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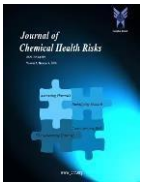

Correlating Salivary Alkaline Phosphatase Level with Cervical Vertebral Maturation, Dental Age and Chronological Age - A Cross-Sectional Study

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KEYWORDS

S-ALP, Cervical maturation, dental age, modified Demirjian method, Chronological age, Lateral cephalogram, OPG

ABSTRACT:

Introduction: Accurate prediction of the growth spurt is critical for modifying the skeletal malocclusion and in devising the treatment plan. Biochemical markers like saliva renders new non-invasive diagnostic tools for detection of skeletal and dental maturation.

Objectives: To analyse salivary alkaline phosphatase level and relating it with cervical vertebral maturation stages, dental age (modified Demirjian method) and chronological age during pubertal and post-pubertal stages.

Methods: 240 subjects aged between 10 to 18 years were grouped according to their growth phase, pubertal (CVMI 3 & CVMI 4) and post-pubertal (CVMI 5 & CVMI 6) using Bacceti's method from Lateral Cephalogram taken in natural head position in each gender. The S-ALP levels were detected using a commercially available AutoZyme Alkaline Phosphatase (Accurex Biomedical Pvt. Ltd., Mumbai, India). Acharya's Indian specific-formula for dental age estimation using Chaillet and Demirjian (2004) stages (0-9) of tooth formation was used to evaluate dental age.

Results: S-ALP activity was higher in pubertal phase than post-pubertal phase. An early dental maturation in girls as compared to boys in both phases except in CVMI 4 of pubertal phase suggesting a variation in dental maturation in the boys and girls. In addition to it, alkaline

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phosphatase activity is also more in girls as compared to boys. There were variation in the correlation between S-ALP, chronological age, and dental age.

Conclusions: The onset of pubertal & post-pubertal growth phase differs in both genders. S-ALP activity varies across different phases of cervical maturation, with higher levels observed during the pubertal phase compared to post-pubertal phases, which is intricately linked to sexual dimorphism during cervical maturation. Also, dental maturation assessed using Acharya's modified Demirjian method may not always correlate with chronological age.

1. Introduction

Skeletal maturity assessment plays a key role in orthodontic diagnosis and treatment planning. Determining the best time to alter the growth of the jaws is dependent on an accurate prediction of the growth spurt.[1] Due to differences in the timing, duration, and rate of growth, predicting an individual's growth potential is essential.[2] There are several growth markers that can be used to identify the active development phase, such as dental, physiological, skeletal, and chronological age. The diverse patterns of growth make chronological age unreliable. [1,3]

Cervical vertebral maturation staging, which is based on assessing the morphological changes of the six cervical vertebrae (C1–C6), was used to predict the growth stage on lateral cephalograms. [1]

However, variations in chronological ages, skeletal maturation & estimated dental ages have been found by meta-analyses, suggesting the need for population-specific standards to estimate the rate of dental maturation better also.[4] One of the most prevalent methods for estimating dental age is the Demirjian eight-stage system, which scores dental maturation based on objective criteria and relative values rather than absolute measurements. Acharya developed a formula that worked for the Indian population by utilizing Demirjian's method to assess dental age and was noticeably superior to that of the original formulas, supporting the idea that population-specific standards should be created.[5]

In contrast to the previous methods that involve radiation exposure, an alternative biochemical approach has been proposed, to accurately predict maturation stages as they represent substances directly involved in bone growth and remodelling. Originally identified in serum, these biomarkers are now detectable in saliva as well, as it is non-invasive and easier to collect, providing ample quantities for analysis without requiring specialized laboratory equipment.[3] There has been a reported connection between the growth spurt and biomarkers associated with bone metabolism.[2]

Alkaline phosphatase, a membrane-bound glycoprotein is significant for bone metabolism.[6] Serum ALP levels increased during infancy and puberty, according to Turan et al. [7] Increased levels of ALP have been reported in gingival crevicular fluid (GCF) during puberty by Perinetti et al. [8] Growing children showed an increase in salivary ALP levels, as reported by Travade et al. [2]

Therefore, this study is designed to correlate Salivary ALP levels, Cervical vertebral maturation, dental age, and chronological age so as to assess if salivary ALP levels can be used as a reliable maturity indicator.

2. Objectives

The aim of the study was to analyse salivary alkaline phosphatase level and relating it with cervical vertebral maturation stages, dental age (modified Demirjian method) and chronological age during pubertal and post-pubertal stages.

The objectives of the study were -

- To measure S-ALP level and correlate it with Cervical maturation stages – CVMI 3 & CVMI 4 (pubertal stage) and CVMI 5 & CVMI 6 (post-pubertal stage) and chronological age
- To assess dental maturation using Acharya's modified Demirjian method and correlate it with S- ALP level and CVMI stages
- To investigate gender differences for S-ALP level and compare it with dental maturation and CVMI stages.
- To correlate dental maturation with chronological age.

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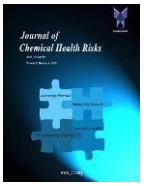


3. Methods

The present cross-sectional study is designed to investigate the correlation between salivary alkaline phosphatase level, chronological age and dental age in pubertal and post-pubertal growth phases.

In this study, subjects included were aged between 10 to 18 years who came to the department. The ethical clearance was obtained from the Institutional Ethics Committee. As all subjects were under 18 years of age, written informed consent was taken from legal guardian for collection of saliva, lateral cephalogram and OPG. Subjects with any systemic medical illness, congenital abnormalities, and under any medication affecting growth were excluded.

Total 240 subjects were grouped according to their growth phase using Bacceti's method [9] from Lateral Cephalogram, taken in natural head position.

The subjects were grouped as:

- **Group A**: Pubertal phase (A1 - CVMI 3 & A2 -CVMI 4)
- **Group B**: Post-pubertal phase (B1 -CVMI 5 & B2- CVMI 6)

Equal number of boys and girls were taken in each group.

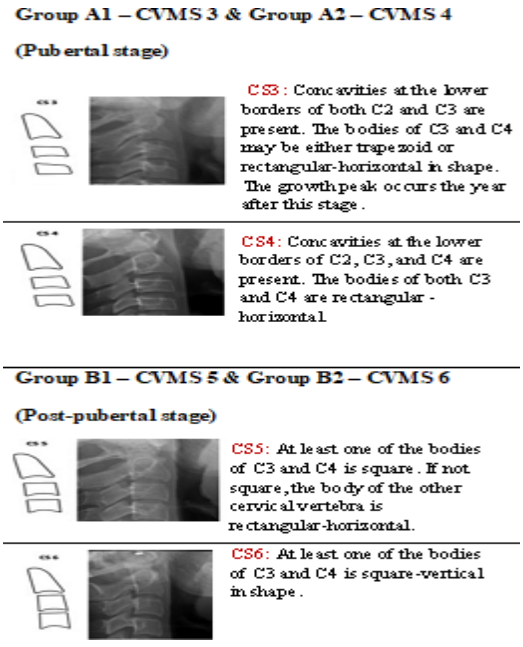
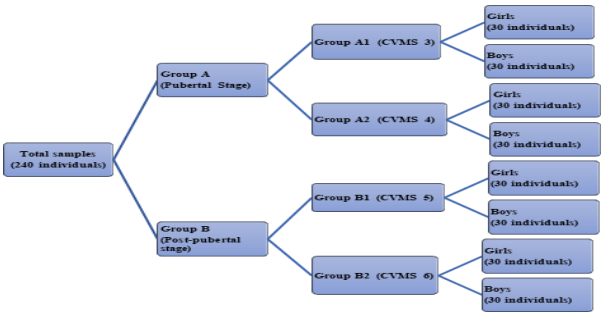
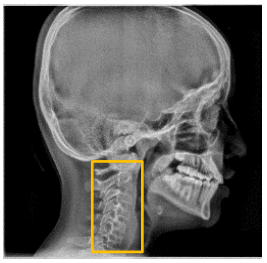


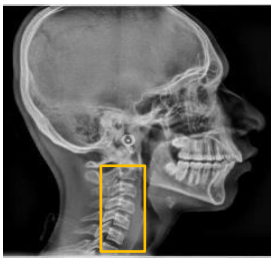
Table 1 : CVMI stages in Pubertal and Post-pubertal stage



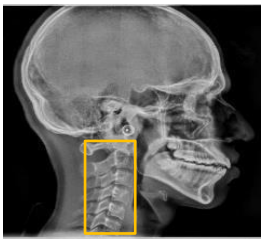
FLOWCHART SHOWING STUDY DESIGN



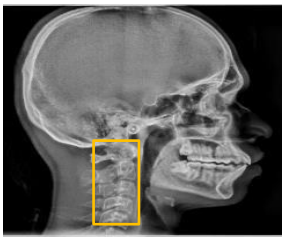
CVMI 3



CVMI 4



CVMI 5



CVMI 6

Fig 1 : Cervical vertebral maturation stages

The same subjects were instructed priorly to not brush, eat or drink 90 minutes before collection of saliva.

Biochemical Analysis of Salivary Samples for salivary alkaline phosphatase level

5 ml unstimulated whole saliva was collected by asking the subject to sit for 5 minutes in an upright position and allow to accumulate saliva in floor of the mouth, which was then transferred to a container of 5 ml by passive drooling method from the oral cavity and was labelled according to the subject's number.

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Fig 2 : Centrifuge and centrifuged samples

The salivary sample was taken to the Research Laboratory, Government Dental College & Hospital, Ahmedabad to detect salivary ALP levels.

The collected whole saliva sample was subjected to centrifuge at 3000 RPM for 10 minutes to separate the supernatant. Salivary alkaline phosphatase was then estimated using the necessary amount of separated salivary sample. The remaining sample was disposed of according to the applicable biomedical waste guidelines.

The S-ALP levels were detected using a commercially available AutoZyme Alkaline Phosphatase (Accurex Biomedical Pvt. Ltd., Mumbai, India) and the samples were assayed according to the kit's instructions.

It is a single reagent system, set for determination of ALP activity based on kinetic method using p-nitrophenyl phosphate (p-NPP).

Working solution was prepared by dissolving 1 tablet of substrate in 5 ml of diluent. 1 ml working solution was mixed with 20 µl saliva sample in Eppendorf tube just before running in autoanalyzer

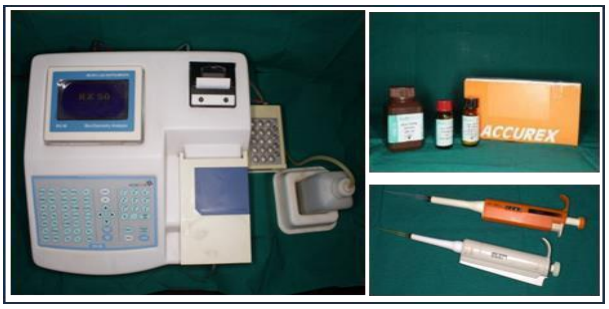

Fig 3 : Accurex AutoZyme Alkaline Phosphatase kit, Micropipettes and RX-50 Bio-chemical analyzer

The components and concentration of the working solution were:

- ✓ Diethanolamine buffer, pH 9.8 1 Mol/L
- ✓ p -nitrophenyl phosphate 10 mMol/L
- ✓ Magnesium chloride 0.5 mMol/L

When samples were added to working solution, the following reaction occurred:

p-nitrophenyl phosphate $\xrightarrow{\text{Alkaline phosphatase}}$ p-nitrophenol +phosphate+H2O

[ALP in a sample hydrolysed para-nitrophenyl phosphate into paranitrophenol and phosphate in the presence of magnesium ions. Under alkaline conditions, a colourless p-nitrophenol was converted to 4- nitrophenoxide, which develops a very intense yellow colour. The rate of increase in absorbance of the reaction mixture at 405 nm due to liberation of para-nitrophenol was proportional to the ALP activity in the sample. The kinetic analysis was performed using a RX-50 Bio-chemical analyzer.]

The following is the estimation process that was used:

- ➢ Before beginning the analysis, the reagents that had been stored were brought to room temperature.
- ➢ The calibration curve method

a) The instrument's automated biochemical analyzer's parameters were set in accordance with the manual's specification. The parameters set were

- Temperature at 37º Celsius.
- Standard concentration: 1000 microliters
- Volume of sample: 20 microliters
- Incubation time: 3 minutes

b) Distilled water was run through the automated biochemical analyzer to reset it to zero.

c) A micropipette was used to transfer 1000 µl of the alkaline phosphatase reagent into the test tube.

d) A 20 µl saliva sample was taken from the centrifuged saliva and combined with the reagent.

e) The sample and reagent were mixed well in a 20:1000 ratio before being aspirated into the analyzer.

f) The machine measured absorbance and automatically plotted the standard curve as soon as the reagent was added.

g) The test sample's salivary alkaline phosphatase concentration was noted.

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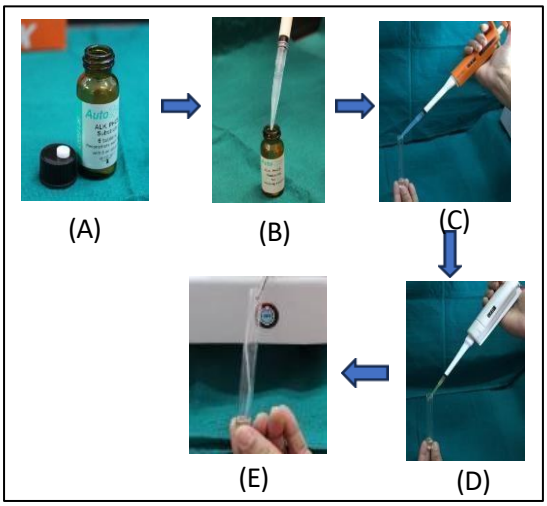

Fig 4 : (A) Substrate, (B) Preparation of working solution, (C) Transfering working solution in test-tube, (D) Addition of Centrifuged Saliva into reagent, (E) Aspiration of Sample-reagent mixture

Fig 5 : Standard curve for absorbance rate

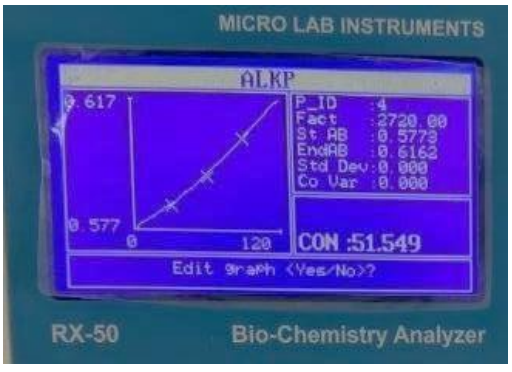

Fig 6 : S-ALP level displayed on RX-50 Bio-chemical analyzer.

A standardized temperature of 37 ° Celsius was used to obtain the S-ALP levels in IU/l in the auto-analyzer, but the fluctuation in temperature range of sample needs to be considered as per the manufacturer's temperature conversion table.

Temperature Conversion

Temperature of assay	Temperature factors		
	25°C	30°C	37°C
25°C	1.0	1.30	1.80
30°C	0.75	1.0	1.35
37°C	0.55	0.74	1.0

Dental age of the same subjects was evaluated, after taking Orthopantomogram (OPG). Stages of tooth formation was assigned to each of the 8 teeth on the mandibular left side using ***Chaillet and Demirjian (2004)***[10] stages (0-9) of tooth formation.

The stages were assigned as,

Stage 0 – No calcification of tooth.

Stage 1 - Visible bony crypt, but no sign of tooth germ.

Stage 2 - Beginning of calcification at superior. Level of crypt without fusion of Calcification points

Stage 3 - Fusion of the calcified points forms one or several cusps, which unite to give a regularly outlined occlusal outline.

Stage 4 –

(a) Enamel formation is complete at the occlusal surface. Its extension and convergence towards the cervical region are seen.

(b) The beginning of dentinal deposit is seen.

(c) The outline of the pulp chamber has a curved shape at the occlusal border.

Stage 5 –

(a) Crown formation is complete to the level of the cementoenamel junction. Root formation has commenced.

(b) The pulp horns are beginning to differentiate, but the walls of the pulp chamber remain curved.

(c) Beginning of root formation in spicule for

Stage 6 –

(a) The root length remains shorter than the crown height.

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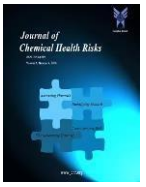


(b) In uniradicular teeth, walls of the pulp chamber are straight, and the pulp horns are more differentiated than in the stage

(c) In multiradicular teeth, initial formation of root border in form of Calcification points or semi-lunar shape

Stage 7 –

In Uniradicular teeth

(a) The walls of the pulp chamber now form a more or less isosceles triangle. The apex ends in a funnel shape.

(b) The root length is equal to or greater than the crown height.

In Multiradicular teeth,

(a) The calcified region of the bifurcation has developed further down from its semilunar stage to give roots a more definitive and distinct outline, with funnel shaped endings.

(b) The root length is equal to or greater than the crown length.

Stage 8 –

(a) The walls of the root canal are now parallel.

(b) The apical ends of the root canals are still partially open.

Stage 9 -

(a) The apical end of the root canal is completely closed.

(b) The periodontal membrane has a uniform width around the root and apex.

- Each developmental stage may have one, two or three criteria. The following rules have to be followed for selecting a particular stage for the given tooth.
- In border line cases, the earlier stage is always considered.
- If only one criterion is given, this must be observed on the radiograph in order to select the stage.
- If two criteria are given, the first one must be observed on the radiograph in order to select the stage.
- If three criteria are given, the first two must be observed on the radiograph in order to select the stage.

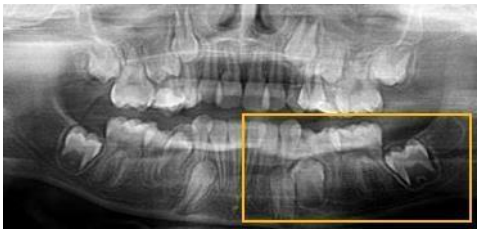
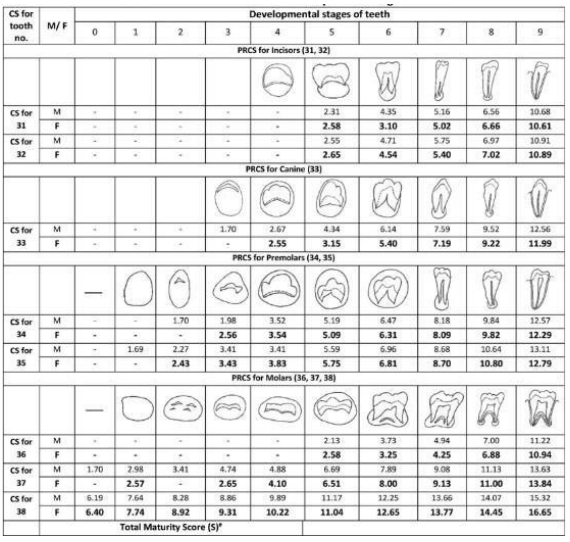


Fig 7 : Eight permanent teeth on left side (31-38) on OPG for dental age estimation.

Corresponding to the selected developmental stage, each tooth is given a numerical score (scoring table). Eight numerical scores are obtained (one score for each tooth)

There is a pictorial representation of the developmental stages & its score for each tooth & gender. (Priyanka Kapoor 2018).[11]



CS for tooth no.	M/ F	Developmental stages of teeth									
		0	1	2	3	4	5	6	7	8	9
PRCS for Incisors (31, 32)											
CS for 31	M	-	-	-	-	-	2.31	4.35	5.16	6.56	10.68
	F	-	-	-	-	-	**2.58**	**3.10**	**5.02**	**6.66**	**10.61**
CS for 32	M	-	-	-	-	-	2.55	4.71	5.75	6.97	10.91
	F	-	-	-	-	-	**2.65**	**4.54**	**5.40**	**7.02**	**10.89**
PRCS for Canine (33)											
CS for 33	M	-	-	-	1.70	2.67	4.34	6.14	7.59	9.52	12.56
	F	-	-	-	-	**2.55**	**3.15**	**5.40**	**7.19**	**9.22**	**11.99**
PRCS for Premolars (34, 35)											
CS for 34	M	-	-	1.70	1.98	3.52	5.19	6.47	8.18	9.84	12.57
	F	-	-	-	**2.56**	**3.54**	**5.09**	**6.31**	**8.09**	**9.82**	**12.29**
CS for 35	M	-	1.69	2.27	3.41	3.41	5.59	6.96	8.68	10.64	13.11
	F	-	-	**2.43**	**3.43**	**3.83**	**5.75**	**6.81**	**8.70**	**10.80**	**12.79**
PRCS for Molars (36, 37, 38)											
CS for 36	M	-	-	-	-	-	2.13	3.73	4.94	7.00	11.22
	F	-	-	-	-	-	**2.58**	**3.25**	**4.25**	**6.88**	**10.94**
CS for 37	M	1.70	2.98	3.41	4.74	4.88	6.69	7.89	9.08	11.13	13.63
	F	-	**2.57**	-	**2.65**	**4.10**	**6.51**	**8.00**	**9.13**	**11.00**	**13.84**
CS for 38	M	6.19	7.64	8.28	8.86	9.89	11.17	12.25	13.66	14.07	15.32
	F	**6.40**	**7.74**	**8.92**	**9.31**	**10.22**	**11.04**	**12.65**	**13.77**	**14.45**	**16.65**
Total Maturity Score (S)#											

- Cs(31 + 32 + 33 + 34 + 35 + 36 + 37 + 38)

Tooth numbering system : federation dentaire internationale

Cs – corresponding score, m – males, f – females, prcs – pictorial representation of calcification

Fig 8 : Score determination based on developmental stage of tooth

The Acharya's Indian specific-formula for dental age estimation using modified Demirjian's method is as follow,

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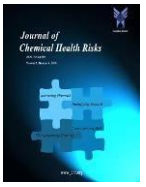

For males:

Age = 27.4351- (0.0097 x S^2) + (0.000089 x S^3)

For females:

Age = 23.7288 - (0.0088 x S^2) + (0.000085 X S^3)

The data obtained from all the parameters were then subjected to statistical analysis.

4. Results

Data was analyzed using the statistical package **SPSS 22.0** (SPSS Inc., Chicago, IL) and level of significance was set at ***p<0.05***. **Descriptive statistics** was performed to assess the mean and standard deviation of the respective groups. Normality of the data was assessed using **Shapiro Wilk test**. **Inferential statistics** to find out the difference between and within the groups was done using **Mann Whitney U TEST (Table 1,2, & 3). One way ANOVA and Post-hoc test (Table 4,5, & 6), Independent t-test (Table 7) and Correlation analysis was done using spearman rho rank correlation (Table 8).**

TABLE 2 – S-ALP (IU/L) IN DIFFERENT CVMI STAGES

		BOYS	GIRLS
CVMI 3	MEAN	50.11	55.72
	MEDIAN	51.5	52.35
	SD	4.22	8.74
	IQR(Q1-Q3)	50.45-51.85	48.4-67.8
	P-VALUE(Mann Whitney U test)	0.03*	
CVMI 4	MEAN	55.85	58.05
	MEDIAN	55.6	57.1
	SD	0.92	5.14
	IQR(Q1-Q3)	54.3-57.7	54.42-58.3
	P-VALUE(Mann Whitney U test)	0.04*	
CVMI 5	MEAN	54.37	57.75
	MEDIAN	52.65	56.3
	SD	4.77	8.89
	IQR(Q1-Q3)	52.18-54.6	52.1-62.5
	P-VALUE(Mann Whitney U test)	0.04*	
CVMI 6	MEAN	48.13	48.19
	MEDIAN	48.05	48.35
	SD	1.74	0.97
	IQR(Q1-Q3)	47.73-48.58	47.5-48.98
	P-VALUE(Mann Whitney U test)	0.77	

*$p<0.05$ IS STATISTICALLY SIGNIFICANT (Mann Whitney U test, $p<0.05$)

TABLE 3 – DENTAL AGE IN DIFFERENT CVMI STAGES

		BOYS	GIRLS
CVMI 3	MEAN	10.89	11.61
	MEDIAN	10.65	11.55
	SD	0.65	1.21
	IQR(Q1-Q3)	10.34-11.32	10.68-11.82
	P-VALUE(Mann Whitney U test)	0.01*	
CVMI 4	MEAN	15.36	13.54
	MEDIAN	15.35	13.8
	SD	0.58	0.91
	IQR(Q1-Q3)	15.2-15.6	13.04-14.17
	P-VALUE(Mann Whitney U test)	0.0001*	
CVMI 5	MEAN	17.14	17.79
	MEDIAN	16.8	17.82
	SD	0.69	0.81
	IQR(Q1-Q3)	16.7-17.35	17.27-18.5
	P-VALUE(Mann Whitney U test)	0.001*	
CVMI 6	MEAN	18.44	18.54
	MEDIAN	18.45	18.5
	SD	0.38	0.44
	IQR(Q1-Q3)	18.23-18.59	18.3-18.88
	P-VALUE(Mann Whitney U test)	0.35	

*$p<0.05$ IS STATISTICALLY SIGNIFICANT (Mann Whitney U test, $p<0.05$)

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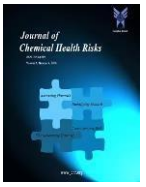

TABLE 4 – CHRONOLOGICAL AGE IN DIFFERENT CVMI STAGES

		BOYS	GIRLS
CVMI 3	MEAN	12.16	11.82
	MEDIAN	12.25	11.85
	SD	0.78	1.32
	IQR(Q1-Q3)	11.67-12.62	10.27-13.1
	P-VALUE(Mann Whitney U test)	0.65	
CVMI 4	MEAN	14.45	14.75
	MEDIAN	14.5	14.4
	SD	0.22	0.68
	IQR(Q1-Q3)	14.3-14.6	14.2-15.1
	P-VALUE(Mann Whitney U test)	0.56	
CVMI 5	MEAN	17.33	17.13
	MEDIAN	17.4	17.4
	SD	0.54	0.78
	IQR(Q1-Q3)	17.02-17.8	16.75-17.68
	P-VALUE(Mann Whitney U test)	0.25	
CVMI 6	MEAN	18.34	18.31
	MEDIAN	18.4	18.45
	SD	0.32	0.49
	IQR(Q1-Q3)	18.2-18.6	18.02-18.7
	P-VALUE(Mann Whitney U test)	0.79	

*$p<0.05$ IS STATISTICALLY SIGNIFICANT(Mann Whitney U test, $p<0.05$)

TABLE 5 – COMPARISON OF S-ALP (IU/L) WITHIN CVMI STAGES

		BOYS	GIRLS
CVMI3		50.11±4.22	55.72±8.74
CVMI4		55.85±0.92	58.05±5.14
CVMI5		54.37±4.77	57.75±8.89
CVMI6		48.13±1.74	48.19±0.97
P-VALUE		0.0001*	0.0001*
POST HOC TEST	CVMI 3 vs CVMI 4	0.0001*	0.54
	CVMI 3 vs CVMI 5	0.0001*	0.65
	CVMI 3 vs CVMI 6	0.10	0.0002*
	CVMI 4 vs CVMI 5	0.0001*	0.99
	CVMI 4 vs CVMI 6	0.0001*	0.0001*
	CVMI 5 vs CVMI 6	0.0001*	0.0001*

*$p<0.05$ IS STATISTICALLY SIGNIFICANT (One way ANOVA test and Post hoc test)

TABLE 6 – COMPARISON OF DENTAL AGE WITHIN CVMI STAGES

		BOYS	GIRLS
CVMI3		10.89±0.65	11.61±1.21
CVMI4		15.36±0.58	13.54±0.91
CVMI5		17.14±0.69	17.79±0.81
CVMI6		18.44±0.38	18.54±0.44
P-VALUE		0.0001*	0.0001*
POST HOC TEST	CVMI 3 vs CVMI 4	0.0001*	0.0001*
	CVMI 3 vs CVMI 5	0.0001*	0.0001*
	CVMI 3 vs CVMI 6	0.0001*	0.0001*
	CVMI 4 vs CVMI 5	0.0001*	0.0001*
	CVMI 4 vs CVMI 6	0.0001*	0.0001*
	CVMI 5 vs CVMI 6	0.0001*	0.0001*

*$p<0.05$ IS STATISTICALLY SIGNIFICANT (One way ANOVA test and Post hoc test)

TABLE 7 – COMPARISON OF CHRONOLOGICAL AGE WITHIN CVMI STAGES

	BOYS	GIRLS
CVMI3	12.16±0.78	11.82±1.32
CVMI4	14.45±0.22	14.75±0.68
CVMI5	17.33±0.54	17.13±0.78

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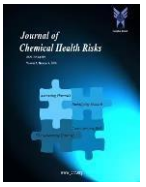

CVMI6		**18.34±0.32**	**18.31±0.49**
P-VALUE		**0.0001***	**0.0001***
POST HOC TEST	**CVMI 3 vs CVMI 4**	**0.0001***	**0.0001***
	CVMI 3 vs CVMI 5	**0.0001***	**0.0001***
	CVMI 3 vs CVMI 6	**0.0001***	**0.0001***
	CVMI 4 vs CVMI 5	**0.0001***	**0.0001***
	CVMI 4 vs CVMI 6	**0.0001***	**0.0001***
	CVMI 5 vs CVMI 6	**0.0001***	**0.0001***

**p<0.05* IS STATISTICALLY SIGNIFICANT (One way ANOVA test and Post hoc test)

TABLE 8 – GENDER DIFFERENCE IN S-ALP (IU/L), DENTAL AGE [DA] & CHRONOLOGICAL AGE [CA] WITH DIFFERENT CERVICAL STAGES

		BOYS	GIRLS	P-VALUE
CVMI 3	**S-ALP**	**50.11±0.65**	**55.72±0.72**	**0.03***
	DA	**10.89±0.65**	**11.61±1.21**	**0.01***
	CA	**12.16±0.78**	**11.82±1.32**	**0.65**
CVMI 4	**S-ALP**	**55.85±0.58**	**58.05±0.81**	**0.04***
	DA	**15.36±0.58**	**13.54±0.91**	**0.0001***
	CA	**14.45±0.22**	**14.75±0.68**	**0.56**
CVMI 5	**S-ALP**	**54.37±0.69**	**57.75±0.91**	**0.04***
	DA	**17.14±0.69**	**17.79±0.81**	**0.0001***
	CA	**17.33±0.54**	**17.13±0.78**	**0.27**
CVMI 6	**S-ALP**	**48.13±0.38**	**48.19±0.41**	**0.77**
	DA	**18.44±0.38**	**18.54±0.44**	**0.35**
	CA	**18.34±0.32**	**18.31±0.49**	**0.59**

**p<0.05* IS STATISTICALLY SIGNIFICANT (Independent t-test,*p<0.05*)

TABLE 9 - CORRELATION OF CA-DA, S-ALP-DA, S-ALP- CA IN DIFFERENT CVMI STAGES

		BOYS	GIRLS	OVERALL
CVMI 3	**CA–DA**	**0.55 (P=0.0001*)**	**0.47 (P=0.008*)**	**0.50 (P=0.001*)**
	S-ALP – DA	**-0.03 (P=0.86)**	**0.19 (P=0.31)**	**0.11 (P=0.31)**
	S-ALP – CA	**0.28 (P=0.12)**	**-0.55 (P=0.001*)**	**-0.32 (P=0.01*)**
CVMI 4	**CA–DA**	**-0.13 (P=0.33)**	**0.31 (P=0.08)**	**0.21 (P=0.15)**
	S-ALP – DA	**-0.28 (P=0.13)**	**0.14 (P=0.43)**	**0.11 (P=0.36)**
	S-ALP – CA	**-0.13 (P=0.46)**	**0.26 (P=0.15)**	**0.23 (P=0.07)**
CVMI 5	**CA – DA**	**0.38 (P=0.002*)**	**0.43 (P=0.01*)**	**0.39 (P=0.01*)**
	S-ALP – DA	**0.65 (P=0.0001*)**	**0.03 (P=0.87)**	**0.24 (P=0.06)**
	S-ALP – CA	**0.04 (P=0.81)**	**0.30 (P=0.10)**	**0.22 (P=0.08)**
CVMI 6	**CA – DA**	**0.16 (P=0.19)**	**-0.01 (P=0.94)**	**0.10 (P=0.78)**
	S-ALP – DA	**-0.25 (P=0.17)**	**-0.05 (P=0.76)**	**-0.15 (P=0.23)**
	S-ALP – CA	**0.01 (P=0.95)**	**-0.43 (P=0.01*)**	**-0.22 (P=0.08)**

5. Discussion

It is an orthodontist's primary duty to assess growth status and residual growth to successfully treat malocclusions

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with skeletal etiology, recording the likely course, size, and potentially even the timing of growth is crucial.[6]

Numerous approaches for cervical vertebral maturation (CVM) have been studied.

The stage of tooth eruption or formation can be used to determine dental maturity. Dental maturation is a complex sequence of events from initial mineralization of a tooth, crown formation, root growth, eruption of tooth into the mouth, and root apex maturation.[12] But tooth eruption is unpredictable due to a variety of factors that can affect it, including available space, ankylosis, and the timing of primary teeth's exfoliation.

Biomarkers offer a novel avenue for predicting maturation stages without the need for invasive X-ray radiation. A biomarker refers to any substance, structure, process, or its products that can be measured in the body.[13] Total serum ALP activity has historically been used for assessing osteoblastic activity. Peak ALP levels are found during childhood's rapidly growing stages, like infancy and puberty.[7] Saliva also contains biomarkers such as alkaline phosphatase (ALP), growth hormone (GH), insulin-like growth factor 1 (IGF-1), and creatinine.[1]

ALP is an enzyme involved in the metabolism of bone.[2] One of these isoenzymes is specifically linked to bone growth. This particular isoenzyme plays a role in hydrolyzing inorganic pyrophosphate, which, in turn, influences the function of osteoblasts and the mineralization of bone. Salivary levels of bone-specific ALP can vary gender-wise, during growth spurts in the process of bone remodelling.

In the present cross-sectional study, S-ALP levels in pubertal and post-pubertal growth phases and its correlation with skeletal maturation by evaluating cervical vertebral maturation stages, dental age and chronological age were assessed.

Mann Whitney U test depicts gender differences for S-ALP in different CVMI stages with significant difference which was more in girls in all CVMI stages except CVMI 6. (Table 1)

The results of this study strengthened previous findings reporting higher alkaline phosphatase activity in the pubertal phase (CVM 3 & CVM 4) as compared to post-pubertal phases (CVM 5 & CVM 6) and it was also noted higher in girls than boys as per cervical maturation. A decrease in ALP levels was found with completion of cervical maturation in CVMI 6.

In a study performed by Fadhlina [3] in 2017 observed highest ALP levels at the pubertal phase (CVMI 3 and 4). Nora Alhazmia [1] in 2019 also reported a significant difference of S-ALP in various CVMS between boys and girls. Tarvade SM et al. [2] in 2015 also showed higher levels of salivary ALP during the growth spurt in their study.

On comparing dental age in different CVMI stages between boys and girls (Table 2) using Mann Whitney U test shows a statistically significant difference between boys and girls in Pubertal phase at CVMI 3 with advanced dental maturation in girls than boys at 10.8 ± 0.6 years whereas highly significant difference (p=0.0001) with dental age of 13.5 ± 0.9 years in girls having delayed dental maturation than boys at 15.3 ± 0.5 years was found in CVMI 4 of pubertal phase.

A significant gender difference with advanced dental maturation was found in girls of post-pubertal phase with dental age of 17.7 ± 0.8 years and 17.1 ± 0.6 years in boys at CVMI 5, whereas it was non-significant in CVMI 6 with dental age of 18.5 ± 0.4 years and 18.4 ± 0.3 years in girls & boys respectively.

Sushil Kumar et al. [14] in 2012 in his comparative study between dental maturation (using original Demirjian method) & CVMI concluded dental maturation to be more advanced in male as compared with female in relation to CVMI stages. George et al. [15] reported dental age in males to be more advanced than that in females in respect to CVMI stages. *In the present study, modified Demirjian method was used for the dental maturation assessment which revealed a variation showing advanced dental maturation in girls in respect to all cervical maturation except CVMI 4.*

Regarding chronological age, Mann Whitney U test did not show statistically significant difference between girls and boys with respect to all CVMI stages (p>0.05). (Table 3)

One-way ANOVA test showed statistically significant difference (p<0.05) on comparing S-ALP within CVMI stages with respect to boys and girls.

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On comparing S-ALP levels between cervical maturation stages of pubertal and post-pubertal phase, a highly significant difference was found between all CVMI stages except between CVMI 3 & CVMI 6 in boys whereas in girls a highly significant (p=0.0002) difference was found between CVMI 3 & CVMI 6 and a highly significant difference (p=0.0001) between CVMI 4 & CVMI 6. (Table 4)

On comparing Dental age within CVMI stages with respect to boys & girls by One-way ANOVA test, showed statistically highly significant difference (p<0.05) in dental maturation with an increase in the dental age in boys as well as girls of both pubertal and post-pubertal phase. (Table 5) *which revealed a simultaneous advancement in the dental and cervical maturation.*

Sheron et al. [16] in their study reported significant difference between the skeletal & dental age in girls & boys. Nilima Thosar [17] in 2020 also observed significant difference.

Comparison of chronological age within CVMI stages by One-way ANOVA test (Table 6) showed statistically highly significant difference (p=0.0001) in boys as well as girls. Post hoc test also reported highly significant (p=0.0001) difference between all CVMI stages of pubertal and post-pubertal phase showing an increase in the cervical maturation with the chronological age.

The independent t-test (Table 7) reported a statistically significant (p=0.03) difference in Pubertal phase with S-ALP between boys and girls, with 50.11 ± 0.65 IU & 55.72 ± 0.72 IU (CVMI 3), 55.85 ± 0.58 IU & 58.05 ± 0.81 IU (CVMI 4) (p=0.04) in boys and girls respectively with girls having higher S-ALP levels suggesting higher enzymatic activity. A significant difference (P=0.04) was also found in CVMI 5 of the post-pubertal phase, with high levels of S-ALP in girls with S-ALP levels 54.37±0.69 & in boys was 57.75±0.91. No significant difference was found in the alkaline phosphatase activity between boys and girls of CVMI 6.

At the threshold of pubertal growth spurt non-significant difference in S-ALP levels between males and females in stage 3 and stage 4 as reported by Snigdha et al. [18]. Tulika et al. [19] reported statistically significant difference in BALP levels between boys & girls was observed at CVMI stages 1 (p<.05), CVMI 2 (p< 0.01), CVMI 5 (p<0.001) and CVMI 6 (p<0.01).

Pubertal phase showed a significant (p=0.01) difference in dental age between girls and boys, with advanced dental maturation in girls with dental age at 11.6 ± 1.2 years than boys at 10.8 ± 0.6 years at CVMI 3 where as a highly significant (p=0.0001) difference was found at CVMI 4 with advanced dental maturation with dental age at 15.3 ± 0.5 years in boys than in girls at 13.5 ± 0.9 years. CVMI 5 of post-pubertal phase showed a highly significant difference (p=0.0001) with advanced dental age in girls at 17.7 ± 0.8 years than boys at 17.1 ± 0.6 years. No significant difference was found in CVMI 6.

According to Sushil Kumar [14] in 2012, with respect to CVMI stages DI in male subjects was more advanced than in female subjects. George et al. [15] also reported dental age in males to be more advanced than in females. Sharon et al. [16] showed an early maturation in Gujarati girls than boys when assessed between dental & skeletal age.

Independent t-test was also performed regarding chronological age, in which no significant difference was found between girls and boys at cervical maturation stages of pubertal and post-pubertal phase.

The present study revealed an early dental maturation in girls as compared to boys in pubertal and postpubertal phase except in CVMI 4 of pubertal phase suggesting a variation in dental maturation in the boys and girls. In addition to it, alkaline phosphatase activity is also more in girls as compared to boys.

Spearman Rho correlation (Table 8) for S-ALP reported significant negative correlation with CA in CVMI 3 girls. A non-significant positive correlation of S-ALP was also found with DA in girls of Pubertal phase at CVMI 3 & CVMI 4. A non-significant negative correlation of S-ALP with DA was found in boys at CVMI 3 & CVMI 4 whereas a non-significant positive correlation of S-ALP with CA was found at CVMI 3 but a negative correlation in CVMI 4. A highly significant positive correlation (p=0.0001) was found for S-ALP with DA among boys in CVMI 5 whereas a negative non-significant correlation in CVMI 6.

A non-significant positive correlation was found for S-ALP with CA among boys of CVMI 5 as well as CVMI

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6 and also in girls of CVMI 5 whereas, a significant negative correlation was found in girls of CVMI 6

An overall Correlation for S-ALP with Chronological age showed a significant negative correlation in CVMI 3 where as a non-significant positive correlation in CVMI 4 as well as CVMI 5. CVMI 6 had a non-significant negative correlation.

A non-significant positive overall correlation was found for S-ALP with DA in CVMI 3, CVMI 4 and CVMI 5 where as CVMI 6 showed a non-significant negative correlation. (Table 9)

Snigdha et al. [18] found moderate correlation between S-ALP & Chronological age. Nora Alhazmi et al. [1] reported both ALP activity & age to be significant in predicting CVMI stages. Harryanto Wijaya [20] reported a significant positive association between chronological age and puberty phase.

A non-significant positive correlation of S-ALP and dental age among girls of pubertal phase (CVMI 3 & 4) with the S-ALP levels at 55.72 ± 8.74 IU/L and 58.05 ± 5.14 IU/L and dental age at 11.6 ± 1.2 and 13.5 ± 0.9 years respectively & non-significant negative correlation among boys with S-ALP levels at 50.11 ± 4.2 IU/L & 55.85 ± 0.92 IU/L and dental age at 10.8 ± 0.6 & 15.3 ± 0.5 years respectively.

Post-pubertal phase showed a significant positive correlation among boys in CVMI 5(P=0.0001) showing a direct relation between the dental age and S-ALP with the dental age at 17.1 ± 0.6 years and S-ALP levels were 54.37 ± 4.77 IU/L where as non-significant positive correlation was seen among girls with S-ALP level 57.75 ± 8.89 IU/L at dental age 17.7 ± 0.8 years. A negative correlation was seen in CVMI 6, between S-ALP & Dental age, 48.13 ± 1.74 IU/L & 18.44 ± 0.38 (boys) and 48.19 ± 0.97 IU/L & 18.54 ± 0.44 (girls) respectively (p>0.05).

Sindy et al [21] reported a significant negative correlation of S-ALP with dental age.

However, in the present study there were variation in the correlation between S-ALP and dental age.

Correlation of S-ALP and chronological age reported a significant negative correlation in girls of CVMI 3 in the Pubertal phase with the S-ALP levels at 55.72 ± 8.74 IU/L at chronological age 11.82 ± 1.32 and in boys non-significant positive correlation with S-ALP levels at 50.11 ± 4.22 IU/L at chronological age 12.16 ± 0.78 suggesting high levels of S-ALP in girls of CVMI 3 whereas, in CVMI 4 a non-significant positive correlation was found in girls but negative in boys.

In Post-pubertal, S-ALP was non-significant but positively correlated to chronological age in CVMI 5 with S-ALP levels at 54.37 ± 4.7 IU/L at chronological age of 17.33 ± 0.54 years in boys and 57.75 ± 8.89 IU/L chronological age of 17.13 ± 0.78 years in girls where as in CVMI 6, girls showed a significant negative correlation between S-ALP and chronological age with the S-ALP levels at 48.19 ± 0.41 IU/L at chronological age of 18.31 ± 0.49 years but a non-significant positive correlation was seen among boys with S-ALP at 48.13 ± 1.74 IU/L at chronological age of 18.34 ± 0.32 years.

In total number of subjects, CVMI 3 showed a significant negative correlation of S-ALP and chronological age where as a negative but non-significant correlation was seen in CVMI 6. A non-significant positive correlation was seen in CVMI 4 & CVMI 5.

In a study done by Sindy et al. [21] in 2021 between chronological age & S-ALP, moderate negative correlation was observed. Whereas, Nora Alhazmi [1] reported a strong positive association between chronological age and CVMS. Wijaya et al. [20] reported a significant positive association of chronological age with the puberty phase suggesting that chronological age can contribute to the salivary BALP in predicting the phase of puberty.

In the present cross-sectional study, S-ALP varies with different maturity indicators, as physiological variations like – age, hormones, genetics, etc. may be the contributing factors.

Correlation analysis between chronological age vs dental age reported significant positive correlation regarding CVMI3 & CVMI 5 (p<0.5).

In pubertal phase, cervical maturation at CVMI 3 and CVMI 4, a variation was found between chronological and dental age in boys and girls. Cervical maturation stages CVMI 5 and CVMI 6 of post-pubertal phase showed non-significant variation in boys and girls.

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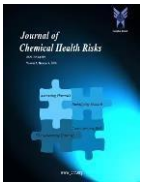

Sindy et al. [23] in 2021 also reported a strong positive correlation between chronological age and dental age. (p<0.001). Skeletal maturity increased together with the increase of chronological and dental ages. (George et al. [15]), Vignesh R et al. [22] also reported a significant correlation in both males and females. Sharon et al. [16] in their study reported moderate (0.3 < r < 0.7) correlation between assessed skeletal and dental age. In a study performed by Hegde et al. [23] a significant positive correlation between chronological and dental age was found (r = 0.985) (p< 0.001) for females. Asma Sookhania et al. [24] reported a strong positive correlation between chronological age and CVMI stages. Safavi et al.[25] reported a low correlation between CVMI stages and chronological age.

6. Conclusion:

- The onset of the pubertal and post-pubertal phases differs between boys and girls. These findings underscore the importance of considering gender-specific patterns in cervical vertebral maturation assessments for accurate developmental evaluations.
- The study demonstrates that alkaline phosphatase (ALP) activity varies across different phases of cervical maturation, with higher levels observed during the pubertal phase compared to post-pubertal phases, with a notable decrease in ALP levels in CVMI 6.

These findings suggest that ALP activity is intricately linked to sexual dimorphism during cervical maturation. S-ALP may not serve as a useful marker for monitoring skeletal development.

- The complex relationship between dental and chronological ages, suggests that dental maturation assessed using Acharya's modified Demirjian method may not always correlate with chronological age. And also, the gender differences in dental maturation across different stages of the CVMI.

Based on the validation results, the data sets a landmark for future longitudinal studies with more ethnical and socioeconomically diverse & larger sample size to investigate the potential use of salivary ALP activity as biomarkers for skeletal maturity and to get standardized values at different stages of skeletal development with less variation.

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