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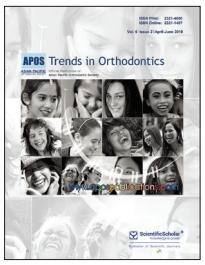

Original Article

Serum osteocalcin – A biochemical marker for pubertal growth assessment

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ABSTRACT

Objectives: Growth evaluation methods have made great strides in the shift from radiologic to non-radiologic biomarkers. Osteocalcin (OC), a bone protein, has been reportedly used as a biomarker for osteoblastic bone formation. The present study aimed at evaluation of serum OC in Class II skeletal patterns for accurate assessment of pubertal growth spurt to facilitate functional jaw orthopedics during the growth period.

Material and Methods: Eighty subjects, comprising 38 males and 42 females with skeletal Class II malocclusion in the age range of 11–18 years, were recruited for the study. Human serum OC was quantitatively assessed with enzyme-linked immunosorbent assay. The cervical vertebral stages were assessed from lateral cephalograms. Statistical analysis for gender-wise comparison of mean serum levels of OC at each cervical stage (CS) and in the intervals of the CSs was carried out using Kruskal–Wallis test and for intergroup comparisons, Mann–Whitney U-test with Bonferroni's correction was done.

Results: Gender-wise comparison of mean serum OC levels revealed that it was highest in CS2 in both males (72.24 ng/mL) and females (74.71 ng/mL) with another discernible peak in CS5 in males (66.82 ng/mL) and in CS6 in females (63.78 ng/mL), exhibiting thereby a circadian rhythm in bone modeling during the entire adolescent growth spurt.

Conclusion: Despite a pre-pubertal and a late pubertal spike in both the genders, the mean OC serum levels actually exhibited a circadian rhythmicity across all the CSs, exhorting thereby the importance of bone remodeling during the complete circumpubertal growth period.

Keywords: Osteocalcin, Skeletal Class II patterns, Cervical vertebral stages, Biochemical markers, Pubertal growth spurt

INTRODUCTION

A paradigm shift in the use of non-radiologic biomarkers to assess skeletal maturity has been evidenced in recent years. Menarche and appearance of secondary sexual characteristics, increase in body height, chronologic age, and tooth mineralization stages have been reported to being used as predictors of skeletal maturity.[1-5] On the other hand, radiologic biomarkers such as middle phalanx of third finger (MP3), hand-wrist radiograph, and cervical vertebral maturation indicators though used extensively in clinical practice are plagued with inter and intraobserver reliability and validity.[6-9] Non-radiologic biologic markers such as testosterone, estrogen, thyroid hormones, growth hormone (GH), insulin-like growth factor-1 (IGF-1), insulin-like growth factor-binding protein-3, parathyroid hormone-related protein, bone alkaline phosphatase

(ALP), and glucocorticoids have been the other promising biomarkers used in skeletal maturity assessment.[10-16]

Osteocalcin (OC), a bone protein dependent on vitamin K otherwise known as gamma-carboxyglutamic acid, has been reported to be routinely used as an osteoblastic bone formation biomarker. Produced by the osteoblasts, it is a 5.6 kDa protein comprising 46–50 amino acids that facilitate the mineralization of bone due to its affinity for calcium. It binds to the hydroxyapatite in the bone as it undergoes the process of carboxylation[17] and serves as a marker of late osteoblast differentiation.[18] It is also produced by the odontoblasts and hypertrophic chondrocytes and the small part of it that is released into circulation is detected by immunoassays. GH stimulates skeletal growth by inducing production of OC in osteoblasts. OC is excreted in urine and has a diurnal variation with a decrease in morning, an increase in the afternoon and early evening with a notable nocturnal peak. It regulates cognition, whole body metabolism and reproduction as also acts as a hormone. Tarallo *et al*.,[19] reported that non-steroidal anti-inflammatory drugs and menstrual cycle in girls had no effect on serum OC levels. Extensive studies have shown that it paralleled the growth velocity curve and reflected the activity of the skeleton as a whole.[20,21]

Hence, the aim of this study was to assess the mean serum levels of OC in skeletal Class II malocclusion subjects and compare them with the cervical vertebral stages as assessed from lateral cephalograms to provide insight into precise skeletal growth assessment during puberty.

MATERIAL AND METHODS

This cross-sectional study was conducted on 80 subjects (42 females and 38 males) with Class II skeletal patterns in the 11–18 years age range, selected from the outpatient department of orthodontics and dentofacial orthopedics of the institution. The Human Institutional Ethics Committee of Sree Balaji Dental College and Hospital, Chennai, India, reviewed and approved the study design. (Approval No: SBDCH/IEC/08/2017/2 dated, 25/10/2017). The participant and their parent/guardian duly gave in their assent in the informed consent form in both English and in the local vernacular. Those with growth abnormalities, history of facial trauma/injury, bleeding, and other endocrinal disorders were excluded from the study. Case history, extraoral, and intraoral clinical examination was done. The 2nd, 3rd, and 4th cervical vertebrae (CV) were visually assessed on lateral cephalograms by two observers as per the method advocated by Baccetti *et al*.,[22] and confirmation of the skeletal Class II pattern was done with lateral cephalometric analysis. Sample size calculation was done at a power of 80% and at a 5% significance level ($\alpha = 0.05$).

Two mL of blood was collected and the serum was separated and stored at −80°C. Quantitative assessment of Human serum Osteocacin/Bone carboxyglutamic acid (OC/BGP) was performed using enzyme-linked immunosorbent assay (ELISA) kit (E-EL-H1343, Elabscience®, USA) based on Sandwich-ELISA principle.

A micro-plate reader (TECAN, InfiniteM200PRO, Switzerland) was used to read the absorbance (optical density, OD) at 450nm within 10 min of addition of Stop Solution. The OD of the standards corresponding to human OC/BGP concentration ranged from 1.25 to 80 ng/mL.

From each standard and patient's sample, the average of the duplicate OD values was first calculated and the average zero standard OD was subtracted from it. The standard curve was so plotted such that the standard concentration was denoted by the X-axis and the OD values by the Y-axis. OC concentration in each patient's sample was calculated using the average absorbance value of each sample.

Statistical analysis was carried out with SPSS (Version 24.0). The normality test with Shapiro-Wilk test revealed that the OC data were not normally distributed, and hence, non-parametric tests were carried out. Statistical analysis for gender-wise comparison of OC mean serum levels at each cervical stage (CS) and in the intervals of the CSs was carried out using the Kruskal–Wallis test and for intergroup comparisons, Mann–Whitney U-test with Bonferroni's correction was done ($P < 0.05$ was considered statistically significant). Intraobserver and interobserver reliability of CVM assessment was measured using Kappa statistics.

RESULTS

Gender-wise comparison of serum OC levels at each CS is depicted in [Table 1]. In males, the highest mean OC levels was 72.24 ± 6.94 ng/mL at CS2 with a gradual decline from CS3 to CS5, to being lowest in CS6 (62.29 ± 17.03 ng/mL) though statistically not significant ($P = 0.052$). In females, the highest mean OC levels were 74.71 ± 0.57 ng/mL at CS2 with a gradual decline from CS3 to CS5 with an appreciable increase in CS6 (63.78 ± 14.33 ng/mL). The lowest mean OC level was at CS4 (55.17 ± 24.58 ng/mL). However, it was also not statistically significant ($P = 0.359$) [Table 1a and b, Figure 1]. To evaluate intergroup comparisons of OC mean serum levels with different CSs, Mann–Whitney U-test with Bonferroni's correction was carried out [Table 2]. The results showed none of the variables were statistically significant [Table 2a and b].

Gender-wise comparison of mean serum OC levels in the four intervals of CSs is depicted in [Table 3]. In males, the highest OC levels was 70.04 ± 10.73 ng/mL in CS2–CS3 with a gradual decline in CS3–CS4, CS4–CS5, and CS5–CS6, to being the lowest in CS4–CS5 (63.27 ± 8.87 ng/mL). Statistically too, it was significant ($P = 0.030$). In females, the highest mean OC levels were 66.97 ± 13.41 ng/mL in CS2–

Table 1: Gender-wise distribution of OC (ng/mL) serum levels at each CS.

CSs	Mean OC (ng/mL)±SD	SEM	95.0% Confidence interval for mean		Median	OC (ng/mL)	
			LL	UL		Min.	Max.
a: OC (ng/mL) serum levels of male subjects at each CS							
CS2	72.24±6.94	2.46	66.44	78.05	75.20	55	75
CS3	68.27±13.13	4.15	58.88	77.66	75.20	42	75
CS4	61.69±7.98	2.66	55.56	67.83	57.90	51	73
CS5	66.82±10.98	5.49	49.36	84.29	71.09	51	74
CS6	62.29±17.03	6.44	46.54	78.04	71.27	29	75
b: OC (ng/mL) serum levels of female subjects at each CS							
CS2	74.71±0.57	0.28	73.80	75.62	74.73	74	75
CS3	56.65±16.10	9.29	16.66	96.64	52.51	43	74
CS4	55.17±24.58	7.77	37.58	72.75	67.57	8	75
CS5	56.39±22.53	5.17	45.53	67.25	63.59	−14	75
CS6	63.78±14.33	5.85	48.74	78.81	68.98	36	75

Kruskal–Wallis test: *P*=0.052 (NS). Kruskal–Wallis test: *P*=0.359 (NS). CS: Cervical stage, OC: Osteocalcin, SEM: Standard error of mean, LL: Lower limit, UL: Upper limit

Table 2: Intergroup comparison of OC (ng/mL) serum levels with different CSs in both males and females.

a: Intergroup comparison of serum OC levels with different CS in male subjects			b: Intergroup comparison of serum OC levels with different CS in female subjects		
CS	Compared stage	*P*-value	CS	Compared stage	*P*-value
CS2	CS3	0.965 (NS)	CS2	CS3	0.229 (NS)
	CS4	0.008 (NS)		CS4	0.036 (NS)
	CS5	0.109 (NS)		CS5	0.162 (NS)
	CS6	0.152 (NS)		CS6	0.067 (NS)
CS3	CS4	0.053(NS)	CS3	CS4	0.811 (NS)
	CS5	0.188 (NS)		CS5	0.718 (NS)
	CS6	0.270 (NS)		CS6	0.714 (NS)
CS4	CS5	0.330 (NS)	CS4	CS5	0.910 (NS)
	CS6	0.408 (NS)		CS6	0.562 (NS)
CS5	CS6	0.927 (NS)	CS5	CS6	0.687 (NS)

Mann–Whitney U-test with Bonferroni's correction for ten comparisons, alpha value set at 0.05/10=0.005; (*P*<0.005). CS: Cervical stage, Sig.: Significant, NS: Not significant

Table 3: Gender-wise distribution of OC (ng/mL) serum levels at each interval of CS.

CSs	Mean OC (ng/mL)±SD	SEM	95.0% Confidence interval for mean		Median	OC (ng/mL)	
			LL	UL		Min.	Max.
a: OC (ng/mL) serum levels of male subjects at each interval of CS							
C2–C3	70.04±10.73	2.53	64.70	75.38	75.20	42	75
C3–C4	65.16±11.22	2.57	59.75	70.56	69.04	42	75
C4–C5	63.27±8.87	2.46	57.91	68.63	62.30	51	74
C5–C6	63.94±14.68	4.43	54.08	73.80	71.27	29	75
b: OC (ng/mL) serum levels of female subjects at each interval of CS							
C2–C3	66.97±13.41	5.07	54.57	79.37	74.25	43	75
C3–C4	55.51±22.29	6.18	42.04	68.98	67.16	8	75
C4–C5	55.97±22.82	4.24	47.29	64.65	64.75	−14	75
C5–C6	58.16±20.83	4.17	49.56	66.76	64.75	−14	75

Kruskal–Wallis test: *P*=0.030 (Sig.). Kruskal–Wallis test: *P*=0.646 (NS). CS: Cervical stage, LL: Lower limit, UL: Upper limit

CS3 stage with a decline in CS3–CS4 and CS4–CS5 with a not so significant increase in the levels in CS5–CS6; the lowest being in CS3–CS4 (55.51 ± 22.29 ng/mL). However, it was statistically not significant ($P = 0.646$) [Table 3a and b,

Figure 2]. To evaluate intergroup comparisons of serum OC levels with different CSs intervals, Mann–Whitney U-test with Bonferroni's correction was carried out [Table 4]. The results showed that none of the variables were statistically significant except CS2–CS3 which in comparison to CS4–CS5 was statistically significant ($P = 0.004$) [Table 4a and b]. Kappa statistics showed that the intra- and interobserver assessment was 98.4% and 96.8%, respectively.

DISCUSSION

Radiologic biomarkers though widely used in the clinical scenario are rift with high subjectivity, poor reproducibility, and radiation exposure.[6,23,24] The commonly used cervical vertebral maturation indices too are subject to wide variability as it has been reported that the CV differ in various skeletal malocclusions with their height being altered in response to posture of the body and morphology of the facial components.[25,26] A lateral cephalometric radiograph is routinely taken for diagnosis and treatment planning in orthodontics and since the cervical vertebral stages could be easily assessed from the same, this method was adopted for comparison with the mean serum OC levels of the skeletal Class II malocclusion subjects. McNamara Jr. and Franchi[27] had opined that reliable determination of the CV staging was largely dependent on the experience of the clinical assessor. Literature is evidence of the fact that molecular signatures of the skeletal growth process have gained greater importance in determination and assessment of skeletal maturation.[28]

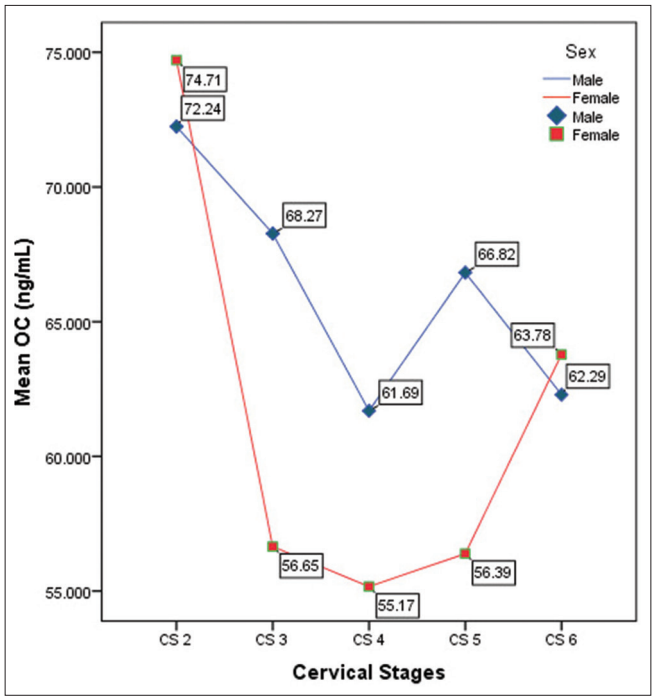

Figure 1: Mean serum osteocalcin levels at each cervical stage.

OC was chosen as a skeletal maturity indicator in our study as literature has proven time and again that it served as a reliable marker for bone mineralization, bone formation, and bone turnover.[17,29] GH induces the production of OC in osteoblasts.[30] OC invariably contributes to serum calcium homeostasis. The use of mean serum OC levels to predict the optimal treatment timing of skeletal Class II malocclusion has not been reported in literature till date. Johansen *et al.*[20] had reported the use of radioimmunoassay (RIA) in the estimation of serum OC in subjects in the age range of (8–20 years). On the contrary, we used the sandwich-based ELISA as RIA would encumber the preparation and handling of radioactive antigen which may prove hazardous in some situations. Our sample was a specific sample of skeletal Class II patterns in whom the identification of the pubertal growth spurt for addressal of the skeletal Class II jaw discrepancies was an added benefit of clinical significance.

Our results showed that mean serum OC levels in males increased in CS2, dipped in CS3 and CS4, and then increased in CS5 to decline in CS6. In females too, an increase was observed in CS2 with a decline in all the other stages with the exception of a peak in CS6. This circadian rhythmicity that was observed was similar to the findings of another study where a diurnal variation

Table 4: Intergroup comparison of OC (ng/mL) serum levels with different CS intervals in both males and females.

a: Intergroup comparison of serum OC levels with different CS intervals in males			b: Intergroup comparison of serum OC levels with different CS intervals in females		
CS	**Compared stage**	***P*-value**	**CS**	**Compared stage**	***P*-value**
CS2–CS3	CS3–CS4	0.118 (NS)	CS2–CS3	CS3–CS4	0.183 (NS)
	CS4–CS5	0.004 (S)		CS4–CS5	0.306 (NS)
	CS5–CS6	0.044 (NS)		CS5–CS6	0.370 (NS)
CS3–CS4	CS4–CS5	0.305 (NS)	CS3–CS4	CS4–CS5	0.809 (NS)
	CS5–CS6	0.800 (NS)		CS5–CS6	0.649 (NS)
CS4–CS5	CS5–CS6	0.361 (NS)	CS4–CS5	CS5–CS6	--

Mann–Whitney U-test with Bonferroni's correction for ten comparisons, alpha value set at 0.05/6=0.008; (P<0.008). CS: Cervical stage, S: Significant, NS: Not Significant

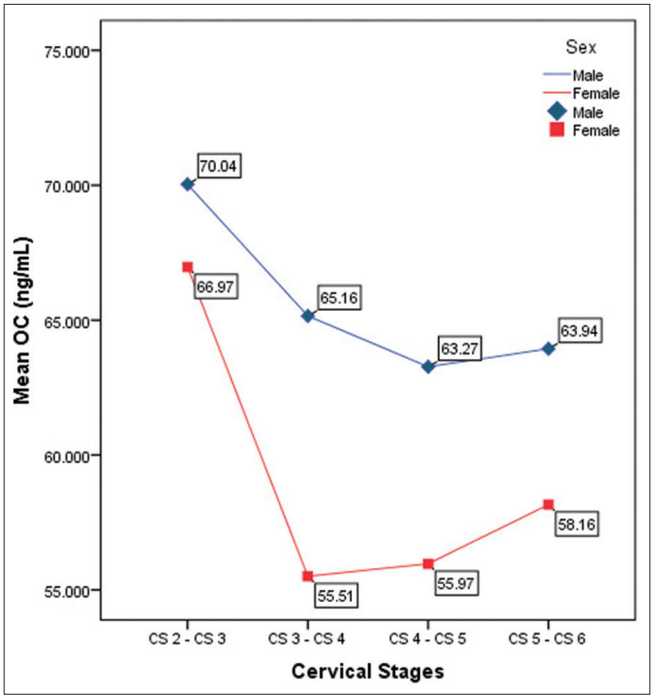

Figure 2: Mean serum osteocalcin levels in the four intervals of cervical stages.

with a circadian rhythmicity and a two-fold fluctuation over a 24 h period was observed.[31] In our study, CS2–CS3 interval witnessed peak levels of OC in both males and females with insignificant interstage differences at all the other intervals in tune with similar findings of yet another study.[32] Our data also showed that in males, the mean serum OC levels in CS2–CS3 in comparison with CS4–CS5 were also statistically significant. It has been reported that in comparison to females, males exhibited greater thickness of the cortical bone due most probably to higher OC peak levels.[33] Our findings too revealed that the mean OC levels in males were increased in comparison to females at each of the CSs. Literature reports have underlined the fact that in comparison to cancellous bone, OC is found to be higher in cortical bone.[34] Serum OC in comparison to serum ALP is considered to be a more sensitive biomarker as its levels correlate well with bone formation rate as also it promotes osteoclastic activity with increasing age.[35] Carter *et al.*,[36] too had reiterated that serum OC was a better determinator of bone mineralization than serum ALP. Serum OC has also been reported to correlate well with skeletal development with low levels in adults and high in children suggesting mineralization and maximal bone matrix formation in boys at puberty.[37] Our study too was in concert with yet another study by Choi *et al.*[38] wherein they had reported that OC levels in the whole period of childhood were predominantly higher than adult values, reflective of bone modeling during the entire active growth period.

Diurnal variations in the concentration of OC are quite common. Hence, collection of the blood sample should be carried out at specified time periods in a day. Technical and qualitative risks need to be taken into consideration when one is dealing with immunoassays. As CVM is also not a perfect rating system to identify the pubertal growth spurt, the use of a non-radiologic biomarker would go a long way in identifying the accurate pubertal growth spurt and duration of the same. Longitudinal studies with a larger sample would help glean more information on the estimation of the intensity and timing of the pubertal growth spurt. Reference ranges of OC mean serum levels at different CVM stages pertaining to a South Indian population to assess peak pubertal growth could also be a step in the right direction, thus negating the need for repeated radiographic exposures. The results of this study suggest that there occurs a gradual change of serum OC levels "during growth," highlighting thereby the importance of bone remodeling during the entire circumpubertal growth period. Hence, the estimation of the serum OC levels could plausibly serve as a potential biomarker of pubertal growth assessment.

CONCLUSION

1. Mean OC serum levels in male subjects were found to be highest in CS2 and then in CS5 and in the female subjects, it was highest in CS2 and then in CS6, exhibiting a circadian rhythm in bone modeling during the entire adolescent growth spurt
2. Comparison of mean OC serum levels in the four intervals of cervical staging showed that in males and females, it was highest in CS2-CS3. However, a constant pattern was maintained in both the sexes in all the stages from CS2 to CS6, emphasizing the important role of bone remodeling during the complete active growth period
3. As a discernible spike of OC was observed around 13 years in males and 12 years in females and a second spike in late post-pubertal stages, growth modulating appliances could be advocated during both pubertal and post pubertal stages of growth.

Declaration of patient consent

The Institutional Review Board (IRB) permission obtained for the study.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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