Bone and Arthrosurgery Science, 2023, Volume 1, Issue 1
http://ojs.bbwpublisher.com/index.php/BAS

Impact of Skeletal Maturation on Bone Metabolism Biomarkers and Bone Mineral Density in Healthy Brazilian Adolescents

Carla C Silva[1], Tamara BL Goldberg[2]*, Hong S Nga[3], Cilmery S Kurokawa[2], Renata C Capela[2], Altamir S Teixeira[4], Jose C Dalmas[5]

[1]Department of Physical Education, State University of Northern Paraná (UENP), Rio de Janeiro 86400-000, Brazil

[2]Department of Pediatrics, Botucatu Medical School (UNESP), Botucatu 18618-687, São Paulo, Brazil

[3]National Council for Scientific and Technological Development (CNPq), Brasília, Rio Grande do Norte, Brazil

[4]Department of Tropical Diseases and Image Diagnosis, Botucatu Medical School (UNESP), Botucatu 18618-687, São Paulo, Brazil

[5]Department of Applied Mathematics, State University of Londrina (UEL), Londrina 86057-970, Paraná, Brazil

***Corresponding author:** Tamara BL Goldberg, tamara@fmb.unesp.br

Abstract: This study was conducted to evaluate the behavior of biomarkers in the bone formation and resorption in Brazilian adolescents according to their biological maturation. Eighty-seven volunteers were selected and divided into three groups according to their bone age (BIA); 10-12 years (n=25), 13-15 years (n=36) and 16-18 years (n=26). Few parameters and biomarkers such as, the weight (kg), height (m), body mass index (kg/m^2), 3-day calcium intake (mg/day), assessment of pubertal events by Tanner criteria, levels of the biomarkers [osteocalcin (OC) (ng/mL), bone alkaline phosphatase (FAO) (U/L), and serum carboxy–terminal telopeptide (S–CTx) (ng/mL)] and their correlation with bone mineral density (BMD) (g/cm^2) were measured by dual–energy X–ray attenuation of the lumbar spine, proximal femur and total body in each volunteer. The results showed that, all the biomarkers have similar behaviors, showing a higher median value for 13 to 15 years (FAO=154.71 U/L, OC=43.0 ng/mL, S–CTx=2.09 ng/mL; $p<0.01$) at the pubertal stage G4, and the median is decreased with the advancing IO and sexual maturation level. Additionally, the biomarker levels showed a parallelism with a peak velocity in the stature level, and interestingly, the training biomarkers indicated a negative correlation with BMD where high BMD values correlated with the low biomarker values. In conclusion, this is the first study were conducted in Brazilian adolescents with strict, and careful inclusion and exclusion criteria to assess the correlation between the bone markers and BMD against the indicators of biological maturation. The results of this study may help to understand and monitor the bone turnover and bone metabolism respectively.

Keywords: Bone biomarkers; Adolescents; Bone mineral density; Bone age

Online publication: April 20, 2023

1. Introduction

The bone tissue extends throughout the body, and it is traditionally evaluated in a static and punctual way by imaging methods. As it is radiopaque, the structure can be analyzed by using qualitative techniques such as plain X–rays, and for more accurate results dual energy X–ray absorptiometry (DXA) or quantitative

tomography can be used [1]. However, metabolic, physiological, or pathological imbalances may affect the radiopaque bone structure, subsequently interfering with the detection. Therefore, the use of more dynamic methods in the detection tends to contribute to the initial stage detection of bone mass reduction more significantly, thereby helping in better understanding of mechanisms related to the prevention of this process [1]. Additionally, based on the literature search the use of bone metabolism biomarkers as a dynamic method for assessing bone turnover is a better option for this study [2-5].

Childhood and adolescence are the only periods of longitudinal physical growth, with high rates of bone matrix undermining [6,7], where 25% of the bone mass is incorporated in 2 years surrounding the maximum height velocity peak [1]. The development of the bone remodeling process is based on two antagonistic processes which are the bone formation and bone resorption, further, these two processes enable the bone modeling and remodeling, which are completely interconnected however, during puberty the bone formation process is more important than bone resorption.

However, the use of bone metabolism biomarkers during puberty is still limited, as it is difficult to establish normalized standards, where the results are often influenced by the intense bone growth and remodeling that occur during that period of time, and are also susceptible to variations in biomarker function that is observed in puberty [3,10]. Gordon reported that imaging method and biomarkers can be used together for monitoring the skeletal remodeling during childhood and adolescence [2]. Further, the scientific literature also suggests that biomarker levels decrease after this phase of life, although there is a continuous increase in the body size and bone mineral density (BMD) that continues for few more years [11].

The interest in choosing the dynamic and accurate assessment of bone tissue during puberty is based on the fact that puberty is a sensitive period for increasing and reducing in the bone re-services and future bone loss respectively [3]. It is known that bone mass decreases from the age of 30 years by 1% to 2% in women, meanwhile 0.3% to 1% in men. Additionally, the bone mass is greater in men than in women, because men have larger skeletons, and the period of bone loss starts much later in men than in women, about a decade later [12]. Studies have shown that, one of the main factors in preventing chronic diseases such as, osteoporosis or subsequential bone fractures in the future, is the attempt to reach the ideal peak of bone mass during adolescence or at the end of skeletal maturation [13,14]. Although the prevalence of osteoporosis in men is reported to be lower than in women, however, the overall incident rate is still high in both genders. According to the data published in the United Stated of America (USA) reveal that around 1 to 2 million men was reported to have osteoporosis, while 8 to 13 million have osteopenia, and the report also showed a fracture risk of 13.5% in men aged 50 years and 25.6% in those aged 60 [15].

The above-mentioned issues have seriously worried the public health organizations; therefore, they start to encourage the prevention of bone mineral capital loss by performing the bone mass tests, enabling the early identification of individuals presenting slightly altered BMD [16]. Additionally, follow-up of bone mass incorporation during childhood and adolescence by using DXA analysis, especially in the second decade of life when practically 95% of bone mass is incorporated, tend to be an adequate method for monitoring the bone mineral deposits, which represent a “reserve source” for bone health in future adult life.

Multiple factors are involved in the interpretation of the results of the assessment of bone biomarkers during puberty therefore, it is essential to disseminate what is known about the subject and its applicability in clinical practice as one of the tools for understanding the bone metabolism. Based on these concepts, the aim of this study was to evaluate the behavior of some bone formation and resorption biomarkers to represent the growth and skeletal maturation in a sample of healthy Brazilian male adolescents, relating biomarkers with BMD assessed by DXA of the lumbar spine, proximal femur and total body.

2. Casuistry and methods

Healthy white male adolescents aged between 10 and 18 years participated voluntarily in this cross-sectional study. They were students from Sao Paulo school, belongs to the high socioeconomic class. Of the 497 total students enrolled in the selected school, 87 adolescents who met the inclusion criteria were included in the study, further participated in all the evaluations. The project was approved by the Ethics Committee of the Faculdade de Medicina de Botucatu, Universidade Estadual Paulista (UNESP), protocols nº 261/2004-CEP and 52/2007-CEP. All the participants received the informed consent form, and the form was signed by both the adolescent and his/her parents or guardian.

The inclusion criteria are stated as below [17,18]:

(1) Adolescents between the 10th and 90th percentile weight for each age group
(2) Adolescents between the 10th and 97.5th percentile height for each age group
(3) Adolescents with age-appropriate body mass index (BMI)
(4) Adolescent who consumes dairy products daily

The exclusion criteria were [19]:

(1) Adolescents with a history of prematurity or low birth weight
(2) Adolescents who had any of the following diseases: diabetes mellitus, acute or chronic malnutrition, congenital or acquired bone diseases, gastrointestinal diseases accompanied by malabsorption, history of nephropathy with or without chronic renal failure, endocrinopathies, early or late puberty, chronic drug abuse, cystic fibrosis, celiac disease
(3) Adolescents who use drugs that negatively affect bone metabolism, such as anticonvulsants or antacids with aluminum
(4) Adolescents who followed an exclusively vegetarian diet, with high intake of fibers, caffeine or soft drinks,
(5) Adolescents who did not consume dairy products on a daily basis

The data collection is started from the school. Firstly, the adolescents were randomly selected, and those who did not present any dysfunction or disease as mentioned in the exclusion criteria were invited to have their weight and height measured subsequently, students who met the above inclusion criteria were then inquired about their smoking and alcohol consumption habits. Further, the students with secondary sexual characters were evaluated, and the results were compared with Tanner's criteria [20]. The skeletal maturation and bone age (SIA) were measured using the Greulich Pyle method [21], subsequently, the food characterization in the selected students was determined by using a 3-day food record.

Further, blood samples were collected from the volunteers by a qualified biomedical assistant. The biological samples consisted of 5mL of blood in a dry tube to determine the bone biomarkers level in the serum. The volunteers were fasted for a minimum of 8 hours, followed by blood collection which is conducted between 7am and 9 am. The collected serum was stored and preserved in the Experimental Research Laboratory in the Department of Pediatrics at -70°C until further use. The bone formation biomarkers such as, bone alkaline phosphatase (FAO) expressed in U/L and osteocalcin (OC) expressed in ng/mL was measured by quantitative immunoassay using the monoclonal anti–FAO antibody (Metra BAP, Metra^{TM} Biosystems) with 5% intra and 6% inter assay coefficients of variation and Metra^{TM} competitive immunoassay kit (Metra^{TM} Biosystems) with 8% intra and 7.6% inter assay coefficients of variation respectively. Additionally, for the resorption marker, serum carboxy–terminal telopeptide (S–CTx) expressed in ng/mL were measured by electrochemical luminescence using the commercial kit ß–Cross Laps serum (Roche) and the analyzer Elecsys 1010 (Roche) with 5% inter assay coefficient of variation.

The groups were formed based on OI, according to the following limits; Group 1: 10 to 12 years, 11 months and 29 days (n=25); Group 2: 13 to 15 years, 11 months and 29 days (n=36); and Group 3: 16 to 18 years, 11 months and 29 days (n=26). In this sense, the choice of analyzing biomarkers as a function of

the skeletal growth is assessed by OI rather than pubertal stages results, from the fact that both showed a high correlation value by Spearman's linear correlation coefficient (R=0.93) with p<0.01.

BMD assessment was performed by DXA, using a Hologic QDR 2000 device, and appropriate bone mass assessment was obtained by using the pediatric software, with the BMD results expressed in g/cm^2. Evaluations were performed in the lumbar spine between L1-L4, in the total proximal femur (collofemoral, trochanteric and intertrochanteric regions, and Ward's area) and total body.

The data were further analyzed using the Statistica Version 6 software. The evaluation values obtained from the descriptive statistics (mean ± standard deviation) were included in the analysis of variance and the Scheffe test. Additionally, Kruskal-Wallis variance analysis were performed for comparing the OIs and bone biomarkers, and Shapiro-Wilk test were used to verified that the total variables is not presented in the normal data distribution. Spearman's correlation coefficients were calculated between bone biomarkers and BMD results at the assessed region, lastly a minimum statistical difference of 5% was considered significant.

3. Results

The general characteristics of the 87 adolescents are presented in **Table 1**, which shows the anthropometric indicators (body weight, height, and BMI), the average daily intake of calcium, and the osseous mineralization indicators (BMD in the lumbar spine region between L1–L4, in the proximal femur and in the whole body) in relation to the OIs.

Table 1. Mean and standard deviation of anthropometric indicators, calcium intake and indicators of bone mineralization in relation to the groups classified according to OI (n=87)

	IO (years)		
Variables	**IO 1 (10–12) (n = 25)**	**IO 2 (13–15) (n = 36)**	**IO 3 (16–18) (n = 26)**
Weight (kg)	38.4 ± 7.9*	52.1 ± 8.2*	62.9 ± 8.6*
Height (m)	1.48 ± 0.08*	1.64 ± 0.08*	1.74 ± 0.06*
IMC (kg/m^2)	17.3 ± 2.09*	19.1 ± 1.88*	20.6 ± 2.51*
Calcium intake (mg/day)	802.1 ± 202.0	747.1 ± 255.0	911.0 ± 264.2
BMD (g/cm^2)–column	0.63 ± 0.08*	0.78 ± 0.15*	0.94 ± 0.10*
BMD (g/cm^2)–femur	0.78 ± 0.04*	0.90 ± 0.13*	1.03 ± 0.11*
BMD (g/cm^2)–whole body	0.84 ± 0.03*	0.92 ± 0.09*	1.06 ± 0.07*

Note: BMD = bone mineral density; BMI=body mass index; OI = bone age; Scheffe test for localization of differences between IOs (p<0.01); *IO 1<IO 2<IO 3.

The results show that the values increase with age and present significant differences when the means are compared by variance analysis and the differences are identified by the Scheffe test. The significant level increases in body weight, height, and BMI are observed with age, a typical event consistent with the natural process of intense physical growth that occurs at puberty. Further, BMD also shows a tendency to increase significantly with advancing skeletal maturation in all the analyzed sites. Calcium intake assessed by 3-day dietary report show similarity in the different age groups. This finding is in accordance with the inclusion criteria of the study, which emphasize the daily consumption of dairy products.

Figure 1 shows the medians of FAO, OC and S–CTx bone biomarkers in relation to the OIs. Kruskal–Wallis analysis of the variance revealed a significant (p<0.01) value for all the tested biomarkers. Although the non-parametric statistical analysis did not identify differences between OIs, it also can be observed that the group with OI between 13 and 15 years presents a higher medians value than the other OIs for all the

tested bone biomarkers, for bone formation (FAO and OC) and bone resorption (S–CTx). Importantly, the last OI (16–18 years) presents a considerably lower medians value compared to the younger groups.

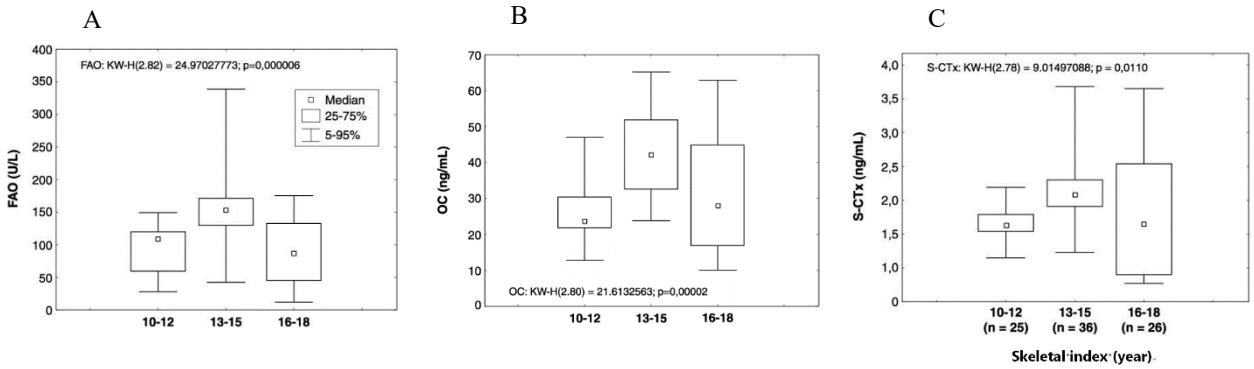

Figure 1. A: Median FAO, B: OC and C: S–CTx according to groups classified by bone age (n=87).
FAO=bone alkaline phosphatase; KW–H=Kruskal-Wallis analysis; OC=osteocalcin; S–CTx=serum carboxy-terminal telopeptide.

Finally, Spearman's correlation coefficients were analyzed between bone markers and BMD in the lumbar spine, proximal femur and whole body.

The **Table 2** showed that the bone formation biomarkers (FAO and OC) were significantly correlated, however, the bone resorption biomarker (S–CTx) showed very low correlation scores, indicating that there was no association between S–CTx and bone mass acquisition in the adolescents analyzed. An interesting finding is that, the bone formation biomarkers indicated negative values; in other words, lower values of formation biomarkers correlated with higher bone densities in the respective sites (**Table 2**).

Table 2. Correlation coefficients between bone biomarkers and indicators of bone mineralisation in the lumbar spine (L1–L4), proximal femur and whole body (n=87)

	S–CTx (ng/mL)	OC (ng/mL)	FAO (U/L)
BMD–column (g/cm^2)	-0.22 (p=0.28)	-0.13 (p=0.51)	-0.37 (p=0.04)*
BMD–femur (g/cm^2)	-0.02 (p=0.93)	-0.45 (p=0.02)*	-0.57 (p=0.00)*
BMD–whole body (g/cm^2)	-0.02 (p=0.91)	-0.47 (p=0.00)*	-0.69 (p=0.00)*

BMD=bone mineral density; FAO=bone alkaline phosphatase; OC=osteocalcin; S-CTx=serum carboxy-terminal telopeptide; *Significant correlations.

4. Discussion

Studies on adolescence and bone health is an important aspect in the international research scenario. Understand the mechanisms which are involved in bone mineralization, especially occurring during the puberty, may be a response to the development of a good quality bone mass, which may result in an active life during aging, through the achievement of a dignified life, from the standpoint of autonomy, independence, and physical capacity [8,22].

Several researchers have highlighted the importance of understanding BMD in children and adolescents, demonstrating that BMD values increase with age [2,7,8,14], however, this growth does not present a linear distribution, which is greater during adolescence. The same observations were reported in Brazil by Silva et al., demonstrated that the critical period for a bone mass increase in healthy male adolescents was between 13 and 15 years of age, during the G4 pubertal development stage [23,24].

Additionally, the literature has reported that the adolescent period is marked by a significant rate of bone formation, as bones are characterized by the metabolically active tissue that is subject to a continuous process of true remodeling.

The results of this study as presented in **Table 1** are similar to the report presented in the specialized literature. In our sample of adolescent males, there is a significant increase in bone mass in the analyzed regions with the advancing skeletal age, especially from 14 years onwards, with the highest averages between the ages of 16 and 18 years, similar results were demonstrated in previous studies [7,10,23]. Box plots showed the medians of biomarkers of the groups classified according to skeletal age, is from 10 to 18 years of age. Statistical treatment by Kruskal-Wallis analysis of variance indicates $p<0.01$ for the biomarkers of bone formation (FAO and OC) and bone resorption (S–CTx). The graphs showed that the medians of the group between 13 and 15 years were considerably higher and then decreased in the group between 16 and 18 years. The lowest biomarker concentrations were observed at the end of puberty, a similar behavior pattern already highlighted by other authors, who reported the values in 18-year-old individuals similar to those found in adults [12].

Additionally, Tuchman et al., found a strong correlation between bone biomarkers and the peak height velocity (PVE), indicating that there is a parallel correlation between the elevated levels of markers and an increase in growth velocity [25]. Moreover, although BMD continues to increase with age until reach a peak bone mass, a reduction in growth velocity was observed as adolescents approach their final height, which is in line with the behavior of bone markers, a fact that reinforces the relationship between the two events.

From this perspective, Van Coeverden et al., assessed the magnitude of the relationship between bone turnover, indicated by the level of bone biomarkers, and EWP by measuring the level of sex steroids, insulin-like growth factor 1 (IGF–1), and insulin-like growth factor binding protein 3 (IGF–BP–3) [7]. The authors also conducted a semi longitudinal study in 155 boys and 141 girls aged between 8.2 to 15.7 years. The results showed that the rapid growth in height was concomitant with the incorporation of bone mass, but not with bone turnover. At the end of puberty, a decrease in estradiol levels was observed, which inhibits chondrocyte proliferation. As a consequence, the authors observed a decrease in the growth rate and in the levels of bone biomarkers. However, bone mass subsequently increased, which was probably influenced by sex steroids, IGF–1 and IGF–BP–3 [7].

The data found in our study (**Table 2**) revealed a significant and negative correlation between the biomarkers OC, FAO, and S–CTx on BMD in the lumbar spine, proximal femur and whole body among all the adolescents. These results differ from those presented for males in the study by van Coeverden et al., who found no significant differences in the values of bone markers between youths in pubertal stages G4 and G5. This is probably due to the fact that the sample consisted of individuals with a maximum age limit of 15.7 years, and because adolescents who were in the G5 stage had a mean age of 13.8±0.9 years. In addition, the authors correlated the results with bone mass content, not with BMD data, and observed a significant correlation, but not a negative correlation as demonstrated in our study. This is probably influenced by the age which is used as a cutoff point, which did not include the entire age range encompassing adolescence, thus not presenting the lower values of bone markers found at the end of this phase of life, as observed in our study.

This research is the first Brazilian study to analyze healthy white male adolescents using a strict inclusion and exclusion criteria, similar to those reported by Yilmaz et al., who showed a reduction in the concentration of biomarkers at the end of puberty only in female adolescents, while BMD continued to increase, revealing a negative correlation between the bone turnover and BMD [26]. In the same study, the authors did not observe a negative correlation between BMD and bone formation markers in male adolescents. However, they evaluated only boys between the age of 10 to 15 years, leading to the decrease in the sensitivity of the test, besides not being able to analyze the complete evolutionary process, because

markers of bone formation and bone turnover show a reduction in the years after the maximum age limit analyzed, as observed in our study, which evaluated adolescents with OIs compatible with the age of 16, 17 and 18 years.

Regarding the relationship between the biomarkers of bone formation and resorption with the secondary sexual characters, our data reveal that biomarkers showed higher medians when the analyzed adolescents reached the G4 pubertal stage (FAO 148, 51 U/L, OC 43.58 ng/mL, S–CTx 2.10 ng/mL), a moment coinciding with the maximum peak of stature velocity, and when they had OI between 13 and 15 years (FAO=154.71U/L, OC=43.0ng/mL, S–CTx=2.09ng/mL; p<0,01). During the G5 stage, the lowest medians were observed for the analyzed biomarkers (FAO 62.21U/L, OC 20.45ng/mL, S–CTX 1.21ng/mL). A statistically significant association was observed between the biomarkers and secondary sexual characters, based on the study of correlation coefficients (data not shown).

Other researchers compared biomarkers of bone formation and bone resorption in children (n=86), with a mean age of 10 years, and adults (n=30), with a mean age of 28 years, in both the genders. Results showed higher levels of FAO and N telopeptide cross–linking (NTx), a marker of bone resorption, in the children group (FAO=170.1±131.4 ng/mL and NTx=89.8±38.9 ng/mL) compared to adults (FAO=20.2±7.5 ng/mL and NTx=15.3±2.5 ng/mL; p<0.01). The authors stated that their results were consistent with the specialized literature, which highlights a considerable increase in the bone metabolic activity in children and adolescents during physical growth. The results also indicated that after the long period of growth, FAO and NTx values showed a considerable decrease [9].

The clinical importance of bone metabolism biomarkers is due to their rapid production during bone remodeling, compared to resulting BMD assessments by traditional methods. The scientific literature has given a specific importance to biomarkers, especially in relation to osteoporosis, which is considered one of the main causes of fragility fractures. Bone markers are proven to be dynamic and effective tool for the evaluation of patients with osteoporosis, and for the follow-up of the effects of medications used for the treatment in these patients, however, the use of biomarkers for diagnosis purposes are not recommended. In prospective studies with postmenopausal women, the increase in resorption markers doubled the risk of fractures was reported. However, it is important to note that the marker responses related to the skeleton as a whole and not only to specific sites, thus the results obtained reveal a risk of probable fracture but not in a specific site [27].

Therefore, tests with biochemical markers of bone remodeling provide an important information for understanding the dynamics of bone metabolism, and can be repeated in short periods of time. However, the great individual variability in the concentration of biomarkers and their release in various anabolic and catabolic processes prevents them from being used alone for diagnosis. Therefore, despite the importance, bone biomarkers are still used in a limited way in the clinical practice and considered as a complementary method to bone densitometry [28], in situations involving the evaluation and follow-up of osteoporosis. The data presented in this study confirm that the assessment of bone mass should be performed using bone biomarkers as a complement to the assessment of BMD. The study and follow-up of biomarkers favor a qualitative evaluation of bone formation and resorption, resulting from the high anabolism observed during puberty. However, the analysis of biomarkers should be complemented by the study of bone densitometry, translation of the timing and pattern of the formation and resorption indexes [29,30]. The combination resulting from the evaluation of various biomarkers of bone formation and resorption is useful for understanding and investigating bone turnover both in healthy children and adolescents and in those with some disease, and also for monitoring the effects resulting from the treatment of diseases that affect bone metabolism.

Disclosure statement

The authors declare no conflict of interest.

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