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Evaluation of reliability of salivary alkaline phosphatase in comparison to other methods of assessing the skeletal maturation

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ABSTRACT

Introduction: Evaluation of skeletal maturation is crucial to growth determination which in turn influences orthodontic treatment planning. Various methods employed for assessment are chronological age, hand wrist maturation, cervical vertebral maturation, and dental calcification. Recently, salivary Bone specific alkaline phosphatase (BALP) have been introduced due to its role in the bone mineralization process.
Aims and Objectives: To test the alternate hypothesis that salivary alkaline phosphatase is a reliable indicator of skeletal maturity in comparison with other methods of assessment of skeletal maturation.
Materials and Methods : Total 112 subjects were selected out of 150 subjects on the basis of inclusion and exclusion criteria, for participation in the study. For each subject, personal details including chronological age was noted. Height and weight measurements were done for calculation of body mass index (BMI). Further, Lateral Cephalogram and Orthopantomogram were obtained for assessment of cervical vertebrae maturation stages and Demirjian index. Then, an unstimulated salivary sample was collected for evaluation of alkaline phosphatase activity by colorimetric method. The data obtained was analyzed using SPSS software.
Results: The results of the present study showed that the highest correlation for skeletal maturation assessment was found with alkaline phosphatase activity.
Conclusion: The hypothesis is accepted. Salivary Alkaline Phosphatase proved to be reliable biomarker for assessment of skeletal maturation.

1. Introduction

The determination of growth status of an individual holds great significance in clinical orthodontics. It not only helps in diagnosis but also decides the optimal time of intervention for certain malocclusion as well as predicts the outcome of the orthodontic treatment.

The chronological age of the patient is easily known from the diagnostic database of the patient but that does not necessarily correlate with the morphological and skeletal maturity status of an individual. Various other methods are available for skeletal maturity assessment from conventional radiographs to recently available methods like analysis of biological mediators in saliva representing the bone metabolism that takes place during the period of growth spurts.[1] These biomarkers have advantage over radiographic methods of being reliable, reproducible, accessible and inexpensive. Utilising saliva as diagnostic fluid over GCF and serum make the collection non-invasive and simplifies storage and manipulation. Hence, skeletal maturity assessment using these biomarkers is gaining more popularity now-a-days. Certain examples of such

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biochemical molecules are Alkaline Phosphatase, Insulin-like growth factor-1, Dehydroepiandosterone Sulphate (DHEAS).[2]

Bone specific Alkaline Phosphatase is one such biomarker which is highly specific & sensitive to bone metabolism which is taking place during the accelerated growth period of an individual.[3]

Hence, the aims & objectives of our study were to test the alternate hypothesis that salivary alkaline phosphatase is a reliable indicator for assessment of skeletal maturation.

2. Materials and Methods

The study was conducted in Department of Orthodontics and Dentofacial Orthopaedics, Department of Microbiology and Department of Biochemistry. Informed consent was taken from the patient or their parents/guardian. Ethical approval for the study was taken from Institutional Research Ethical Committee (vide no. HDC/ethical /ortho/2020/14) Out of 150 patients who visited the department of Orthodontics seeking orthodontic treatment, 112 subjects were selected for participation in the study. The gender wise distribution for the subjects used as participants in the study shown in Table 1. The subjects were selected based on the following inclusion criteria: 1) Age group between 7 to 17 years. 2) Patients with good oral hygiene having plaque or gingival index ≤25% and probing depth less than 4mm. 3) Patients with good general health assessed with patient history and records. The exclusion criteria were as follow: 1) Use of anti-inflammatory agents/antibiotics in the month preceding sample collection.2) History of congenital or developmental disturbances. 3) Previous history of trauma or injury to the face as well as oral habits. 4) History of any oral habit. 5) Radiographs with supernumerary or missing teeth (except third molars). 6) Radiographs of low quality and with artefacts. 6) Subjects with history of/currently undergoing orthodontic treatment.

2.1. Methods

For each patient, personal chronological age was noted in months. The height and weight data were recorded by making the patient stand upright looking straight ahead on a digital weighting scale without taking any support. At the same time, the free running horizontal headboard of stadiometer was adjusted to touch the top of the head of the patient and the measurement was noted on the rigid vertical board and weighing scale.[4]

The formula used for calculation of BMI was as follows:

BMI (kg/m^2) = Weight (kilograms) / (Height(meters))^2

Then the saliva was collected from each subject. They were asked to refrain from smoking, eating, drinking or tooth-brushing for 1-2 hours. The sample collection was done between same time period for all patients, from 9:00 am to 12:00 pm to eliminate circadian changes. Prior to sample collection, patients were made to rinse mouth thoroughly to prevent debris contamination. Spitting method for collection of unstimulated saliva was employed. The patient was made to sit upright with the head slightly tilted forward and the eyes open. Saliva was allowed to accumulate in the floor of the mouth and the subject spitted it out into the test tube every 60 seconds until 2 ml of quantity of saliva was obtained.[5] Collected saliva samples were taken immediately to laboratory and centrifuged at 3000 rpm for 15 minutes to separate debris and supernatant. In another test tube, 20 μl of the supernatant obtained was mixed with 1000μl of working reagent from Erba Mannheim Alkaline Phosphatase Assay Kit. The yellow-coloured solution obtained was aspirated into a cuvette which was then placed in the cuvette holder of the colorimeter. The colorimeter had already been calibrated with distilled water at a wavelength of 405 nm before placing the test solution. After initial delay of 60 seconds, change in absorbance for the test solution was calculated at the same wavelength. The rate of change in absorbance at 405nm was proportional to the activity of ALP.[6](Figure 1)

Activity of ALP at 37°C (Units/Litre) = $\frac{(\Delta A405 / min) \, x \, T.V. \, X \, 103}{S.V. \, X \, Absorptivity \, x \, P}$

where,

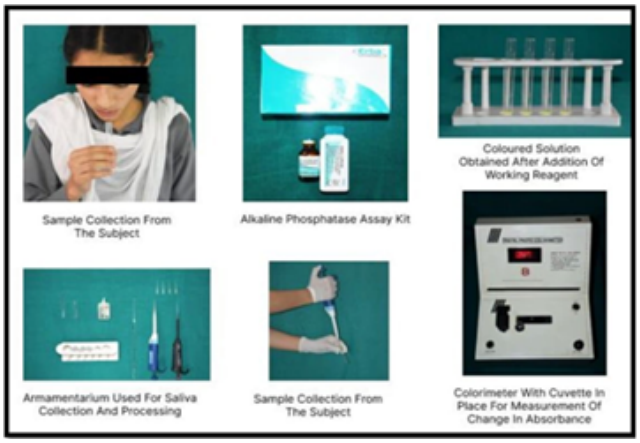


Figure 1: Salivary collection and processing

T.V. = Total Reaction Volume in μl

S.V.= Sample Volume in μl

Absorptivity = millimolar absorptivity of p-nitrophenyl phosphate at 405 nm = 18.8

P = cuvette lightpath = 1 cm

Activity of ALP at 37°C (Units/Litre) = (ΔA_{405} / min) x Factor (2713)

Further radiographs were obtained and traced for assessment. The technical specifications for Lateral

Table 1: Gender wise distribution of the subjects participated in the study

Skeletal Age	Total	Males	females	N(%)	Males(%)	Females(%)
CS1	11	9	2	9.8	8	1.8
CS2	16	12	4	14.3	10.8	3.5
CS3	17	14	3	15.2	12.5	2.7
CS4	36	11	25	32.1	9.8	22.3
CS5	20	8	12	17.9	7.2	10.7
CS6	12	4	8	10.7	3.5	7.2
Total	112	58	54	100	51.8	48.2

Table 2: The lateral cephalogram and panoramic radiograph specifications (PLanmeca X-Ray Machine)

Generator	**Constant potential, microprocessor controlled operating frequency 80 Hz**
X-ray	D-052 SB
Focal spot size	0.5×0.5mm (According to IEC 336)
Total Filtration	2.5mm ALk
Anode Voltage	68KVp
Anode current	12mA DC
Exposure time	0.6 sec and 18 sec
Film Size	8” x 10”/ 6” x 12”
Cassette	Flat
System identifier	163-170cm / 64”-67”
Magnification	1.08-1.13
Line Voltage	100/117/220-230/270V;50-60Hz
Regulation	Automatic, ± 10%
Color	White (RAL 9016)

Table 3: Descriptive statistics of chronological age (years), body mass index (KG/M^2) Alkaline phosphatase activity (units/litre) and dental ages (Years) in all the cervical vertebrae maturation stages

Cervical stages	Chronological age (years)	Body mass index (kg/m2)	Alkaline phosphatase activity (units/litre)	Dental age (years)
CS1	11.90±1.19	15.20±1.98	193.20±35.20	10.70±0.95
CS2	12.40±0.88	17.30±2.02	197.31±34.51	11.50±0.79
CS3	13.30±1.23	18.45±2.12	311.20±28.96	12.55±0.69
CS4	14.20±1.02	18.72±2.88	315.57±33.03	13.44±1.05
CS5	15.20±1.09	20.65±2.62	108.52±36.29	14.78±1.01
CS6	16.17±0.78	20.95±3.65	89.53±55.82	15.47±0.55

Table 4: Spearman correlation of chronological age(years), body mass index (kg/m^2), alkaline phosphatase activity (units/litre), dental age (YEARS) and cervical vertebrae maturation stages.

Predictors	Parameters	Correlation coefficient (r)	p Value
Alkaline phosphatase activity	CVMI	0.920**	0.000**
	Dental Age	0.763**	0.000**
	Chronological Age	0.304**	0.001**
	Body Mass Index	0.064	.504
Cvmi stage	Alkaline Phosphatase Activity	0.920**	0.000**
	Dental Age	0.838**	0.000**
	Chronological Age	0.763**	0.000**
	Body Mass Index	0.470**	0.000**
Dental age	Alkaline Phosphatase Activity	0.763**	0.000**
	CVMI stage	0.838**	0.000**
	Chronological Age	0.551**	0.000**
	Body Mass Index	0.316**	0.001**
Chronological age	Alkaline Phosphatase Activity	0.304**	0.001**
	CVMI stage	0.763**	0.000**
	Dental Age	0.551**	0.000**
	Body Mass Index	0.278**	0.003**

$p<0.05$ * statistically significant; p<0.01** statistically highly significant

Table 5: Multinominal logistic regression analysis forcervical vertebrae maturation stage 1

CVMI Stage	Predictor	Standard Error	z-Score	p Value
2 – 1	Age (Years)	0.90	0.44	0.66
	BMI(Kg/m2)	0.36	2.20	0.90
	ALP (Units/Litre)	0.03	-1.32	0.19
	Dental age (Years)	1.36	0.08	0.94
3 – 1	Age (Years)	0.44	0.17	0.87
	BMI(Kg/m2)	1.74	0.01	0.10
	ALP (Units/Litre)	0.14	2.94	<.001**
	Dental age (Years)	0.68	6.28	< .001**
4-1	Age (Years)	0.43	-1.07	0.28
	BMI(Kg/m2)	1.74	-0.24	0.81
	ALP (Units/Litre)	0.14	3.02	<.001**
	Dental age (Years)	0.68	9.24	< .001**
5-1	Age (Years)	0.54	-147.99	< .001**
	BMI(Kg/m2)	0.10	-162.43	< .001**
	ALP (Units/Litre)	0.01	-382.48	< .001**
	Dental age (Years)	0.63	222.17	< .001**
6-1	Age (Years)	0.54	-144.79	< .001**
	BMI(Kg/m2)	0.10	-159.83	< .001**
	ALP (Units/Litre)	0.01	-383.58	< .001**
	Dental age (Years)	0.63	221.21	< .001**

<0.05 * statistically significant; p<0.01** statistically highly significant

Table 6: Multinominal logistic regression analysis for cervical vertebrae maturation stage 2

CVMI Stage	Predictor	Standard Error	z-Score	p Value
3 – 2	Age (Years)	1.18	-2.41	0.06
	BMI(Kg/m2)	1.29	-3.23	.001**
	ALP (Units/Litre)	0.11	5.35	< .001**
	Dental age (Years)	0.92	18.86	< .001**
4 – 2	Age (Years)	1.13	-2.01	0 .10
	BMI(Kg/m2)	1.30	-3.54	< .001**
	ALP (Units/Litre)	0.11	5.46	< .001**
	Dental age (Years)	0.99	19.51	< .001**
5 – 2	Age (Years)	0.54	-77.68	< .001**
	BMI(Kg/m2)	0.10	137.03	< .001**
	ALP (Units/Litre)	0.12	-22.51	< .001**
	Dental age (Years)	0.64	99.72	< .001**
6 – 2	Age (Years)	0.54	-74.46	< .001**
	BMI(Kg/m2)	0.10	139.68	< .001**
	ALP (Units/Litre)	0.12	-22.51	< .001**
	Dental age (Years)	0.64	98.74	< .001**

p<0.05 * statistically significant; p<0.01** statistically highly significant

Cephalogram and orthopantomogram machine have been specified in Table 2. The 3 parts of cervical vertebrae were traced on Lateral Cephalogram according to Baccetti, Franchi and McNamara (2005). The profile changes of second, third and fourth cervical vertebrae were assessed and assigned the cervical vertebrae maturation stages. (Figure 2)[7]

In the panoramic radiographs, central incisors to second molars were traced in the left mandibular quadrant according to Demirjian A, Goldstein H and Tanner JM (1973).(Figure 2). Eight stages of calcification (A-H) for each tooth described by a written criteria accompanied by photographic illustrations and schematic drawings. Each stage for the seven teeth was allocated a biologically weighed score and the sum of scores provided an estimate of the subject's dental maturity measured on a scale from 0 – 100. The overall maturity score was then converted to dental age by using available tables and/or percentile curves.[8]

For error assessment in the study, lateral cephalogram tracings were evaluated twice by the same examiner with an interval of one week difference. Assessment of intra-examiner reliability was done using Kappa statistics which

Table 7: Multinominal logistic regression analysis for cervical vertebrae maturation stage 3

CVMI Stage	Predictor	Standard Error	z-Score	p Value
4 – 3	Age (Years)	0.80	-0.59	0.55
	BMI (Kg/m2)	0.23	-1.89	0.06
	ALP (Units/Litre)	0.02	0.73	0.46
	Dental age (Years)	1.08	1.84	0.07
5 – 3	Age (Years)	4.36	-2.60	0.01*
	BMI (Kg/m2)	1.01	13.28	< .001**
	ALP (Units/Litre)	0.48	-4.38	< .001**
	Dental age (Years)	3.35	7.18	< .001**
6 – 3	Age (Years)	4.36	-2.21	0.03*
	BMI (Kg/m2)	1.01	13.49	< .001**
	ALP (Units/Litre)	0.48	-4.39	< .001**
	Dental age (Years)	3.34	7.00	< .001**

<0.05 * statistically significant; p<0.01** statistically highly significant

Table 8: Multinominal logistic regression analysis for cervical vertebrae maturation stage 4

CVMI Stage	Predictor	Standard Error	z-Score	p Value
5 – 4	Age (Years)	2.44	-5.39	< .001**
	BMI(Kg/m2)	0.64	17.86	< .001**
	ALP (Units/Litre)	0.25	7.64	< .001**
	Dental age (Years)	1.97	13.89	< .001**
6 – 4	Age (Years)	2.44	-4.68	< .001**
	BMI(Kg/m2)	0.64	18.21	< .001**
	ALP (Units/Litre)	0.25	-7.67	< .001**
	Dental age (Years)	1.97	13.58	< .001**

<0.05 * statistically significant; p<0.01** statistically highly significant

Table 9: Multinominal logistic regression analysis for cervical vertebrae maturation stage 5

CVMI Stage	Predictor	Standard Error	z-Score	p Value
6 – 5	Age (Years)	1.07	1.56	0.12
	BMI(Kg/m2)	0.20	1.25	0.20
	ALP (Units/Litre)	0.01	-0.58	0.56
	Dental age (Years)	1.26	-0.52	0.61

<0.05 * statistically significant; p<0.01** statistically highly significant

Table 10: Malesto centre aligned between mean and SD Females to centre aligned between mean and SD Overall to centre aligned between mean and SD

Cervical	Males		Females		Overall	
Stage	Mean	SD	Mean	SD	Mean	SD
CS1	189.91	27.13	230.60	57.55	197.31	34.51
CS2	192.09	37.5	196.69	25.98	193.20	35.20
CS3	308.13	31.22	325.56	0.00	311.20	28.96
CS4	335.42	32.72	307.48	30.10	315.57	33.03
CS5	101.74	49.71	113.04	25.43	108.52	36.29
CS6	27.130	0.00	105.13	51.50	89.530	55.82

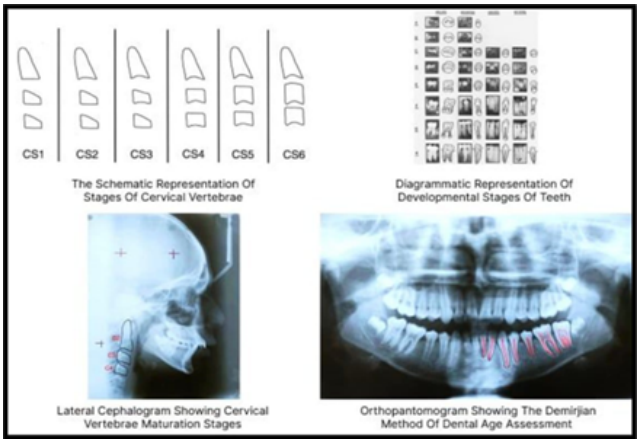
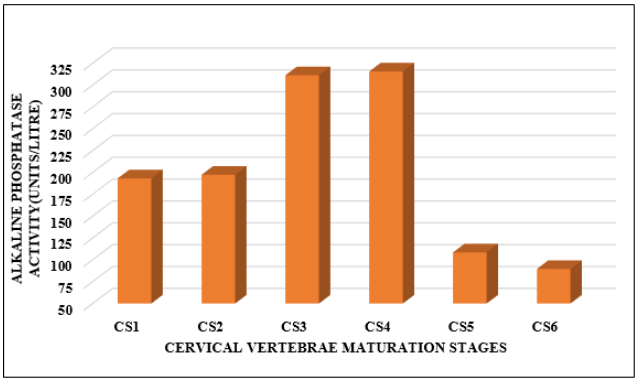


Figure 2: Radiographic assessment



Graph 1: Bargraph representingthe mean alkaline phosphatase level (Units/litre) among thecervical vertebrae maturation stages

showed perfect agreement (Kappa = 0.80-1.00, p<0.001).

The data was statistically analyzed using SPSS (Statistical package for social sciences for Window) software (version 21). The relation of salivary alkaline phosphatase in comparison to other methods was done using Spearman Correlation test. The level of significance was set at p<0.05.

3. Results

The increased alkaline phosphatase activity from pre-pubertal cervical stages (CS1 and CS2) to circum-pubertal cervical stages (CS3 and CS4) and further fall thereof in post-pubertal cervical stages (CS5 and CS6) was shown in Table III and. The highest correlation of cervical maturation was found with the alkaline phosphatase activity, followed by Demirjian Index and chronological age was shown in Table 4. The multinominal logistic regression analysis for cervical vertebrae maturation of cervical stages with salivary alkaline phosphate levels and other methods of skeletal maturation assessment was shown in Tables 5, 6, 7, 8 and 9. The salivary alkaline phosphate levels according to the cervical stages 1-6 in males and females was shown in Table 10.

4. Discussion

The most reliable predictors of skeletal maturation are considered to be radiographic maturity indicators as they aid in predicting the timing of pubertal growth, estimating growth velocity as well as the amount of growth remaining. But the associated radiation exposure and subjective outcome are its limitations. Lately, biomarkers like salivary alkaline phosphatase (ALP), has been employed. Alkaline phosphatase levels are expected to peak during puberty and decline post-puberty, implying that it can be explored as an indicator of skeletal growth.[3] Hence, the present study was done to evaluate the reliability of salivary alkaline phosphatase as a skeletal maturity indicator and compare it with other methods for assessment of skeletal maturation.

The results of the present study showed that the levels of alkaline phosphatase peaked at cervical stage 4 and declined thereafter as shown in Table 3 and Graph 1. This might be because high bone metabolism occurred during rapid growth phases of infancy and puberty leading to a marked increase in salivary alkaline phosphatase activity and further decline in activity of alkaline phosphatase levels during the cervical stages 5 and 6 might be attributed to deceleration in skeletal maturation. These results were in accordance with the study done by Travade S M et al. (2015).[9]

In addition, when correlation was done between alkaline phosphatase activity and other methods of assessment of skeletal maturation, it was found that dental age, cervical vertebrae maturation stages and chronological age were significantly correlated (p<0.05) as shown in Table 4. This might be because progression of skeletal maturity towards puberty led to increased bone metabolism and osteoblastic activity which resulted in increased activity of salivary alkaline phosphatase. This was in accordance with the study done by Sookhakian A et al. (2022).[10]

Furthermore, the statistically significant correlation between dental age and alkaline phosphatase activity was due to maturation of calcification stages of teeth (canine, premolar, molar) which showed marked alkaline phosphatase activity owing to increased bone metabolism during this phase. These results were in accordance with the study done by Nelwan SC et al. (2021).[11] There was also a statistically significant correlation found between chronological age and alkaline phosphatase activity which might be because, increased chronological age was associated with increased levels of alkaline phosphatase up to puberty. Whereas, in post pubertal age, the levels of alkaline phosphatase declined. These results were in accordance with the study done by Irham F et al. (2017)[12] and Alhazmi N et al. (2019).[13] Moreover, the correlation of alkaline phosphatase activity with body mass index was

found to be statistically insignificant. (p=0.504)

Further, the results of the present study showed that when the multinominal logistic regression analysis for the CVMI stages with ALP and other methods of assessment of skeletal maturation was done; the statistically significant results of ALP with cervical stage 1, 2, 3 and 4 were found ($p<0.05$) as shown in Tables 5, 6, 7, 8 and 9. The statistically significant difference in levels of alkaline phosphatase in cervical stage 3 and 4 might be because, as an individual reached puberty, bone turnover increased. This increased the activity of alkaline phosphatase, which was required to create a pH suitable for hydroxyapatite crystal formation in the bone matrix. Whereas, in cervical stage 5 and 6 subjects were near the completion of maturation, thus there was retarded bone metabolism and resultant decreased alkaline phosphatase activity. These results were in accordance with the study done by Sonwane S and Bhad W et al. (2022)[3] and Khade DM et al (2023)[14] who reported that salivary biomarkers can be used as an adjunct for growth prediction along with the other methods of skeletal maturation assessment.

Moreover, the results of the present study showed that the salivary alkaline phosphatase level (U/L) peaked in cervical stage 4 (335.42±32.72) in males and cervical stage 3 (325.56±0.00) in females as shown in Table 10.

Though the results showed salivary alkaline phosphatase as an acceptable biomarker but there were some limitations of the present study, such as differences in evaluation methods of skeletal maturity, discrepancies in the number, age, and racial background of the studied subjects which was conditioned by ethnic origin, climate, nutrition and socioeconomic status, and industrialization.

Thus, Alkaline Phosphatase is a reliable predictor by itself as well as together with other predictors which aid in estimation of skeletal maturation. Therefore, the identification of alkaline phosphatase levels can allow the orthodontist to properly determine the treatment plan as well as identify the proper timing for its implementation in order to perform orthopaedic functional treatment and initiate orthognathic surgery.

5. Conclusion

1. The salivary alkaline phosphatase, a non-invasive biomarker proved to be reliable for assessment of skeletal maturation.
2. The salivary alkaline phosphatase was found to be the most reliable among all other methods for assessment of skeletal maturation followed by dental age, chronological age and body mass index.

- Ethical approval for the study was taken from Institutional Research Ethical Committee. (vide no. HDC/ethical /ortho/2020/14)
- Informed consent was taken from the patient or their parents/guardian.

6. Source of Funding

None.

7. Conflict of Interest

None.

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