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## **Serum osteocalcin levels compared with cervical maturational stages as growth indicators: An in-vivo study**

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**Abstract**--OBJECTIVE: To determine whether serum osteocalcin levels can be used as a marker for assessing skeletal maturity. MATERIALS AND METHODS: The sample consisted of 60 patients aged 08-20 years (30 males, 30 females) reporting to Department of Orthodontics and dentofacial orthopedics, and Department of Pedodontics, Faculty of dental sciences, Ramaiah university of Applied sciences, Bangalore. Lateral cephalometric radiographs were obtained from all the subjects. Cervical staging of orthodontic patients satisfying the inclusion criteria were evaluated on their respective lateral cephalograms by using the Hassel and Farman method. After cervical vertebral maturation evaluation, 60 subjects selected for the

study from the screened patients were grouped into 6 cervical stage (CS) groups of 10 per group (5 males, 5 females). 1ml blood sample was collected from each patient after taking informed consent. The collected blood was centrifuged for 20 min at room temperature and then stored at -20°C until analysis. The serum samples were subjected to ELISA test for determining levels of Osteocalcin. One – way ANOVA test was used to determine whether there are any differences between the means of serum osteocalcin levels in different CVMI stages Independent –t test was done to compare and determine whether there is statistical evidence that the serum osteocalcin level means are significantly different. RESULTS: There was a significant rise in the mean serum osteocalcin levels from pre-pubertal to pubertal phase and a gradual decline in the mean serum osteocalcin levels from the pubertal to post pubertal phase. CONCLUSION: Serum Osteocalcin can be used as a biomarker to determine the skeletal maturity of an individual.

**Keywords**---Osteocalcin, CVMI, Skeletal, ELISA, Pubertal.

## **Introduction**

Skeletal maturation is an integral part of an individual's pattern of growth and development. Accurate determination of skeletal maturity and remaining growth is crucial for many orthodontic, orthognathic and dental implant decisions. The chronologic timing of puberty and the adolescent growth spurt demonstrate much variation and are affected by both genetic and environmental factors. The degree of skeletal development is a reflection of the level of physiological maturation of a subject. Bone age was shown to be as important as chronological age in evaluating an adolescent's physical development<sup>44</sup>. The determination of skeletal age indicates how much further growth, a child will attain. Certain skeletal developmental stages of the hand and wrist have been shown to be closely associated with the pubertal growth spurt and these radiographs have been used as an indirect method for the assessment of somatic maturity stage. However, the routine use of hand wrist radiographs has lately been questioned from the radiation hygiene, safety point of view. Cervical vertebral stages and hand-wrist radiographs are currently used to identify the peak mandibular bone growth. These are highly subjective techniques that not only involve radiographic exposure but also lack the ability to determine the intensity of the growth spurt and the end of growth.

Various markers of bone formation have been described including their bone specificity and age-related changes which includes osteocalcin, alkaline phosphatase and its skeletal isoenzyme, procollagen I extension peptides. Osteocalcin, also known as bone gamma-carboxyglutamic acid protein ,is a vitamin K –dependent protein of the bone ,it has been shown to be a potential biomarker ,which can predict growth status with the development of more sensitive assays<sup>57</sup>. This study is an attempt to correlate serum osteocalcin levels with different stages of cervical vertebrae maturation index (CVMI) in determining the skeletal age.

## Materials and Methods

This study was carried out on patients who reported to the Department of Orthodontics and dentofacial orthopedics, and Department of Pedodontics, M.S Ramaiah dental college and hospital Bangalore. The inclusion criteria involved age 08-20 years, with good general health. Written informed consent was obtained from all patients or the parents. The exclusion criteria involved patients with systemic illness, those who had antibiotic therapy and used anti-inflammatory drugs 1-2 weeks prior to the test. The effect of antibiotics is mediated through the biomarkers, the level of serum biomarkers increases during antibiotic therapy. The sample consisted of 60 patients aged 08-20 years (30 males, 30 females) . Lateral cephalometric radiographs were obtained and cervical staging was assessed using Hassel and Farman method. 1ml blood sample was collected from each patient after taking informed consent. The collected blood was then centrifuged for 20 min at room temperature and then stored at -20°C until analysis.

For ELISA test 100ul sample was added to each well and incubated for 90 minutes at 37 degree and then 100ul biotinylated detection Ab was added followed by incubation for 1 hour at 37 degree, followed by 100 ul HRP conjugate,90ul substrate reagent and 50 ul stop solution. After addition of every substrates aspiration was done along with washing and finally after addition of stop solution reading was done at 450 nm and results were calculated. One –way ANOVA test was used to determine whether there are any differences between the means of serum osteocalcin levels in different CVMI stages . Independent –t test was done to compare and determine whether there is statistical evidence that the serum osteocalcin level means are significantly different.The Independent t test is a parametric test .

## Results

Table 1

Genderwise comparison of Mean Serum Osteocalcin levels [ng/ml] in different stages of Cervical Vertebral Maturation stages using Independent Student test								
CVMS	Gender	N	Mean	SD	S.E.M	Mean Diff	t	P-Value
Stage 1	Males	5	13.84	2.19	0.98	1.18	0.944	0.37
	Females	5	12.66	1.73	0.78			
Stage 2	Males	5	20.12	0.95	0.43	-0.22	-0.252	0.81
	Females	5	20.34	1.71	0.76			
Stage 3	Males	5	31.20	2.56	1.15	-0.70	-0.350	0.74
	Females	5	31.90	3.67	1.64			
Stage 4	Males	5	24.58	1.08	0.48	0.02	0.020	0.99
	Females	5	24.56	1.99	0.89			
Stage 5	Males	5	21.54	0.93	0.42	1.04	1.176	0.27
	Females	5	20.50	1.74	0.78			
Stage 6	Males	5	18.72	1.12	0.50	5.10	7.770	<0.001*
	Females	5	13.62	0.95	0.43			

In the present study, the relationship between serum Osteocalcin(B GLA) protein

levels and cervical maturational stages in 60 subjects were evaluated. Later, the results of these sixty subjects were tabulated according to CVM stages

CVMS	N	Mean	SD	Std. Error	Min	Max	F	P-Value
Stage 1	10	13.25	1.96	0.62	11.1	16.4	92.076	<0.001*
Stage 2	10	20.23	1.31	0.41	18.2	22.8		
Stage 3	10	31.55	3.01	0.95	28.6	37.9		
Stage 4	10	24.57	1.51	0.48	23.2	28.0		
Stage 5	10	21.02	1.43	0.45	18.3	22.7		
Stage 6	10	16.17	2.86	0.90	12.0	20.2		

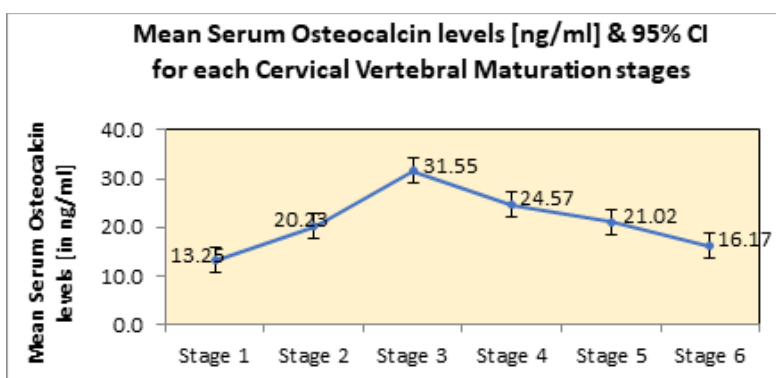


Fig 1.

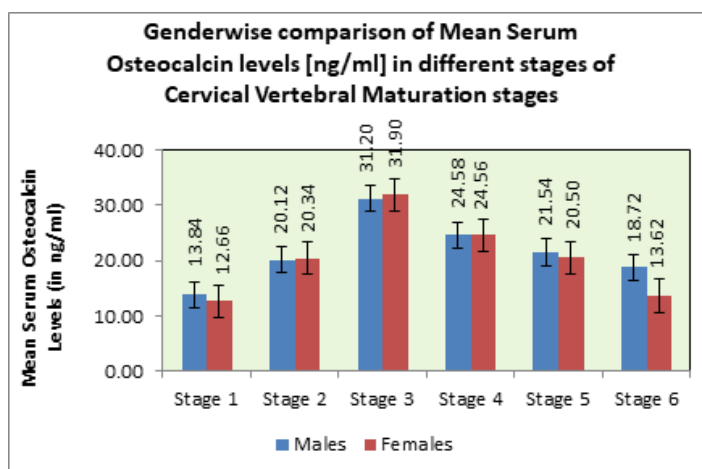


Fig 2.

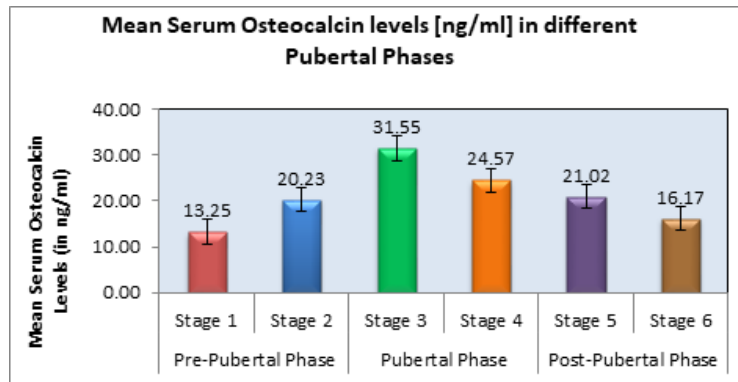


Fig 3.

Table 1 shows the Gender wise comparison of mean serum osteocalcin levels [ng/ml] in different stages of Cervical Vertebral Maturation stages using Independent Student test. The mean serum osteocalcin level for males and females in CVM stage 1 was 13.84 ng/ml and 12.66 ng/ml, stage 2 was 20.12 ng/ml and 20.34 ng/ml, stage 3 was 31.20 ng/ml and 31.90, stage 4 was 24.58 ng/ml and 24.56 ng/ml, stage 5 was 21.54 ng/ml and 20.50 ng/ml, and stage 6 was 18.72 ng/ml and 13.62 ng/ml respectively. Fig 2, shows the Gender wise comparison of mean serum osteocalcin levels [ng/ml] in different stages of Cervical Vertebral Maturation stages

Table 2 represents the comparison of mean serum osteocalcin levels(ng/ml)between different cervical maturational stages using one-way anova test .The mean serum osteocalcin in Cvmi stage 1 was 13.2513.25 ng/ml, stage 2 was 20.23 ng/ml, stage 3 was 31.55 ng/ml, stage 4 was 24.57 ng/ml, stage 5 was 21.02 ng/ml and stage 6 was 16.17 ng/ml. These inter -CVMI stage differences revealed that osteocalcin levels gradually rise from CVMI 1-3 in both males and females and then gradually decline from CVMI stage 4 to CVMI stage 6. Fig 3 shows the mean serum osteocalcin levels in different pubertal phases. Fig 1 show that there was a gradual increase in the mean serum osteocalcin level from CVM stage 1 to stage 3 where the mean serum osteocalcin reaches its peak in CVMI stage 3 and there was a decrease in mean serum osteocalcin level from CVMI stage 4 to stage 6.

## Discussion

The degree of skeletal development is a reflection of the level of physiological maturation of a subject. Bone age was shown to be as important as chronological age in evaluating an adolescent's physical development<sup>44</sup>. The assessment of skeletal maturity is a vital technique in the assessment, development and timing of therapy in children with growth disorders, such as constitutional growth retardation and growth hormone deficiency.<sup>26</sup> The cervical vertebral maturation as an indicator for skeletal maturity has had its share of defenders and adversaries. In an ongoing report, Perinetti et al reasoned that visual appraisal of the CVMI stages was exact and repeatable to a satisfactory level. In spite of the fact that the legitimacy of the CVMI has been addressed every so often, numerous

creators have proposed its viability in surveying development potential when utilized with other diagnostic tools.<sup>49</sup>

Hassel and Farman expressed that since skeletal development is a constant procedure, one diagnostic tool ought not be depended on too intensely. In this way, the different development markers must be utilized together while thinking about orthodontic rectifications to guarantee exactness.<sup>15</sup> Baccetti et al stated that an “ideal” biologic indicator of individual mandibular skeletal maturity should be characterized by the following features: (1) efficacy in detecting the peak in mandibular growth, (2) no need for additional x-ray exposure, (3) ease in recording, and (4) consistency in the interpretation of the data.<sup>28</sup> The various methods to determine the skeletal maturity are CVMI staging by Hassel and Farman, Baccetti et al, Hand wrist radiographic method by Fishman, MP3 staging by Hagg and Taranger and Serum IGF-1 by Masoud et al, which are invasive methods having either radiation exposure or collection of blood samples.<sup>6, 7, 15, 37</sup> Biomarkers have the advantage of avoiding unwanted radiation exposure. They can be measured from various biologic fluids such as blood, saliva, and urine, thereby overcoming the subjectivity associated with radiographs.<sup>54</sup>

The highest concentrations of bone markers (such as serum bone-specific alkaline phosphatase, carboxy- and amino-terminal propeptide of type 1 collagen and osteocalcin) and of bone resorption markers (such as serum cross-linked carboxy terminal telopeptide of type 1 collagen and urinary excretion of collagen cross-links) are reported in the early stages of puberty or at midpuberty indicating that pubertal stage is a main determinant of bonemarker levels.<sup>20</sup> Determination of maturation and subsequent evaluation of growth potential are extremely important. Our goal was to evaluate the validity of Serum Osteocalcin levels in different cervical maturational stages to assess the skeletal maturation. According to Kirmani et al serum osteocalcin significantly increased with age, body age, body weight, height and bone age until age 12-13 years in girls and 14-15 years in boys. It is found by Kirmani et al that serum osteocalcin increased early in puberty and peaked at 14 years of age but declined after the age of 14 years<sup>44</sup>. This is the reason osteocalcin is considered a potential biomarker and can predict growth status with the development of more sensitive assays. In our study, we used the ELISA technique for determination of level of serum osteocalcin because it has increased analytical sensitivity and specificity when compared with radioimmunoassay and immunoradiometric assay<sup>48</sup>.

Osteocalcin also known as bone gamma-carboxyglutamic acid (Gla) protein, is Vitamin K –dependent protein of the bone. It is produced by osteoblasts, odontoblasts and hypertrophic chondrocytes and binds to hydroxyapatite after releasing from osteoblasts. Larger part is integrated into extracellular bone matrix and smaller part is released into circulation, available for detection by immunoassays. The results of the present study could not be compared directly with any other studies, as this was the first study to be conducted on serum for osteocalcin estimation. Therefore there is a necessity for determination of the correlation between osteocalcin and CVMI which can provide a precise, non-radiographic indicator of skeletal maturity. Determination of maturation and subsequent evaluation of growth potential are extremely important. Our goal was to evaluate the validity of Salivary Osteocalcin levels in different cervical

maturational stages to assess the skeletal maturation.

The comparison of mean Serum Osteocalcin levels [ng/ml] between different Cervical Vertebral Maturation stages showed statistically significant values ( $p < 0.001$ ). There was a gradual increase in the mean Serum Osteocalcin levels from CVM stage 1 to stage 3 where the mean levels reach its peak in CVMI stage 3 and there was a decrease in mean Serum Osteocalcin level from CVMI stage 4 to stage 6. This result was similar to the results found by Mohit et al<sup>54</sup> in 2016 where the mean serum and urine IGF-1 levels increases from CVMI stage 1 to stage 4 and decreases from CVMI stage 4 to stage 6. Table 2 and Fig 1 ,2 shows the Comparison of Mean serum osteocalcin levels [ng/ml] between different Cervical Vertebral Maturation stages using One-way ANOVA test. The mean serum osteocalcin in CVMI stage 1 was 13.25 ng/ml, stage 2 was 20.23 ng/ml, stage 3 was 31.55 ng/ml, stage 4 was 24.57 ng/ml, stage 5 was 21.02 ng/ml and stage 6 was 16.17 ng/ml. These inter -CVMI stage differences revealed that osteocalcin levels gradually rise in both males and females and then gradually decline from CVMI stage 4 to CVMI stage 6. Such variation in osteocalcin during puberty suggests some sex hormone regulation of osteocalcin levels. This result was similar to the results found by Sinha et al in 2016 where the mean serum and urine IGF-1 levels increases from CVMI stage 1 to stage 4 and decreases from CVMI stage 4 to stage 6. The mean serum and urine IGF-1 level reaches its peak in CVMI stage 3.

There are previous reports about the influence of sex steroids on the serum levels of systemic and local factors. Increase in sex steroids with pubertal development increase the level of local and systemic factors in young men and women.<sup>10</sup> This phenomenon of influence of sex steroids on serum levels of systemic and local factors probably influences and is responsible for elevated serum levels of osteocalcin during peak high velocity. This decrease in serum osteocalcin level in post pubertal stage could be because of lesser contribution of replicated mesenchymal cells to chondrocytes, thus explaining the slowed cartilaginous growth with age<sup>48</sup>. Though the collection of serum sample is not typical practice in orthodontics but it was easy for us to convince the patients by making them understand the association between growth and development and treatment alternatives. Finally, we found that the subjects were more than willing to take part in our investigation and know the outcomes.

The field of bone turnover markers has developed considerably in the past decade. Biochemical monitoring of bone metabolism depends upon measurement of enzymes and proteins released during bone formation and of degradation products produced during bone resorption. Various biochemical markers are now available that allow a specific and sensitive assessment of growth status of the patient. Although these markers are not recommended for use as a skeletal maturity indicator yet, they appear to be useful for the screening process prior to growth modulation therapy which may help us to avoid unwarranted radiation exposure. The present study results can be further validated considering a larger sample size to yield a generalizable norm/range for different CVMI stages. More research is necessary to validate these results in a different population and with a larger sample. We propose longitudinal studies to confirm the utility of Serum Osteocalcin, in accurately determining the timing and possibly the intensity of a

patient's growth spurt and to determine whether Serum Osteocalcin is a good predictor of skeletal maturity

## Conclusion

The conclusions drawn from this study are:

- This study indicates that there is a significant association between mean Serum Osteocalcin levels and cervical vertebral maturational stages.
- There is a significant association between mean Serum Osteocalcin levels at different pubertal phases.
- There is no significant association between gender and mean serum Osteocalcin levels in different cervical maturational stages.
- The findings of the study suggest that serum Osteocalcin levels could be used as a biomarker to measure the skeletal maturity of an individual.

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