations, which allowed us identify the names and addresses of all girls born in Denmark with Danish citizenship, aged 10.0–11.0 years, living in the municipalities of Copenhagen and Frederiksberg (n = 1755). The oldest were recruited first and we continued until 225 had agreed to participate, corresponding to 22% of the 1009 who were asked to participate by mail. Of the 225 girls participating in the study, genotypes were successfully obtained for 224 girls, and these were included in the present work. The distribution of Tanner score and the mean age, weight, height, and body mass index (BMI) for the group are shown in Table 1. The study was approved by the Research Ethical Committee of Copenhagen and Frederiksberg [J.nr (KF) 01-129/01]. Informed consent was obtained from the parent/guardian of each participant. Girls were excluded on the basis of the following criteria: chronic diseases, intake of drugs that could influence bone metabolism, and daily intake of calcium supplements or vitamin/mineral supplements.

Table 1. Baseline characteristics of Danish girls

n		224
Age (years)		11.42 ± 0.01
Height (m)		1.51 ± 0.58
Weight (kg)		41.6 ± 0.6
BMI (kg/m^2)		18.2 ± 0.5
Tanner score		
1 (%)		8.93
2 (%)		41.52
3 (%)		48.66
4 (%)		0.89
RFLP	Genotype	*n* (%)
VDR:		
*Fok*I	*FF*	89 (39.7)
	Ff	109 (48.7)
	ff	26 (11.6)
*Taq*I	*TT*	72 (32.1)
	Tt	115 (51.3)
	tt	37 (16.5)
ERα:		
*Pvu*II	*PP*	62 (27.7)
	Pp	114 (50.9)
	pp	48 (21.4)
*Xba*I	*XX*	35 (15.6)
	Xx	110 (49.1)
	xx	79 (35.3)

Values are mean ± SE
BMI, body mass index; RFLP, restriction fragment length polymorphism; VDR, vitamin D receptor, ERα, estrogen receptor-α

Design

This was an association study that examined the relationship between bone status indices (BMD, BMC, and serum and urinary-based biochemical markers of bone turnover) and restriction fragment length polymorphisms (RFLP) that define the human VDR and ERα (i.e., candidates genes for low bone density) in a cohort of healthy young Danish girls aged 10.9–11.9 years. Subjects were instructed to collect urine samples between 0800 and 1000 after an overnight fast. In addition, after an overnight fast, two blood samples (10 ml) were also taken between 0800 and 1000 from each subject. On the same day as blood and urine sampling, each girl, with the help of their parent/guardian and a trained researcher, completed various questionnaires, including a general health and lifestyle questionnaire, a food frequency questionnaire (FFQ), a physical activity questionnaire, and a pubertal status questionnaire. Anthropometric measurements (weight and height) were also taken at this time. Measurement of BMD, BMC, and urinary and serum-based biochemical marker levels were made independently of the RFLP analysis, and results were stored in a coded fashion.

Assessment of dietary calcium and vitamin D

The subjects filled in, together with their parents/guardians, a standardized food frequency questionnaire (FFQ) with 12 questions covering nine food items that ascertained the foods (including fortified foods) contributing to 95% of the vitamin D intake and 75% of the calcium intake determined from the most recent dietary intake studies in Denmark.

The questionnaire had nine predetermined possible frequencies (ranging from "less than one time per month" to "4–5 times per day or more"). A trained dietician checked the completed FFQ and requested additional information or clarification on entries, if necessary. The Danish Institute for Food and Veterinary Research (DFVF) performed the intake calculations using the General Intake Estimation System (GIES), a system developed at DFVF [23]. The FFQ and calculations used have been reported earlier [24].

Pubertal status

The stage of puberty was assessed in each subject ad modum Marshall and Tanner [25] by a validated self-assessment protocol based on the evaluation of breast development by pictures and timing of menarche (Müller et al. 1998; unpublished data).

Physical activity

Physical activity was recorded using a 24-h recall questionnaire (Habitual Activity Estimation Scale, HAES) [26], as used previously in young Danish girls [27]. In brief, the HAES questionnaire records the hours per day spent on each of four activity levels corresponding to supine position (I), sitting (II), walking (III), and running (IV). In the current study, hours per day spent on high activity (level IV) were used as a measurement for activity level, as this has been shown to correlate well with mean daily activity computer (Caltrac) measurement (counts/day) during a week (7 days) [27]. Activity was recorded during 2 weekdays and 1 weekend day, and the mean of the 3 days activity (level IV) was used in the analyses.

Bone mineral density and bone mineral content

Bone mineral content (BMC, measured in grams of hydroxyapatite), bone size [expressed as anteroposterior projected bone area (BA), measured in cm^2], and bone mineral density (BMD = BMC/BA in g/cm^2), as well as percentage fat and lean body mass content, were measured by dual-energy X-ray absorptiometry (DEXA) using the Hologic 1000/W scanner (Hologic, Waltham, MA, USA). The skeletal sites assessed were the whole body and the lumbar spine (L2–L4). Subjects wore only underpants and a cotton T-shirt during the scan. For whole-body and lumbar analyses, software version V5.73 and V4.76P, respectively, were used. For quality control, a spine phantom was scanned daily. The coefficient of variation (CV%) for these BMC, BMD, and BA measurements on the spine phantom over a period of 2 years ($n = 429$) was 0.35%, 0.32%, and 0.32%, respectively. In adults examined within 8 weeks, an interval of reproducibility expressed as CV% was 1.6%, 2.2%, 0.9%, 2.3%, and 1.9% for whole-body BMC, BA, BMD, percentage fat, and lean body mass, respectively (Hansen D, Astrup A, 1998; unpublished data). The effective dose for a whole-body and a lumbar DEXA scan was not more than 10 μSv in total, equal to about daily background radiation in Denmark.

Collection and preparation of samples

Subjects were supplied with suitable collection containers for urine samples and asked to collect morning void urine samples between 0800 and 1000 after an overnight fast. Portions of urine were stored at –20°C from the morning of collection until required for analysis. Blood was collected by venepuncture into vacutainer tubes with no additive or ethylene diamine tetraacetic acid (EDTA). Blood samlpes collected in tubes with no additive were processed to serum, which was immediately stored at –80°C until required for analysis. Genomic DNA was extracted from blood samples collected in EDTA-containing vacutainer tubes using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). This isolation technique resulted in high molecular weight DNA (>20 kb) that was free of RNA contamination and had a 260/280 absorbance ratio >1.7. The isolated DNA was then stored at –20°C until required for analysis.

Restriction fragment length polymorphisms

RFLP in the VDR and ERα genes were determined by polymerase chain reaction (PCR) techniques followed by digestion of the amplified PCR products with *Taq*I [28], *Fok*I [29], (VDR) and *Pvu*II and *Xba*I [30] (ERα) restriction endonucleases (New England Biolabs, Beverly, MA, USA), as described previously.

Experimental techniques

Serum intact parathyroid hormone and osteocalcin. Serum intact parathyroid hormone (iPTH) levels were measured in serum using an enzyme-linked immunoassay (ELISA) (OCTEIA intact parathyroid hormone; Immuno Diagnostic Systems, Boldon, UK). The intra- and interassay CV was 3.0% and 5.4%, respectively. Osteocalcin levels were measured in serum samples using an ELISA (Metra Osteocalcin EIA Kit; Quidel, Santa Clara, CA, USA). The intra- and interassay CV was 6.0% and 7.6%, respectively.

Serum 25-hydroxyvitamin D. Serum 25-hydroxyvitamin D [25(OH)D] levels were measured by a HPLC-based method, as described in detail elsewhere [24].

Urinary creatinine, pyridinoline, and deoxypyridinoline. Creatinine was determined in urine samples using a diagnostic kit (Metra Creatinine Assay Kit; Quidel). Urine samples were analyzed in duplicate for pyridinoline (Pyr) and deoxypyridinoline (Dpyr) using an automated HPLC system, as described in detail elsewhere [31–34].

Statistical methods

Data for all variables were normally distributed and allowed for parametric tests of significance. Data are presented as means with their standard errors. To test whether the allelic distributions of the various candidate gene RFLP satisfied Hardy–Weinberg equilibrium, a chi-squared (χ^2) test was used to compare the observed and expected frequencies of the various alleles for each RFLP within a separate candidate gene, separately. For each pair of markers within the candidate genes, linkage disequilibrium was evaluated using Halpoview software (www.hapmap.org). The association between the candidate gene RFLP and physical characteristics (age, weight, height, BMI) as well as dietary calcium intake, serum 25(OH)D levels, and physical activity score were assessed by analysis of variance (ANOVA) for each candidate gene RFLP separately. Differences in unadjusted BMD and unadjusted BMC as well as biochemical indices of bone turnover in relation to each RFLP within a gene, as well as the cross-genotypes in that gene (i.e., *Fok*I and *Taq*I cross-genotypes and *Xba*I and *Pvu*II cross-genotypes), were assessed by ANOVA, with Bonferroni's correction of multiple comparisons. Gene-by-gene interaction effects were tested for VDR and ERα in models that contained two RFLP and their product, which represented the interaction effect. Furthermore, differences in biochemical indices of bone turnover in relation to candidate gene RFLP were further assessed by multiple regression analysis that controlled for pubertal status and other variables (Tanner score, age, weight, and height). The relationships between candidate gene RFLP/cross-genotypes and BMD and BMC were also investigated after all size-related continuous variables (BMC, BA, BMD, height, weight) were converted to the natural logarithm before the analysis [35]. To investigate the influence of candidate gene RFLP on BMC adjusted for bone and body size (size-adjusted BMC), BA, height, and weight were included in multiple linear regression equations as recommended by Prentice et al. [35]. Furthermore, differences in BMD and BMC (adjusted and unadjusted) in relation to candidate gene RFLP were further assessed by multiple regression analysis that controlled for other potentially confounding effects of Tanner score, dietary calcium, vitamin D status, and physical activity score.

Results

Baseline physical characteristics and VDR and ERα genotype frequencies

Baseline physical characteristics of the girls are presented in Table 1. The majority (~90%) of girls in the present study were of early- to midpubertal status.

The percentage frequencies for *Pvu*II and *Xba*I ERα and *Fok*I and *Taq*I VDR genotypes for the group of young Danish girls are also shown in Table 1. There was no evidence of deviation from Hardy–Weinberg equilibrium for any of the four polymorphisms ($P > 0.05$). Although there was strong linkage disequilibrium between *Pvu*II and *Xba*I ERα polymorphisms ($D' > 0.95$), there was no linkage disequilibrium between *Fok*I and *Taq*I VDR polymorphisms ($D' < 0.05$).

Baseline physical characteristics, dietary calcium, vitamin D status, and physical activity score in Danish girls stratified by VDR and ERα genotype

Age, weight, height, and BMI of the girls were similar among VDR *Taq*I and ERα *Xba*I and *Pvu*II genotype groups (Tables 2, 3). Although age, weight, and BMI were not associated with the VDR *Fok*I genotype, girls with the *ff* genotype were significantly ($P < 0.05$) taller than those with either the *Ff* or *FF* genotype (Table 2). The mean (SD) daily dietary intakes of vitamin D and calcium by the girls were 2.6 (1.4) μg and 1030 (617) mg, respectively. The mean (SD) serum 25(OH)D concentration of the girls was 43.2 (17.1) nmol/l. Dietary calcium intake and physical activity score for the girls did not vary by RFLP genotype group within either of the two candidate genes investigated (Tables 2, 3). Although serum 25(OH)D concentration did not vary among *Fok*I, *Pvu*II, or *Xba*I polymorphism groups (Tables 2, 3), there was a tendency ($P = 0.067$) for serum 25(OH)D concentration to vary among *Taq*I polymorphism groups (see Table 2).

Effect of VDR and ERα genotype on biochemical indices of bone turnover

Serum PTH and osteocalcin as well as urinary pyridinium cross-links (Pyr and Dpyr) levels were unaffected by VDR *Fok*I or *Taq*I genotype (see Table 2). Serum PTH was not associated with ERα *Pvu*II or *Xba*I genotype (see Table 3). Urinary pyridinium cross-link and serum osteocalcin levels were ($P \leq 0.05$) or tended to be ($P = 0.063$; osteocalcin among *Xba*I ERα genotype groups) associated with ERα *Pvu*II or *Xba*I genotypes (see Table 3). However, although the associations between urinary pyridinium cross-link ($P < 0.05$) and ERα *Pvu*II or *Xba*I genotypes remained after adjustment for pubertal status (Tanner score), age, weight, and height, the associations between serum osteocalcin levels and ERα *Pvu*II or *Xba*I genotypes disappeared (data not shown). *XX* and *PP* genotypes appeared to be associated with reduced levels of urinary Pyr and Dpyr compared to those of the other two genotypes (see Table 3). There were no significant gene–gene interaction effects between polymorphisms at the ERα and the VDR on bone marker levels (data not shown).

Effect of VDR and ERα genotype on unadjusted and adjusted BMD and BMC, including control of potential dietary and confounding environmental factors

Unadjusted BMD of the lumbar spine or whole body was not associated with *Xba*I, *Pvu*II, *Fok*I, or *Taq*I

Table 2. Vitamin D receptor (VDR) genotype and bone health indices of 224 Danish girls

	VDR *Fok*I genotype				VDR *Taq*I genotype			
	ff	*Ff*	*FF*	*P* value	*tt*	*Tt*	*TT*	*P* value
n (%)	26 (11.60)	109 (48.66)	89 (39.74)	–	37 (16.52)	115 (51.34)	72 (32.14)	–
Age (years)	11.49 ± 0.04	11.43 ± 0.02	11.40 ± 0.02	0.107	11.44 ± 0.03	11.41 ± 0.02	11.45 ± 0.03	0.371
Height (m)	1.54[a] ± 0.01	1.50[b] ± 0.01	1.50[b] ± 0.01	0.012	1.50 ± 0.12	1.51 ± 0.06	1.50 ± 0.09	0.585
Weight (kg)	44.6 ± 1.6	40.8 ± 0.8	41.7 ± 1.0	0.136	43.0 ± 1.6	41.6 ± 0.7	40.8 ± 1.1	0.464
BMI (kg/m^2)	18.7 ± 0.6	18.0 ± 0.2	18.4 ± 0.3	0.520	19.0 ± 0.6	18.1 ± 0.3	18.0 ± 0.3	0.217
Tanner stage (%1/2/3/4)	3.8/30.8/65.4/0	6.4/43.2/49.5/0.9	13.5/42.7/42.7/1.1	–	10.8/29.7/56.8/2.7	10.4/36.5/52.2/0.9	5.6/55.5/38.9/0	–
Physical activity (h/d)	2.77 ± 0.31	2.80 ± 0.14	2.83 ± 0.15	0.984	2.92 ± 0.27	2.80 ± 0.13	2.77 ± 0.19	0.888
S-25 OH D (nmol/l)	45.5 ± 3.6	43.5 ± 1.5	42.0 ± 1.9	0.621	44.6 ± 2.9	40.6 ± 1.5	46.4 ± 2.1	0.067
Ca intake (mg/d)	1156 ± 149	990 ± 57	1048 ± 63	0.447	1065 ± 94	1048 ± 63	992 ± 65	0.785
U-Pyr (nmol/mmol Cr)	91.5 ± 9.7	83.0 ± 4.1	80.2 ± 4.7	0.532	77.6 ± 6.4	82.1 ± 4.1	86.4 ± 5.3	0.616
U-Dpyr (nmol/mmol Cr)	50.2 ± 5.4	45.1 ± 2.3	46.2 ± 2.5	0.639	45.4 ± 4.0	45.8 ± 2.2	46.9 ± 2.8	0.935
S-Osteocalcin (µg/ml)	56.1 ± 3.5	49.6 ± 1.5	52.0 ± 1.7	0.158	50.5 ± 3.2	52.2 ± 1.5	50.2 ± 1.7	0.689
S-PTH (pmol/l)	3.15 ± 0.18	2.98 ± 0.08	3.24 ± 0.10	0.547	3.27 ± 0.14	3.06 ± 0.08	3.08 ± 0.12	0.472
TB BMD (g/cm^2)	0.88 ± 0.01	0.86 ± 0.01	0.87 ± 0.01	0.154	0.87 ± 0.01	0.87 ± 0.01	0.86 ± 0.01	0.907
TB BMC (g)	1411 ± 49	1268 ± 26	1304 ± 32	0.070	1302 ± 51	1306 ± 24	1286 ± 37	0.893
Spine BMD (g/cm^2)	0.72 ± 0.02	0.69 ± 0.01	0.70 ± 0.01	0.292	0.69 ± 0.02	0.70 ± 0.01	0.70 ± 0.01	0.914
Spine BMC (g)	32.11 ± 1.62	28.55 ± 0.68	29.09 ± 0.96	0.083	28.64 ± 1.25	29.67 ± 0.65	28.67 ± 0.91	0.590

Values are mean ± SE
BMI, body mass index; TB, total body; BMD, bone mineral density; BMC, bone mineral content; U, urinary; Pyr, pyridinoline; Dpyr, deoxypyridinoline; S, serum
[a,b]Mean values within a row that have different superscripts are significantly different using Bonferroni's correction for multiple comparisons ($P < 0.05$)

Table 3. Estrogen receptor-alpha (ERα) genotype and bone health indices of 224 Danish girls

	ERα *Xba*I genotype				ERα *Pvu*II genotype			
	xx	*Xx*	*XX*	*P* value	*pp*	*Pp*	*PP*	*P* value
n (%)	79 (35.27)	110 (49.10)	35 (15.63)	–	48 (21.43)	114 (50.90)	62 (27.67)	–
Age (years)	11.42 ± 0.02	11.42 ± 0.02	11.45 ± 0.04	0.735	11.39 ± 0.03	11.43 ± 0.02	11.44 ± 0.03	0.461
Height (m)	1.50 ± 0.01	1.51 ± 0.01	1.50 ± 0.01	0.496	1.49 ± 0.01	1.51 ± 0.01	1.51 ± 0.01	0.346
Weight (kg)	40.8 ± 1.0	42.3 ± 0.9	41.0 ± 1.1	0.468	40.7 ± 1.4	41.2 ± 0.7	43.0 ± 1.2	0.335
BMI (kg/m^2)	18.0 ± 0.3	18.45 ± 0.3	18.1 ± 0.4	0.689	18.1 ± 0.4	18.0 ± 0.3	18.8 ± 0.4	0.232
Tanner stage (%1/2/3/4)	10.1/39.2/50.7/0	8.2/40.9/50.0/0.9	8.6/48.6/40.0/2.8	–	12.5/41.7/24.8/0	6.5/45.4/47.2/0.9	11.3/38.7/48.4/1.6	–
Physical activity (h/d)	2.71 ± 0.15	2.93 ± 0.16	2.66 ± 0.20	0.486	2.70 ± 0.17	2.96 ± 0.16	2.63 ± 0.16	0.309
S-25 OH D (nmol/l)	43.4 ± 2.0	43.5 ± 1.7	41.4 ± 2.6	0.802	42.8 ± 2.4	44.3 ± 1.7	41.3 ± 2.0	0.514
Ca intake (mg/d)	976 ± 60	1068 ± 65	1047 ± 97	0.593	1007 ± 82	1034 ± 59	1049 ± 80	0.938
U-Pyr (nmol/mmol Cr)	88.2[a] ± 5.3	84.6[a] ± 4.2	64.8[b] ± 5.2	0.028	89.3[a] ± 6.8	87.7[a] ± 4.4	69.1[b] ± 4.0	0.013
U-Dpyr (nmol/mmol Cr)	46.7[a] ± 2.4	47.2[a] ± 2.3	41.0[b] ± 3.5	0.038	46.8[a] ± 2.1	48.4[a] ± 2.5	41.3[b] ± 2.7	0.050
S-osteocalcin (µg/ml)	47.8 ± 1.7	53.3 ± 1.6	52.7 ± 2.9	0.063	45.2[a] ± 2.1	53.6[b] ± 1.6	51.8[ab] ± 2.0	0.010
S-PTH (pmol/l)	2.97 ± 0.09	3.19 ± 0.09	3.15 ± 0.17	0.256	2.98 ± 0.11	3.15 ± 0.09	3.11 ± 0.12	0.547
TB BMD (g/cm^2)	0.86 ± 0.01	0.87 ± 0.01	0.86 ± 0.01	0.711	0.86 ± 0.01	0.87 ± 0.01	0.87 ± 0.01	0.462
TB BMC (g)	1268 ± 31	1320 ± 29	1303 ± 40	0.463	1242 ± 43	1298 ± 25	1344 ± 38	0.173
Spine BMD (g/cm^2)	0.69 ± 0.01	0.70 ± 0.01	0.70 ± 0.02	0.712	0.68 ± 0.01	0.70 ± 0.01	0.71 ± 0.01	0.408
Spine BMC (g)	28.59 ± 0.72	29.46 ± 0.73	29.61 ± 1.38	0.672	27.92 ± 1.02	28.98 ± 0.60	30.51 ± 1.13	0.171

Values are mean ± SE
BMI, body mass index; TB, total body; BMD, bone mineral density; BMC, bone mineral content; U, urinary; Pyr, pyridinoline; Dpyr, deoxypyridinoline; S, serum
[a,b]Mean values within a row that have different superscripts are significantly different using Bonferroni's correction for multiple comparisons ($P < 0.05$)

polymorphisms (Tables 2, 3). This lack of association remained even after the BMD data were adjusted for possible confounding effects of weight, height, and Tanner score.

Unadjusted BMC of the lumbar spine or whole body was not associated with *Xba*I, *Pvu*II, or *Taq*I polymorphisms (see Tables 2, 3). Furthermore, size-adjusted BMC (at both sites) was not associated with *Pvu*II or *Taq*I polymorphisms, even after controlling for Tanner score (data not shown). There was a tendency ($P = 0.070$–0.083) for unadjusted BMC (at both sites) to be associated with the *Fok*I VDR genotype (see Table 2). However, this tendency disappeared after the BMC data were adjusted for size (i.e., bone area, height, and weight) and Tanner score (data not shown). Size-adjusted BMC of the whole body, but not lumbar spine, tended ($P = 0.084$) to be associated with the *Xba*I ERα genotype, even after adjustment for Tanner score (data not shown). Inclusion of other potential confounding factors such as physical activity, dietary calcium, and vitamin D status in the regression models did not alter the genotype–BMD/BMC findings (data not shown). There were no significant gene–gene interaction effects between polymorphisms at the ERα locus and the VDR locus on either BMD or BMC (data not shown).

Discussion

Although there have been numerous studies of the association of polymorphisms in the VDR and ERα genes on bone mass in adults, and a more limited number of such studies in prepubertal children or adolescents, the data are conflicting [2–9,10,13,16,18–20]. In the present study, these relationships were investigated in peripubertal children, a group seldom investigated despite the dramatic increases in BMD during this phase of life [22].

The genotype frequencies of VDR (defined by the *Taq*I and *Fok*I endonuclease systems) and ERα (defined by the *Pvu*II and *Xba*I endonuclease systems) in the group of Danish girls in the present study were similar to those previously reported in Danish populations [36,37] and were in Hardy–Weinberg equilibrium.

There were no significant associations evident between BMC and BMD (at whole body and lumbar spine; unadjusted but also adjusted for confounding factors, as suggested by Deng et al. [38]) and VDR (*Taq*I or *Fok*I) or with ERα (*Pvu*II or *Xba*I) genotypes. There have been only three studies published that investigated the effect of ERα genotype on BMD in children, but all three failed to find an association [13,16,20]. The present study, which had a sample size of 224, had sufficient power to detect 7.8%–9.2% differences in spinal BMD, respectively, between the extreme homozygotes for *Pvu*II and *Xba*I ERα genotypes. It should be noted that Willing et al. [39] reported that premenopausal women ($n = 253$) with the *PP* ERα genotype had significantly higher BMD of the spine (6.4%; $P < 0.005$) compared to women with the *pp* ERα genotype. Furthermore, the same authors reported that premenopausal women ($n = 248$) with the *XX* ERα genotype had significantly higher BMD of the spine (6.5%; $P < 0.005$) compared to women with the *xx* ERα genotype [39]. These findings in premenopausal women might suggest the sample size in the studies of children, including the present (which to our knowledge is the largest study of adolescent children in terms of subject number), may have been limited to detect measurable differences in BMD/BMC.

There were no significant associations between the *Taq*I VDR genotype and BMD or BMC in the Danish girls in the present study. A number of studies have investigated the effect of the *Taq*I VDR genotype on BMD in children. Tao et al. [13] reported that adjusted lumbar spine BMD was significantly ($P < 0.05$) lower (by 8.3%) in prepubertal girls (aged 7.0–7.9 years) with the *TT* VDR genotype ($n = 24$) compared to those with the *tt* ($n = 11$) genotype. Lorentzon et al. [15] found that lumbar spine BMD in postpubertal girls (mean age, 16.9 years) with the *TT* genotype ($n = 29$) was significantly lower (5.1%; $P < 0.05$) compared to those with the *Tt* genotype ($n = 44$). The sample size in the present study was sufficient to detect 8.9% and 5.5% differences in lumbar spine BMD between *TT* and *tt* and *TT* and *Tt* VDR genotype groups, respectively.

There were no significant associations between *Fok*I VDR genotype and BMD or BMC in the Danish girls in the present study. A limited number of studies have investigated the effect of VDR genotype on BMD in pre- and postpubertal children. Some studies have reported a lack of association between *Fok*I VDR genotype and BMD in children ($n = 114$; prepubertal) [13]; ($n = 143$; prepubertal) [19]; ($n = 91$; postpubertal) [15]. On the other hand, Ames et al. [14] found that differences of the order of 8.2% and 4.8% in total body BMD exist between *FF* ($n = 30$) and *ff* ($n = 10$), and *FF* and *Ff* ($n = 32$) VDR genotype groups, respectively, in a mixed group of Caucasian, Mexican-American, and African-American children, as well as in Caucasian children only, aged 7–12 years. The sample size in the present study was sufficient to detect 6.0% and 3.3% differences in total-body BMD between *FF* and *ff* and *FF* and *Ff* VDR genotype groups, respectively. However, although the children in the study by Ames et al. [14] were for the most part prepubertal (79% with Tanner score 1; remainder with Tanner scores of 2 and 3), the children in the present study were mainly of early- to midpubertal status (92% Tanner scores 2 and 3, with only 8% Tanner score 1). Ferrari et al. [19] suggested that the significant association found between *Bsm*I VDR genotype and lumbar spine BMD in prepubertal girls ($n = 143$) was not present in peri- and postpubertal girls (age range, 10.0–18.5 years; $n = 54$). However, the small sample size of peri- and postpubertal girls would have limited the potential to detect significant differences in BMD among VDR genotypes in the study by Ferrari et al. [19]. Furthermore, several, but not all, studies report a significant association between VDR genotype and BMD in premenopausal adult women [6], suggesting that genotypic effects on PBM may persist into young to middle adulthood.

It has been suggested that environmental factors such as dietary calcium [19] and physical activity [21] might affect the relationship between BMD/BMC and VDR or ERα gene polymorphisms. However, in the present study, di-

etary calcium intake and high physical activity score did not influence the lack of relationship between genotype and bone mass. Ferrari et al. [19] suggested that a relatively high calcium intake (as was evident in the girls in the present study) and, thus, BMD accrual may be optimized in subjects with certain VDR genotypes, which could mask differences between genotypes.

The rate of bone turnover is also an important determinant of bone mass [40]. The findings of studies in adults that investigated the impact of VDR and ERα genotype on bone turnover, as assessed by biochemical markers, have been conflicting [6,9]. In the present study, VDR genotype had no influence on markers of bone turnover. On the other hand, ERα genotype (*Pvu*II and *Xba*I) influenced markers of bone resorption, but not those of bone formation, in Danish girls in the present study. Unfortunately, there are no available data with which to compare our findings. The reduced rates of bone resorption in girls with *XX* and *PP* *Xba*I and *Pvu*II ERα genotypes, respectively, in the present study might support the findings of low BMD associated with the *pp* ERα genotype in premenopausal women [39] and the findings of a recent meta-analysis that suggested that the *XX* ERα genotype was found to confer a higher BMD, in addition to a protective effect that decreased the risk of fracture [41]. It is possible the apparent effects of ERα genotype on bone resorption in the girls in the present study were translated into measurable effects in BMD or BMC, but, as mentioned earlier, the sample size may have been limited to detect these small differences.

There were no apparent interactions between the VDR and ERα gene polymorphisms on bone mass or turnover in the present study. Willing et al. [42] reported a significant interaction between VDR (*Taq*I) and ERα (*Pvu*II and *Xba*I) genes in relation to bone mass in children, but these were aged 4.5–6.5 years.

In conclusion, the findings of the present study, which to our knowledge is the largest study in adolescent children in terms of subject number, suggest that VDR genotype is not related to BMD, BMC, or bone turnover in healthy young (11- to 12-year-old) Danish girls, even after controlling for certain dietary and lifestyle factors. Although the rate of bone resorption was influenced by ERα genotype, ERα genotype did not appear to influence BMD or BMC in the girls. This finding, however, may have been as a consequence of the study being underpowered to detect small genotype-dependent differences in bone mass. As puberty is a time during which estrogen levels, the ligand for the ERα, change considerably, this may influence the interaction between ERα genotype and bone mass and turnover. Further research on the impact of genotype on bone health indices during the pubertal growth spurt is warranted.

Acknowledgment This work was supported in part by funding made available under The European Commission Fifth Framework Programme (Contract no. QLK1-CT-2000-00623).

References

1. Gueguen R, Jouanny P, Guillemin F, Knutz C, Pourel J, Siset G (1995) Segregation analysis and variance components analysis on bone mineral density in healthy families. J Bone Miner Res 10: 2017–2022
2. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA (1994) Prediction of bone density from vitamin D receptor alleles. Nature (Lond) 367:284–287
3. Eisman JA (1995) Vitamin D receptor gene alleles and osteoporosis: an affirmative view. J Bone Miner Res 10:1289–1293
4. Eisman JA (2001) Pharmacogenetics of the vitamin D receptor and osteoporosis. Drug Metab Dispos 29:505–512
5. Peacock M (1995) Vitamin D receptor alleles and osteoporosis: a contrasting view. J Bone Miner Res 10:1294–1297
6. Wood RJ, Fleet JC (1998) The genetics of osteoporosis: vitamin D receptor polymorphisms. Annu Rev Nutr 18:233–258
7. Gennari L, Becherini L, Falchetti A, Masi L, Massart F, Brandi ML (2002) Genetics of osteoporosis: role of steroid hormone receptor gene polymorphisms. J Steroid Biochem Mol Biol 81:1–24
8. Uitterlinden AG, Fang Y, Bergink AP, van Meurs JB, van Leeuwen HP, Pols HA (2002) The role of vitamin D receptor gene polymorphisms in bone biology. Mol Cell Endocrinol 197:15–21
9. Cusack S, Cashman KD (2003) Impact of genetic variation on metabolic response of bone to diet. Proc Nutr Soc 62:1–12
10. Gunnes M, Berg JP, Halse J, Lehmann EH (1997) Lack of relationship between vitamin D receptor genotype and forearm bone gain in healthy children, adolescents and young adults. J Clin Endocrinol Metab 82:851–855
11. Slemenda CW, Christian JC, Reed T, Reister TK, Williams CJ, Johnston CC Jr (1992) Long-term bone loss in men: effects of genetic and environmental factors. Ann Intern Med 117:268–291
12. Sainz J, Van Tornout J, Loro L, Sayre J, Roe TF, Gilsanz V (1997) Vitamin D receptor gene polymorphisms and bone density in prepubertal American girls of Mexican descent. N Engl J Med 337:77–82
13. Tao C, Yu T, Garnett S, Briody J, Knight J, Woodhead H, Cowell CT (1998) Vitamin D receptor alleles predict growth and bone density in girls. Arch Dis Child 79:488–494
14. Ames SK, Ellis KJ, Gunn SK, Copeland KC, Abrams SA (1999) Vitamin D receptor gene Fok I polymorphism predicts calcium absorption and bone mineral density in children. J Bone Miner Res 14:740–746
15. Lorentzon M, Lorentzon R, Nordstrom P (2001) Vitamin D receptor gene polymorphism is related to bone density, circulating osteocalcin and parathyroid hormone in healthy adolescent girls. J Bone Miner Metab 19:302–307
16. Katsumata K, Nishizawa K, Unno A, Fujita Y, Tokita A (2002) Association of gene polymorphisms and bone density in Japanese girls. J Bone Miner Metab 20:164–169
17. Strandberg S, Nordstrom P, Lorentzon R, Lorentzon M (2003) Vitamin D receptor start codon polymorphism (Fok I) is related to bone mineral density in healthy adolescent boys. J Bone Miner Metab 21:109–113
18. Ferrari S, Rizzoli R, Manen D, Slosman D, Bonjour JP (1998a) Vitamin D receptor gene start codon polymorphisms (Fok I) and bone mineral density: interaction with age, dietary calcium and 3′-end region polymorphisms. J Bone Miner Res 13:925–930
19. Ferrari SL, Rizzoli R, Slosman DO, Bonjour JP (1998b) Do dietary calcium and age explain the controversy surrounding the relationship between bone mineral density and vitamin D receptor gene polymorphisms? J Bone Miner Res 13:363–370
20. Suuriniemi M, Mahonen A, Kovanen V, Alen M, Lyytikainen A, Wang Q, Kroger H, Cheng S (2004) Association between exercise and pubertal BMD is modulated by estrogen receptor alpha genotype. J Bone Miner Res 19:1758–1765
21. Laaksonen MM, Karkkainen MU, Outila TA, Rita HJ, Lamberg-Allardt CJ (2004) Vitamin D receptor gene start codon polymorphism (Fok I) is associated with forearm bone mineral density and calcaneal ultrasound in Finnish adolescent boys but not in girls. J Bone Miner Metab 22:479–485
22. Theintz G, Buchs B, Rizzoli R, Slosman D, Clanien H, Sizonenko PC, Bonjour JPH (1992) Longitudinal monitoring of bone mass

accumulation in healthy adolescents: evidence for a marked reduction after 16 years of age at the levels of lumbar spine and femoral neck in female subjects. J Clin Endocrinol Metab 75:1060–1065
23. Christensen T (2001) Outline of GIES: general intake estimation system. In: Fourth International Food Data Conference: New Trends in the Management and Uses of Food Databases, 24–26 August, 2001, Bratislava, Slovakia (poster)
24. Andersen R, Molgaard C, Skovgaard LT, Brot C, Cashman KD, Chabros E, Charzewska J, Flynn A, Jakobsen J, Karkkainen M, Kiely M, Lamberg-Allardt C, Moreiras O, Natri AM, O'Brien M, Rogalska-Niedzwiedz M, Ovesen L (2005) Teenage girls and elderly women living in northern Europe have low winter vitamin D status. Eur J Clin Nutr 59:533–541
25. Marshall WA, Tanner JM (1969) Variations in pattern of pubertal changes in girls. Arch Dis Child 44:291–303
26. Boucher GP, Lands LC, Hay JA, Hornby L (1997) Activity levels and the relationship to lung function and nutritional status in children with cystic fibrosis. Am J Phys Med Rehabil 76:311–315
27. Molgaard C, Thomsen BL, Michaelsen KF (2001) The influence of calcium intake and physical activity on bone mineral content and bone size in healthy children and adolescents. Osteoporos Int 12:887–894
28. Riggs BL, Nguyen TV, Melton LJ III, Morrison NA, O'Fallon WM, Kelly PJ, Egan KS, Sambrook PN, Muhs JM, Eisman JA (1995) The contribution of vitamin D receptor gene alleles to the determination of bone mineral density in normal and osteoporotic women. J Bone Miner Res 10:991–996
29. Gross C, Eccleshall TR, Malloy PJ, Villa ML, Marcus R, Feldman D (1996) The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in post-menopausal Mexican-American women. J Bone Miner Res 11:1850–1855
30. Kobayashi S, Inoue S, Hosoi T, Ouchi Y, Shiraki M, Orimo H (1996) Association of bone mineral density with polymorphism of the estrogen receptor gene. J Bone Miner Res 11:306–311
31. Pratt DA, Daniloff Y, Duncan A, Robins SP (1992) Automated analysis of the pyridinium crosslinks of collagen in tissue and urine using solid-phase extraction and reversed-phase high-performance liquid chromatography. Anal Biochem 207:168–175
32. Calabresi E, Lasagni L, Franceschelli F, Bartolini L, Serio M, Brandi ML (1994) Use of an internal standard to measure pyridinoline and deoxypyridinoline in urine. Clin Chem 40:336–337
33. Robins SP, Stead DA, Duncan A (1994) Precautions in using an internal standard to measure pyridinoline and deoxypyridinoline in urine. Clin Chem 40:2322–2323
34. Colwell A, Russell RG, Eastell R (1993) Factors affecting the assay of urinary 3-hydroxy pyridinium crosslinks of collagen as markers of bone resorption. Eur J Clin Invest 23:341–349
35. Prentice A, Parsons TJ, Cole TJ (1994) Uncritical use of bone mineral density in absorptiometry may lead to size-related artifacts in the identification of bone mineral determinants. Am J Clin Nutr 60:837–842
36. Bagger YZ, Jorgensen HL, Heegaard AM, Bayer L, Hansen L, Hassager C (2000) No major effect of estrogen receptor gene polymorphisms on bone mineral density or bone loss in postmenopausal Danish women. Bone (NY) 26:111–116
37. Tofteng CL, Jensen JEB, Abrahamsen B, Odum L, Brot C (2002) Two polymorphisms in the vitamin D receptor gene—association with bone mass and 5-year change in bone mass with or without hormone-replacement therapy in postmenopausal women: the Danish osteoporosis prevention study. J Bone Miner Res 17:1535–1544
38. Deng HW, Li J, Li JL, Johnson M, Gong G, Recker RR (1999) Association of VDR and estrogen receptor genotypes with bone mass in postmenopausal Caucasian women: different conclusions with different analyses and the implications. Osteoporos Int 9:499–507
39. Willing M, Sowers M, Aron D, Clark MK, Burns T, Bunten C, Crutchfield M, D'Agostino D, Jannausch M (1998) Bone mineral density and its change in white women: estrogen and vitamin D receptor genotypes and their interaction. J Bone Miner Res 13: 695–705
40. Cashman K, Flynn A (1999) Optimal nutrition: calcium, magnesium and phosphorus. Proc Nutr Soc 58:477–487
41. Ioannidis JP, Stavrou I, Trikalinos TA, Zois C, Brandi ML, Gennari L, Albagha O, Ralston SH, Tsatsoulis A, ER-Alpha Genetics Meta-Analysis (2002) Association of polymorphisms of the estrogen receptor alpha gene with bone mineral density and fracture risk in women: a meta-analysis. J Bone Miner Res 17:2048–2060
42. Willing MC, Torner JC, Burns TL, Janz KF, Marshall T, Gilmore J, Deschenes SP, Warren JJ, Levy SM (2003) Gene polymorphisms, bone mineral density and bone mineral content in young children: the Iowa Bone Development Study. Osteoporos Int 14:650–658