J Bone Miner Metab (2004) 22:64–70
DOI 10.1007/s00774-003-0451-5

Journal of
Bone and
Mineral
Metabolism

Low calcium intake is associated with decreased adrenal androgens and reduced bone age in premenarcheal girls in the last pubertal stages

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Abstract In 50 premenarcheal girls selected from the lowest and highest end of the calcium-intake distribution of a large population sample, we evaluated bone mineral density (BMD), together with the following hormonal-metabolic parameters: androstenedione (ASD), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), testosterone (T), estradiol (E2), the apparent free fractions of T (AFTC) and E2 (AFEC), osteocalcin (OC), parathyroid hormone (PTH), and 25-hydroxyvitamin D. Dietary calcium was assessed by 3-day food records, and BMD was measured at ultradistal (ud) and proximal (pr) radial sites, using dual-energy X-ray absorptiometry. Calcium intake, which was below the recommended levels set for the Italian population and below the recommended daily allowance (RDA) in both subgroups of girls, did not show any apparent relationship with ud- and pr- BMD. However, despite the similar chronological age of the two premenarcheal groups, in the low-calcium consumers, we found lower bone age, delayed pubertal development, and lower circulating adrenal androgens. Of interest, in girls who had a low calcium intake, PTH levels were significantly higher. In all premenarcheals, we observed that DHEA, T, and AFTC were positively correlated with bone age and with bone density at both radial sites. Even though bone density at the two radial sites did not show any apparent relationship to calcium consumption, the increased mean PTH in the girls with low calcium intake seems to underscore the hormonal attempt in maintaining calcium homeostasis. In conclusion, low calcium intake and reduced levels of adrenal androgens, leading to decreased bone age and delayed pubertal development, indicate a link between calcium intake, the hormonal milieu, and skeletal maturation.

Key words calcium intake · adrenal and gonadal androgens · bone mineral density · bone age

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Received: July 25, 2002 / Accepted: April 21, 2003

Introduction

During adolescence, calcium intake is generally known to affect the genetically determined attainment of peak bone mass [1,2]. The acquisition of bone mass in girls parallels body growth, showing the greatest increase throughout pubertal development, when the sharp rise of estrogens with the onset of menarche strongly contributes to skeletal maturation, acting in siynergy with other hormones and growth factors [3–7].

The pivotal role of estrogens in increasing and maintaining bone mass is now generally accepted [8], although some studies report a lack of correlation of estrogen with the risk of bone fracture in elderly women [9]. However, the determination of total estrogen circulating levels without measuring the estrogen free fraction (bioavailable) can be misleading [10]. In the presence of relatively low total serum estradiol levels, estrogen deficiency could be amplified by a concomitant enhancement of sex hormone-binding globulin levels, resulting in reduced hormonal bioavailability to the target tissues [11].

Our understanding of the role of androgens in bone tissue was recently reviewed [12]. The suggestion is that androgens are mainly involved in stimulating periosteal bone growth and enhancing cortical bone remodelling [12,13].

In the context of a European multicenter investigation of bone density and dietary calcium, anthropometric characteristics, puberty, and lifestyle factors [14], we studied the role of adrenal and gonadal steroids, together with osteocalcin and parathyroid hormone (PTH), in modulating bone turnover in 50 premenarcheal girls. In particular, androgens, a potential source of estradiol via the aromatization process, were also evaluated, in relation to skeletal accrual, during the crucial period of bone maturation.

Subjects and methods

All girls (n = 1079; age range, 11–14 years) attending secondary schools in the town of Rende (Cosenza, southern Italy) were invited to participate in the present study.

Subjects were excluded for the following reasons: non-Caucasian origin, chronic systemic disease, use of corticosteroids, vigorous sports activity (more than 7 h per week), and vegetarianism or adherence to any prescribed diet (except for an energy-restricted diet). Subsequently, 722 girls filled out a 20-item food frequency questionnaire (FFQ), adapted for Italian adolescents, in order to select our population sample from the lowest (100 adolescents) and highest (100 adolescents) end of the calcium-intake distribution. All premenarcheals (n = 50) at both the low (n = 22) and high (n = 28) end of the calcium-intake distribution were enrolled in this study.

The definitive values of calcium consumption were re-evaluated in the selected population (n = 50) using a 3-day food-record method. Therefore, subjects were invited to record everything they consumed during a consecutive Wednesday, Thursday, and Friday, the week before their visit to the Institute. Food and quantity, and recipes for composite dishes were recorded, and, if necessary, the parent responsible for meal preparation was invited to assist in checking and compiling the food diaries. Mean daily consumption of food products was converted into calcium intake (mg/day), using the National Food Composition Tables [15].

The local Medics-Ethics Committee approved our study protocol, and parents of all participants gave their written consent.

Morning serum samples were drawn from each subject (after an overnight fast) by arm venipuncture to measure serum levels of hormones. After centrifugation, the serum samples were frozen at −70°C in individual aliquots for later assay in duplicate.

Radioimmunoassays (RIA) were used to determine total testosterone (T; ICN Pharmaceuticals, Costa Mesa, CA, USA; intra- and interassay coefficients of variation [CVs], 10.5% and 8.2%, respectively), total estradiol (E2; Immunotech, Marseille, France; intra- and interassay CVs, 5.6% and 6.7%, respectively), total androstenedione (ASD; Diagnostic Systems Laboratories, Webster, TX, USA; intra- and interassay CVs, 2.8% and 7.0%, respectively), total dehydroepiandrosterone (DHEA; ICN Pharmaceuticals; intra- and interassay CVs, 7.3% and 7.0%, respectively), and total dehydroepiandrosterone sulfate (DHEAS; ICN Biomedicals; intra- and interassay CVs, 8.5% and 7.6%, respectively). Apparent free fractions of T (AFTC) and E2 (AFEC) were measured using the technique of dialysis described by Vermeulen et al. [16]. Briefly, dialysis tubes, 1 cm in diameter and 15 cm long (Viskase, Chicago, IL, USA) were washed and left overnight in distilled water. The tubes were filled with 1 ml of plasma and were sealed at each end with a double knot, bent into a U-shape, and placed in 20-ml scintillation vials with 10 ml of phosphate buffer (pH 7.4) containing about 10 000 cpm ^{3}H-T (specific activity 79 Ci/mmol, obtained from Amersham Life Science, Milan, Italy). Estimation of free steroid fractions was derived after shaking tubes in a water bath at 37°C for 24 h when the equilibrium of dialysis was determined in the closed system, as previously described [16].

Immunoradiometric assays (IRMA) were used to quantify serum osteocalcin (OC; (Diagnostic Systems Laboratories; intra- and interassay CVs, 2.9% and 4.7%, respectively), serum PTH (ICN Pharmaceuticals; intra- and interassay CVs, 3% and 5.2%, respectively), and serum sex hormone binding globulin (SHBG) levels (Diagnostic Systems Laboratories) after 100-fold sample dilution prior to assay (intra- and interassay CVs, 2.8% and 8.8%, respectively). Serum levels of 25-hydroxyvitamin D (25-OH-D) were determined, after extraction with acetonitrile, with an RIA kit (Incstar, Stillwater, MN, USA (intra- and interassay CVs, 7.6% and 8.2%, respectively).

One investigator measured height and weight and assigned pubertal stages to the girls according to Tanner [17].

Bone mineral content (BMC) and bone area were evaluated, by dual-energy X-ray absorptiometry (DXA; Osteoscan; Nederburg, Bunschoten, Netherlands), in the nondominant arm at the ultradistal (ud) and proximal (pr) radius (one-third distal point between the styloid process and the tip of the olecranon of the elbow) representing the trabecular and cortical bone components, respectively [18]. DXA, a rapid, accurate, and reproducible method of assessment of different bone components, with a very low radiation exposure (0.02–0.03 mSV per scan) was used to calculate bone mineral density (BMD; g/cm^2), by dividing the BMC value (expressed in grams) by the projected area of bone. Two technicians collected all densitometric data, calibrating the Osteoscan every day against a reference phantom. The CVs for ten measurements of the same subject (with repositioning) were 2.15% for ud-BMD and 1.75% for pr-BMD.

Left-hand and wrist X-rays were performed in girls to estabilish bone age, by the Tanner-Whitehouse (TW2) method [19].

Statistics

Data values for all variables were presented as means with standard error. Comparisons of means were made using unpaired Student's t-test. Pearson's correlation,

and linear and multiple regression analyses were performed using SIMSTAT 3.5 (Provalis Research, Montreal, Canada). Because variations of BMD could be due to differences in bone size between individuals, we included height, weight, and bone area as covariables on multiple regressions, with BMD as a dependent variable. *P* values of less than 0.05 were considered significant.

Results

Table 1 shows anthropometric characteristics, radial BMD, and calcium intake of girls selected according to the criteria indicated in the "Methods" section. It is worth noting that the dietary calcium consumption was below the recommended levels set for the Italian population, and below the recommended daily allowance (RDA) [20], even in the girls with high calcium intake (Table 1).

No relationship was observed between calcium intake and radial BMD adjusted for height, weight, and bone area, while calcium intake was related to bone age in all girls ($r = 0.34$; $P = 0.018$).

Despite the similar chronological age of the two subgroups, low-calcium consumers exhibited markedly delayed pubertal stage, well fitting with the decreased bone age (Table 1).

It is important to note that, in low-calcium consumers, a decrease in all aromatizable adrenal androgens (DHEA, DHEAS, and ASD) predicted a lower peripheral action of estrogens (Table 2). Furthermore, in the same subgroup, the enhanced SHBG reduced the amount of biologically active estradiol, resulting in a significant lowering of AFEC concentrations in these subjects (Table 2).

It is worth remarking that, in the subjects who had a low calcium intake, PTH levels appearred to be significantly enhanced, while serum levels of OC and 25-OH-D were similar in the two subgroups (Table 3).

Table 1. Anthropometric characteristics, dietary calcium, and ultradistal/proximal-bone mineral density (ud/pr-BMD; X ± SE and *P* values) in premenarcheal girls with low and high calcium intake (Ca I)

	Premenarcheals ($n = 50$)		
	Low Ca I	High Ca I	*P*
Subjects (*n*)	22	28	
Age (years)	12.01 ± 0.10	12.33 ± 0.18	NS
Bone age (years)	11.96 ± 0.30	12.89 ± 0.14	<0.01
Tanner stage (*n*)	3.50 ± 0.21	4.10 ± 0.11	<0.01
Height (cm)	150.68 ± 1.76	152.59 ± 1.18	NS
Weight (kg)	46.06 ± 2.46	45.16 ± 1.49	NS
Body mass index (kg/m^2)	20.22 ± 0.85	19.87 ± 0.52	NS
Dietary calcium (mg/day)	420 ± 12.38	839 ± 20.01	<0.001
ud-BMD (g/cm^2)	0.29 ± 0.01	0.30 ± 0.01	NS
pr-BMD (g/cm^2)	0.61 ± 0.01	0.60 ± 0.01	NS

NS, not significant

Table 2. Levels of testosterone (T), estradiol (E2), androstenedione (ASD), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), sex hormone binding globulin (SHBG), and apparent free fractions of T (AFTC) and E2 (AFEC) (X ± SE and *P* values) in premenarcheal girls with low and high calcium intake (Ca I)

	Premenarcheals		
	Low Ca I ($n = 22$)	High Ca I ($n = 28$)	*P*
T (nM)	0.92 ± 0.07	0.88 ± 0.06	NS
E2 (pM)	136.67 ± 15.94	171.72 ± 12.75	NS
ASD (nM)	3.56 ± 0.25	5.38 ± 0.44	<0.01
DHEA (nM)	21.87 ± 1.97	27.78 ± 1.74	<0.05
DHEAS (μM)	1.67 ± 0.20	2.32 ± 0.19	<0.05
SHBG (nM)	175.72 ± 15.38	119.60 ± 8.45	<0.01
AFTC (pM)	14.90 ± 2.01	14.60 ± 1.01	NS
AFEC (pM)	2.05 ± 0.08	2.41 ± 0.10	<0.01

Of interest, in all premenarcheals, DHEA, T, and its free fraction (AFTC) were positively related to bone age (Fig. 1); moreover DHEA, T, and AFTC were positively related to both radial BMD values (Figs. 2, 3, and 4).

Table 3. Serum osteocalcin (OC), parathyroid hormone (PTH), and 25-hydroxyvitamin D (25-OH-D) levels (X ± SE and *P* values) in premenarcheal girls with low and high calcium intake (Ca I)

	Premenarcheals		
	Low Ca I (n = 22)	High Ca I (n = 28)	*P*
OC (nM)	1.36 ± 0.08	1.44 ± 0.09	NS
PTH (pM)	3.48 ± 0.44	2.39 ± 0.22	<0.05
25-OH-D (nM)	50.82 ± 6.01	49.67 ± 4.47	NS

Discussion

In our investigation, the average dietary calcium intake was below the RDA [20] in premenarcheal girls in the presence of normal body mass index (BMI) and body weight. Even though bone density at both radial sites measured did not show any substantial difference between the two groups of girls, lower calcium intake was associated with lower mean bone age and delayed pubertal development. However, in this regard, we would point out how the bone density detected at the two radial sites may be not adequately representative of the systemic influence of the low calcium intake on bone maturation. Such effects, indeed, appear to be evaluated in a more reliable way by bone age determination, which was positively related to calcium intake in all girls. In low-calcium consumers, a reduction in circulating adrenal androgens suggests that, along with other nutritional factors [21 and references therein], calcium

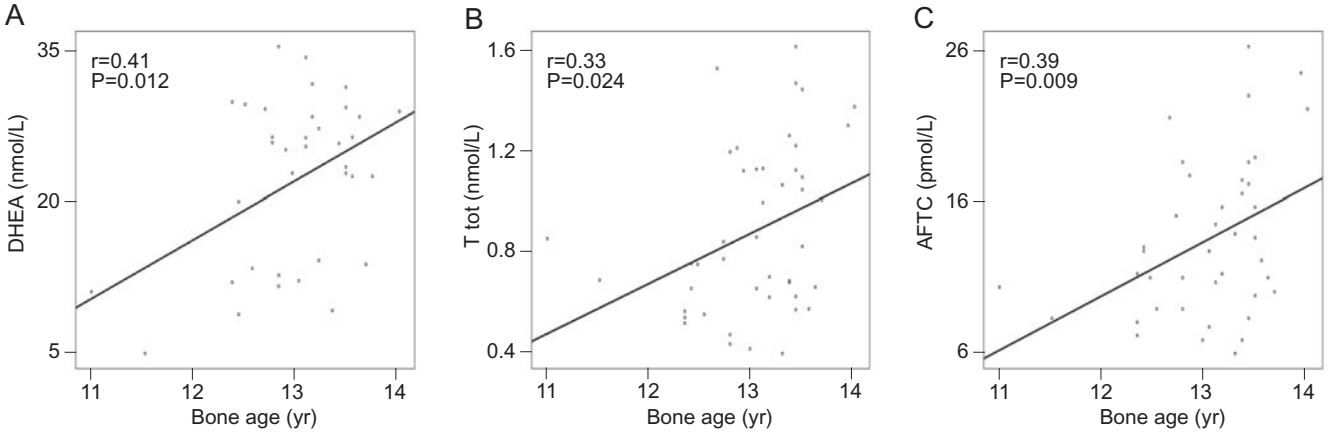

Fig. 1. Correlations between **A** dehydroepiandrosterone (*DHEA*), total and apparent free fraction of testosterone (*T tot* and *AFTC* **B** and **C**, respectively) and bone age in premenarcheal girls. *yr*, years

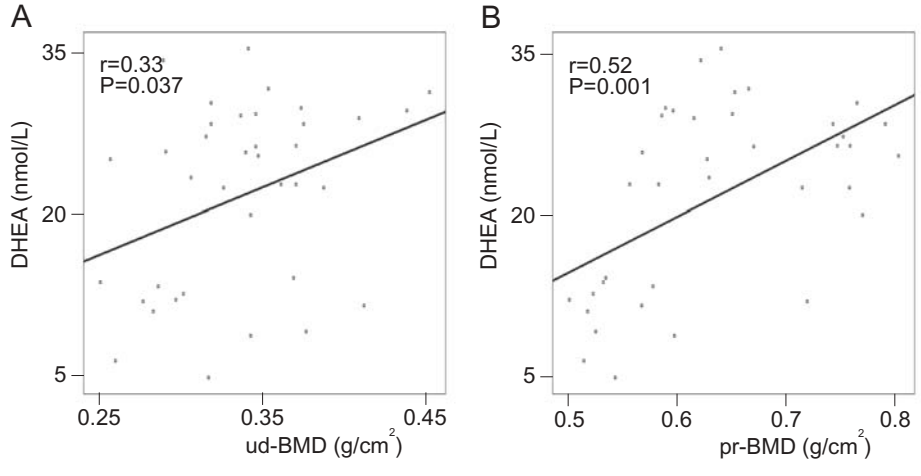

Fig. 2. Correlations between dehydroepiandrosterone (*DHEA*) and ultradistal/proximal bone mineral density (*ud/pr-BMD* **A**/**B**) in premenarcheal girls

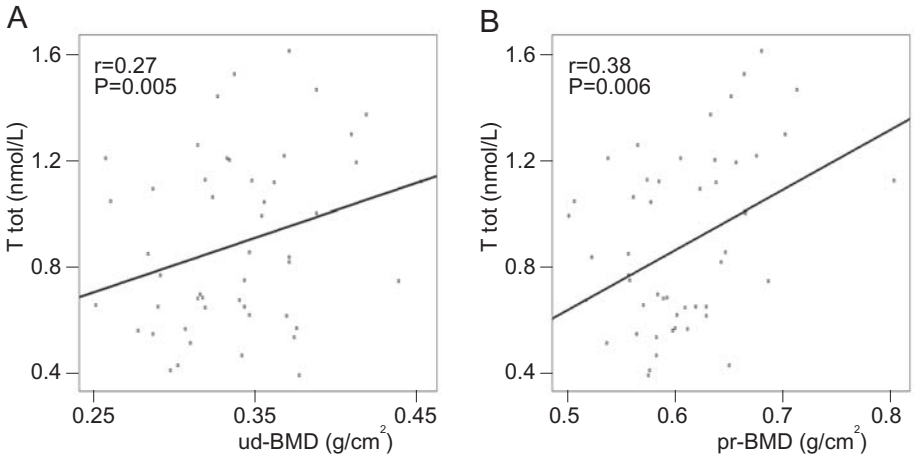

Fig. 3. Correlations between total testosterone (*T tot*) and ultradistal/proximal bone mineral density (*ud/pr-BMD* **A**/**B**) in premenarcheal girls

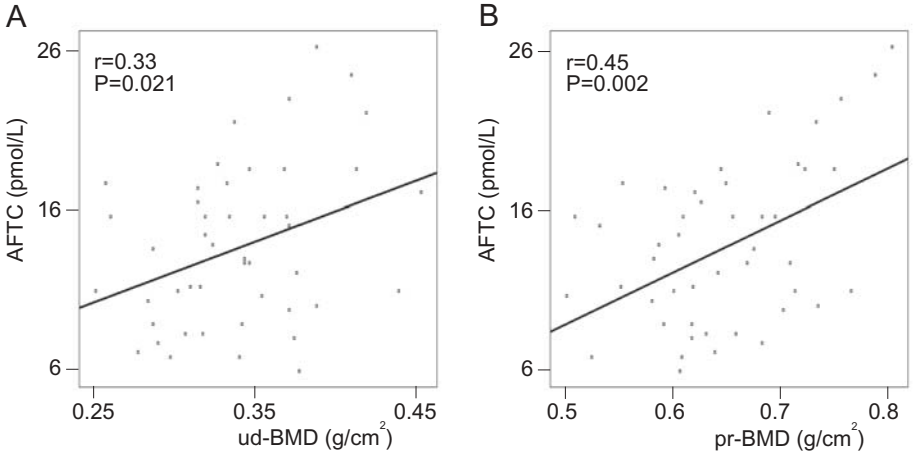

Fig. 4. Correlations between apparent free fraction of testosterone (*AFTC*) and ultradistal/proximal bone mineral density (*ud/pr-BMD* **A**/**B**) in premenarcheal girls

may influence enzymatic adrenal steroidogenesis. On the other hand, there is increasing evidence that anabolic changes are involved in the enhanced bioactivity of insulin and/or insulin-like growth factor-I (IGF-I) [22,23]. As the adrenal cortex expresses insulin and IGF-I receptors [24], and both mitogenic peptides enhance adrenal secretion in human adrenocortical cells [24–26], it becomes plausible to regard nutritional status as an important regulator of adrenarche.

In our premenarcheal girls, both adrenal and gonadal androgens were positively related to bone density. These data fit with the results of previous studies showing that AFTC and DHEA were the androgens more consistently correlated with bone density [27–29]. In the same vein, we observed in the present study that DHEA, T, and AFTC were positively related to bone age, even though the relationship between AFTC and bone age was much more significant than DHEA or T and bone age. For instance, testosterone, the major gonadal androgen, circulates largely bound to albumin and SHBG, while AFTC represents the hormone that is bioavailable to the target tissue. DHEA and ASD are the major circulating adrenal androgens in both women and men [30]. DHEA is metabolized to DHEAS, which has little androgenic activity, and thus, a direct stimulatory effect of DHEAS on bone, although possible, is unlikely. In this regard, we would point out that DHEAS may serve, rather, as a surrogate marker for the effect of DHEA, which can directly stimulate both osteoblast proliferation and differentiation (similar to testosterone and dihydrotestosterone [DHT]) [31] or for the effect of ASD [6].The detection of functional androgen receptors (ARs) in various bone cells [32–35] has implicated bone as a target tissue for androgen action and has fuelled an increase in further investigations of the direct and indirect effects of androgens on bone cells in vitro, as well as on bone metabolism in vivo. In addition, the presence of the enzymes aromatase, 17-β-hydroxysteroid-dehydrogenase, 3-β-hydroxysteroid-dehydrogenase, and 5-α-reductase, detected in osteoblastic cells [36–41], indicates the ability of the bone microenvironment to locally form biologically

potent estrogens and androgens from weak circulating sex steroid hormones such as DHEA, DHEAS, and ASD. Thus, the biological effects of these compounds may result from activation of the ER or AR, as demonstrated in several in vitro and in vivo studies [12 and references therein]. For instance, ERs have been generally demonstrated in cultured bone cells of the osteoblast lineage [42,43]. Besides, there is a large mass of data demonstrating that estrogens decrease bone resorption, promoting the apoptosis of osteoclasts, and that they also increase skeletal mass, reducing apoptosis in osteoblasts and osteocytes [44,45]. Indeed, during skeletal growth and maturation, estrogens, in combination with other hormones, induce major beneficial effects on skeletal development consistent with a combination of increases in bone height, bone diameter, cortical bone width, and cancellous bone mass [6,8]. Moreover, estrogens have indirect actions that affect bone metabolism, which include promoting the intestinal absorption of calcium and decreasing the renal excretion of calcium. In the present study, the increase of SHBG observed in low-calcium consumers produced a significant lowering of AFEC, which may negatively influence bone maturation, as previously evidenced [8,46].

Moreover, in low-calcium consumers, we found an enhancement of PTH, a major osteotropic factor which plays a critical role in calcium homeostasis and in regulating the rate of bone turnover.

In conclusion, our observations support the notion that low dietary calcium consumption in premenarcheal girls is associated with a lower adrenal androgen secretion. Both a low calcium intake and reduced levels of adrenal androgens appear to cooperate in decreasing bone age and in delaying pubertal development in girls with low calcium intake. Testosterone and DHEA may also act through an intracrine conversion into estrogens, to contribute to bone mass acquisition during this important period of skeletal maturation.

Acknowledgments. We thank the operators L. Abbink and G. Huijbers for collecting densitometric data and we thank Drs. L. Bosoni and L. Pecoriello for X-ray assessment. We are grateful to Professor Melvin M. Grumbach for critical prereview of the manuscript.

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