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Effect of gender, puberty, and vitamin D status on biochemical markers of bone remodededeling

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Abstract

Peak bone mass, a determinant of osteoporosis at older ages, is affected by genetic, nutritional, lifestyle, and hormonal factors. Adolescence is a critical time for peak bone mass accrual, and boys achieve a higher peak bone mass than girls. We have reported vitamin D insufficiency in adolescents in our population, but its impact on bone remodeling is unclear. We systematically evaluated the impact of puberty, gender, and vitamin D status on biochemical markers of bone remodeling. Serum osteocalcin (OC), bone alkaline phosphatase (BAP), C-terminal telopeptide of type I collagen crosslinks (S-CTX), and 25 OH vitamin D were measured in 172 healthy students from private schools in the fall of 1999: There were 92 girls and 80 boys, age 10–17 years. In girls, all markers of bone turnover changed significantly with pubertal stage, were maximal at midpuberty, and decreased toward adult levels by Tanner stage V. Conversely in boys, these markers increased during early pubertal stages but had not normalized by Tanner stage V. Levels of all biochemical markers were significantly higher in boys compared to girls even after adjustment for age, body weight, and Tanner stage, $P < 0.0001$. In the subgroup of girls, those with vitamin D insufficiency, serum levels of BAP and S-CTX were highest. However, in multiple regression analyses, gender was the only consistent correlate of all three markers of bone remodeling. In conclusion, after adjusting for age, weight, and Tanner stages, changes in bone remodeling markers were most powerfully affected by gender. The latter may have important implications on gender differences in peak bone mass.

Keywords: Bone alkaline phosphatase; Serum-CTX; Osteocalcin; Puberty; Vitamin D

Introduction

Biochemical markers of bone remodeling provide a means of evaluating skeletal dynamics that complements static measurement of bone mineral density [1]. Although these biologic indices are widely accepted as research tools in population-based studies, their clinical utility in the management of the individual patient remains controversial.

Serum levels of bone-specific alkaline phosphatase and osteocalcin are established bone formation markers in children [2], whereas the main immunoassays for measurement of bone resorption, N-telopeptide to helix in urine (NTX), C-telopeptide-1 to helix in serum (ICTP), and C-telopeptide-2 in urine and serum C-terminal telopeptide of type I collagen crosslinks (S-CTX), have been mostly evaluated in adults and adolescent girls [3].

Peak bone mass is a major determinant of bone mass in later life and therefore of the risk of developing osteoporosis at that time. Men reach a peak bone mass that is 10–15% higher than peak bone mass in women. This difference may be explained by gender differences in body size, muscle mass, as well as possibly in bone remodeling during a critical period for bone mass increment, that is, puberty. Indeed more than one-half of peak bone mass is accrued during this critical period [4]. However, the impact of gen-

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der on bone turnover with concomitant measurement of bone formation and bone resorption in adolescents has not been systematically investigated. During puberty, the growth spurt is accompanied by a large increase in bone remodeling [5,6]. Known determinants of bone remodeling include genetic factors, gender, puberty, and environmental factors such as nutrition and exercise [7]. Most studies evaluating biochemical markers of bone remodeling are, however, from the United State and Europe [2–7].

Similar to findings reported from Europe, we recently reported that a large proportion of adolescents in our country have low vitamin D levels [8,9]. We have demonstrated these levels to be lower in girls compared to boys [8], therefore possibly contributing to their lower peak bone mass. Indeed one recent study has shown that serum 25-hydroxyvitamin D concentrations $\leq$ 40 nmol/L (16 ng/mL) tended to be associated with low forearm bone mineral density (BMD) [10]. Although there is increased evidence for an increase in bone resorption markers in adults with vitamin D insufficiency, this issue has not been previously investigated in children/adolescents [11].

The purpose of this study therefore is to measure biochemical markers of bone turnover in healthy adolescents, and to investigate in our population (1) the effect of puberty on biochemical markers of bone turnover including the least studied in that age group, namely S-CTX; (2) gender differences in bone turnover markers during puberty, which may ultimately contribute to gender differences in bone mass; and (3) to explore the effect of vitamin D insufficiency on biochemical markers of bone turnover at a critical time of bone mass accretion.

Methods

Subjects and protocol

We evaluated 172 healthy adolescents, from age 10 to 17 years, enrolled in a study investigating the prevalence of vitamin D insufficiency in healthy school children [8]. Only students who participated in the fall 1999 study had biochemical markers of bone remodeling measured and thus provided the data for the current study. Students were recruited from three private schools in Beirut, Lebanon, during the fall of the year 1999 (November to December), and were all reported to be healthy and engaged in normal activities for their age. The average duration of daylight hours during that period is 8 h, and temperature ranges between 10°C (50°F) and 17°C (62.6°F) (Observatory Laboratory, American University of Beirut). Students were excluded if they had a chronic illness, or if they were taking any medications known to affect skeletal metabolism. They all answered a detailed 7-day food frequency questionnaire concerning intake of calcium and vitamin D [8]. Exposure to the sun was estimated by the weekly number of minutes spent in the sun as reported by each student, which was divided by 7 to estimate daily sun exposure.

Height and weight percentiles were derived by using growth curves published by the U.S. National Center for Health Statistics [12] because national standards are not available. Sexual development determinations were complemented by a pediatrician (M.N.) and an endocrinologist (M.C.). The grading system of Tanner was used and subjects were classified as Tanner stage I, II, III, IV, or V. The Tanner system included assessment of the pattern of development of pubic hair in both girls and boys. This study was approved by the Institutional Review Board at the American University of Beirut, and written informed consent was obtained from all study participants and/or their parents/legal guardians.

Blood was drawn for serum 25-hydroxyvitamin D (25-OHD), osteocalcin (OC), bone alkaline phosphatase (BAP), and C-telopeptides crosslinks (CTX) in the nonfasting state, before noon. Phlebotomy took place between 8:30 and 12:00 noon. Usually students would have had eaten breakfast; however, specific information on the proportion of students fasting was not available. All blood samples were processed within 2 h of venipuncture, stored at −20°C until assayed, which was performed in duplicates for each parameter.

Hormonal assays

Serum 25-OHD was measured by a competitive protein-binding assay using the Diasorin Incstar Kit (Diasorin, Saluggia, Italy). For 25-OHD, the manufacturer's normal range is 9 to 47 ng/mL, the lower limit is 5 ng/mL, the intraassay coefficient of variation is $<$ 11%, and the interassay coefficient of variation $<$ 13% at a serum concentration of 47 ng/mL. Vitamin D insufficiency was defined as a 25-OHD $<$ 20 ng/mL, as determined from an examination of the 25-OHD/PTH (parathyroid hormone) curves in children/adolescents [2,9].

Serum OC was measured by immunoradiometric sandwich assay RIA (ELSA-osteo kit, CIS Bio International, Gif-Sur-Yvette, Cedex, France). The intra- and interassay coefficients of variation were 3.8% and 5.2% at 22 ng/mL respectively.

Serum BAP was measured with an enzyme immunoassay (Alkphase-B, Metra Biosystems, Mountain View, CA), and the respective intra- and interassay coefficients of variation were 5.2% and 5% at 100 U/L, respectively.

S-CTX was measured by an enzyme-linked immunosorbent assay (Crosslaps Immuno-Biological Laboratories, Hamburg, Germany), with intra- and interassay coefficients of variation of 5.1% and 6.5%, respectively, at 3.5 pmol/mL.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD) unless mentioned otherwise. Comparison of continu-

Table 1
Characteristics of the study group[a]

Characteristics	Girls	Boys	*P* value
N	92	80	
Age (years)	13 (2)	13 (2)	NS
Weight (kg)	48 (12)	50 (14)	NS
Height (cm)	154 (9)	158 (13)	0.01
Weight percentile	52 (27)	54 (30)	NS
Height percentile	47 (27)	55 (25)	NS
BMI (kg/m^2)	20 (4)	20 (4)	NS
Ca intake (mg/day)	642 (364)	792 (394)	0.014
Vitamin D intake (IU/day)	153 (164)	146 (155)	NS
Sun exposure (min/day)	74 (64)	99 (52)	0.008
25-OH D (ng/mL)	19 (7)	24 (6)	<0.001

[a] Numbers are expressed as mean (± SD). BMI, body mess index; Ca, calcium; 25-OH D, 25-hydroxy vitamin D; NS, not significant.

ous variables between various subgroups of participants was performed by using a two-tailed *t* test. One-way analysis of variance (ANOVA) was used to evaluate the effect of pubertal stages on markers within each gender. ANOVA was also used to evaluate the impact of predictors such as gender or vitamin D on markers of bone remodeling, and adjusting for covariates including weight, age, and Tanner stages. The relationships between age, body mass index (BMI), calcium intake, 25-OHD, and bone turnover markers were evaluated by calculating Pearson correlation coefficients.

Further evaluation of all significant correlates of markers of bone turnover on bivariate analysis was performed by using a stepwise multiple regression analysis. The normality of distribution of biochemical markers of bone remodeling was assessed by using the P-P plot and the Kolmogorov-Smirnov test (SPSS Software, Version 10; SPSS, Chicago, IL), and BAP and S-CTX were transformed. OC, 1/BALP, and 1/S-CTX were therefore included as dependent variables in the ANOVA and regression analyses. The respective/additional proportion of variance in markers of bone turnover explained by the significant correlates was therefore derived from the above model. Adjusted R^2 values were presented and a significance level of 0.05 accepted. The analyses were performed by using SPSS Software, Version 10 (SPSS).

Results

Clinical characteristics

We studied a total of 172 students (80 boys and 92 girls). There were no differences in age, height percentile, weight percentile, and BMI between the two genders. Boys were taller, reported longer sun exposure, had higher mean calcium intake, and higher serum 25-OHD levels compared to girls (Table 1).

Markers of bone formation in adolescents

For girls, bone formation markers assessed by measurements of serum level of OC and BAP varied significantly with pubertal stages, and were highest at Tanner stages II and III, $P < 0.001$. They decreased thereafter into the adult range for girls for OC (Fig. 1A), but were still slightly higher than the upper limit for normal for BAP (Fig. 2A). For boys, serum OC and BAP also varied significantly with pubertal stages and were highest in Tanner stages II and III, similar to girls, $P < 0.05$. However, they remained well above the normal range for adults even by Tanner stage V (Figs. 1 and 2B).

Markers of bone resorption in adolescents

S-CTX levels varied significantly with pubertal stage in girls, being higher at midpuberty, and declined in late puberty into the upper limit for premenopausal women, $P = 0.026$ (Fig. 3A). Similarly, for boys, S-CTX started to increase at the early pubertal stages but remained elevated

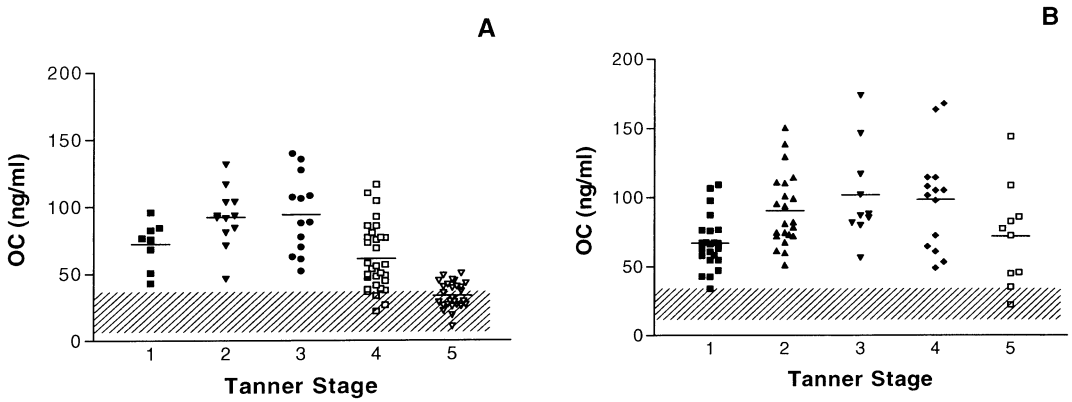

Fig. 1. Serum osteocalcin levels (OC) in 92 girls (A) and 80 boys (B). The bar represents the mean for the bone turnover marker by pubertal stage. The shaded area represents the normal range in premenopausal women (A), and normal range in adult males (B), as determined by manufacturer's kit insert. There were significant differences between mean levels of serum OC of boys and girls in Tanner stages IV ($P < 0.0001$) and V ($P < 0.0001$).

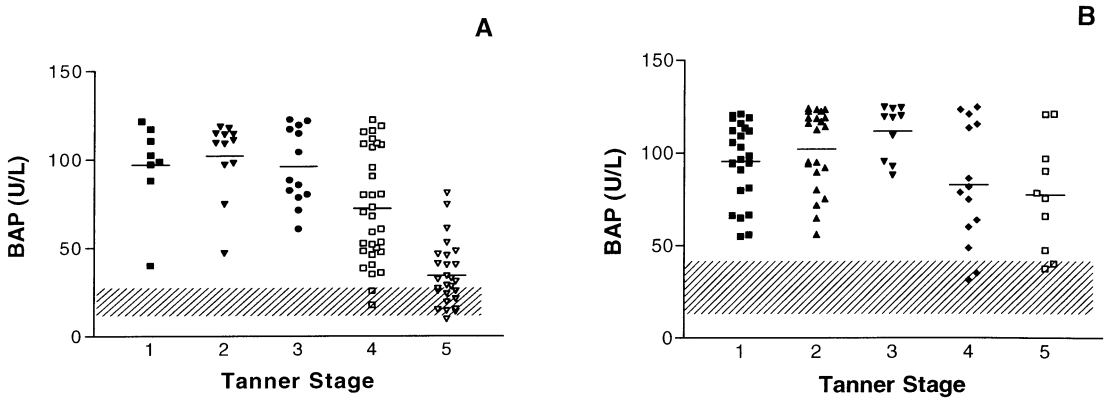

Fig. 2. Bone alkaline phosphatase (BAP) in 92 girls (A) and 80 boys (B). The bar represents the mean for the bone turnover marker by pubertal stage. The shaded area represents the normal range in premenopausal women (A), and normal range in adult males (B), as determined by manufacturer's kit insert. There were significant difference between mean levels of serum BAP of boys and girls in Tanner stage V ($P < 0.0001$).

across all the pubertal stages, with levels well above the adult range, even for Tanner stage V, $P = 0.1$ (Fig. 3B).

Gender difference in markers of bone remodeling

Mean serum levels of OC, BAP, and S-CTX were compared between genders by Tanner stage using two-tailed *t* test. There was a significant effect of gender on these markers, levels being higher in boys than in girls for S-CTX in Tanner stage II, for OC and S-CTX in Tanner stage IV, and for all markers in Tanner stage V (Figs. 1, 2, and 3). Levels of all markers remained higher in boys than in girls at Tanner V (data not shown), $P < 0.05$. To adjust for differences in age, body weight, and Tanner stage between genders, ANOVA analysis was performed with markers as dependent variable, gender as a predictor and age, and weight and Tanner stage as covariates. The analysis revealed significant effect of gender, after adjusting these covariates, on markers of bone remodeling including OC, 1/BAP, and 1/S-CTX ($P < 0.001$).

Vitamin D levels and markers of bone remodeling

Vitamin D insufficiency was observed in 40% (71 of 172) of students in the fall, with a mean level of 15 ± 4 ng/mL for the overall group. There was no significant correlation between vitamin levels and bone remodeling markers in the study group overall, or by subgroup of gender. There were also no significant differences in markers of bone formation or bone resorption in the group overall between the students with vitamin D insufficiency and students group who were vitamin D replete in the group overall. Subgroup analysis revealed that girls with vitamin D insufficiency had higher serum BAP and S-CTX than girls who were vitamin D replete ($P < 0.05$). To further examine this issue, ANOVA with markers as outcome variables, and vitamin D, Tanner stage, and gender as covariates was performed. It revealed no impact of vitamin D on bone remodeling, which was further confirmed in the multivariate analysis (see below).

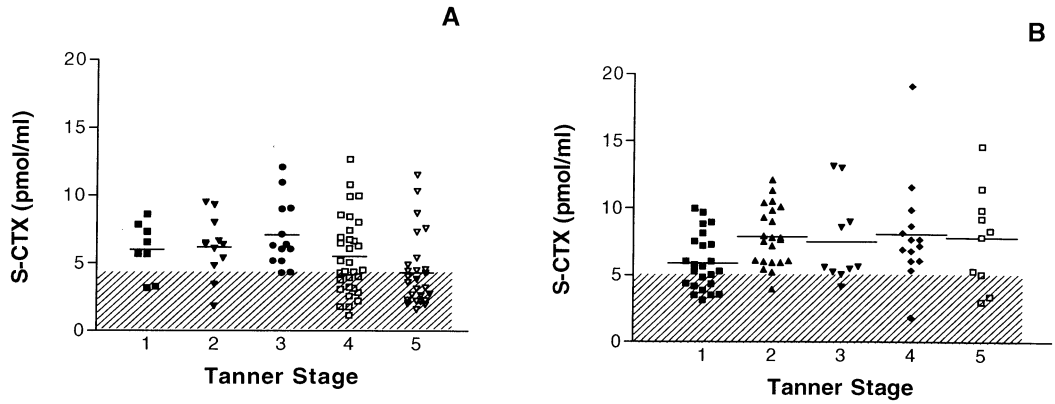

Fig. 3. Serum C-telopeptide crosslinks (S-CTX) in 92 girls (A) and 80 boys (B). The bar represents the mean for the bone turnover marker by pubertal stage. The shaded area represents the normal range in premenopausal women (A), and normal range in adult males (B), as determined by manufacturer's kit insert. There were significant differences between mean levels of serum CTX of boys and girls in Tanner stages II ($P = 0.04$), IV ($P = 0.016$), and V ($P = 0.003$).

Table 2
Beta coefficient and cumulative R^{2*} derived from the stepwise regression model with bone markers as the outcome variables and Tanner stage, gender, age, and BMI as the significant correlates[a]

Correlates	Beta coefficient	R^{2b}	P value
OC			
Gender	21.2	0.1	<0.001
Age	−4.2	0.18	0.002
BMI	−1.6	0.2	0.013
Constant	150		
1/BAP			
Tanner	0.002	0.2	0.054
Gender	−0.007	0.27	0.001
Age	0.002	0.3	0.013
Constant	−0.01		
1/S-CTX			
Gender	−0.07	0.09	<0.001
Constant	0.24		

[a] BMI, body mass index; OC, osteocalcin; BAP, bone alkaline phosphatase; S-CTX, serum C-terminal telopeptide of type I collagen crosslinks.
[b] Cumulative R^2 in each row shown as additional significant variables on bivariate analysis are added to the model.

Clinical and biochemical correlates of bone remodeling markers

The relations between biochemical markers and age, BMI, calcium intake, and vitamin D levels were examined for the overall group (boys and girls) using Pearson correlation analysis. Age and BMI negatively correlated with both markers of bone formation, $R = -0.3, -0.5$, $P < 0.01$, respectively. The correlation was not significant between age, BMI, and 1/S-CTX. There was a negative correlation between 1/S-CTX and mean calcium intake, $R = -0.17$, $P = 0.036$.

Multiple stepwise regression model

In the stepwise multiple regression analysis, the best correlate of OC was gender, accounting for 10% of its variance. The addition of age and BMI accounted for 8% and 2% of OC variance, respectively (Table 2). Gender was also the best single and only significant predictor of 1/S-CTX, accounting for 9% of its variance. Conversely, 1/BAP was best explained by Tanner stage, which accounted for 20% of its variance. The addition of gender and age improved the variance by 7% and 3% for each variable, respectively (Table 2).

Discussion

Our study is one of the few that systematically investigated the effect of gender and puberty, and explored the effect of vitamin D nutrition on biochemical markers of bone remodeling. In girls, remodeling markers were highest in Tanner stages II and III and had decreased into the normal premenopausal normal ranges by Tanner stage V. Conversely, in boys, these markers were significantly elevated in early and midpuberty and remained well above the normal range for normal young men, even by Tanner stage V. They were gender differences in remodeling markers after adjusting for age, weight, and Tanner stages.

Previous studies have shown that BAP and OC, sensitive markers of bone formation, and the peptide-bound forms of resorption markers (ICTP and NTX) increase significantly during puberty, in both boys [2,5,13] and girls [2,14,15]. Our findings on the measured formation markers were consistent with the results of the previously cited studies. Conversely, the clinical use of S-CTX, a peptide-bound resorption marker, is less well studied in children [3]. Our results reveal a change in S-CTX levels throughout the pubertal development in girls similar to the data shown by Lehtonen-Veromaa et al. [3]. In contrast, our data did not show a significant change in S-CTX during pubertal changes in boys. These differences in the profiles of the various biochemical markers of bone resorption in boys may reflect their relative specificity for collagen versus bone. Although samples in our study were drawn in large part in the nonfasting state [16], we do not believe this affected the validity of our results since the profile obtained for markers across puberty are very similar to published data. Few are the studies that systematically compared the effect of puberty and gender on bone remodeling [17–19]. We and others have observed gender differences in bone formation markers, being higher in boys at pubertal stages IV and V compared to girls [17]. Higher bone formation markers have also been noted in adult males compared to female subjects [20]. Mora et al. [19] found that NTX levels were higher in girls at stage II and in boys at stage V. Our study showed an increase in all biochemical markers in boys compared to girls that persisted through Tanner stage V, possibly reflecting a higher bone remodeling that lasted longer in that gender. Interestingly, Bonjour et al. [21] had shown that for a given pubertal stage, males tended to have higher BMD at the femur and a higher bone mineral content at the L2–L4 level with the difference being more pronounced at Tanner stages IV and V. Taken together, these results strengthen the hypothesis that the higher peak bone mass in adult males, compared to females, may be the result of an increased bone turnover that persist longer in boys than in girls [6]. However, this hypothesis would be best examined in a longitudinal study that simultaneously measures changes in these new serum bone markers and BMD during pubertal development in both genders.

Subgroup analysis that revealed an increased BAP and S-CTX in the setting of vitamin D insufficiency in girls in our study may indicate a deleterious effect of such insufficiency on bone remodeling, as has been reported in adults [11], although there is no data to that effect today. However, such impact of vitamin D insufficiency on bone remodeling was not observed in the ANOVA and in the stepwise regression analysis. Furthermore, our analyses were however

limited by the small sample size in the various subgroups. The potential deleterious effect of vitamin D insufficiency on remodeling markers in children must be assessed in a randomized control trial. If confirmed, with a larger sample size and in longitudinal studies, it would present compelling evidence for the consideration of vitamin D insufficiency treatment during adolescence [8,9,10,22]. This would be especially relevant in girls, a gender that is at risk for compromised nutrition in adolescence, that reaches lower peak bone mass, and that is at greatest risk for fractures.

In the regression analyses, gender was the consistent correlate of all three markers of bone remodeling. The gender difference is an interesting finding that was clearly investigated and documented in one previous study [17]. However, close examination of the data from previous studies reveals gender differences in several markers across adolescence, similar to our study, but that were not specifically looked for [2,5,18,19]. This gender difference seemed to be independent of body size as assessed by adjustment for weight in our study. It may reflect gender differences in calcium intake and vitamin D levels as we showed, higher muscle mass in boys, differences in growth rates, or more interestingly the genetic difference, explaining peak bone mass variation [6,7,18]. The gender effect may be also explained by differences in sex steroids secreted during pubertal development, as shown by studies revealing that the best predictors of bone markers were testosterone levels in boys [23,24] and estrogen in girls [4].

In conclusion, our study demonstrates changes in bone remodeling markers that are affected by puberty, gender, and possibly vitamin D status. Gender is a consistent independent correlate of all markers of remodeling measured in this study. This gender difference in remodeling markers during puberty may well reflect the physiologic pathway responsible for the higher peak bone mass achieved in boys compared to girls. That, however, remains to be proven in longitudinal studies.

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