ORIGINAL RESEARCH

Salivary Total Protein and Alkaline Phosphatase Activity as Biomarkers for Skeletal Maturity and Growth Prediction in Healthy Children: An *In Vivo* Study

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Abstract

Introduction: Skeletal maturity assessment involves radiographic analysis and visual inspection of developing bone and their initial appearance or sequential ossification and related changes in size and shape along with the expression of various biomarkers in body fluids.

Aim: To investigate the correlation of biomarkers such as salivary alkaline phosphatase (S-ALP) and salivary total protein (STP) with skeletal maturity assessment and growth prediction in growing children.

Materials and methods: A total of 8–15-year-old 150 healthy children were divided into five groups depending upon radiographic stage maturity of the middle phalanx of the left hand's third finger according to the Hagg and Taranger method. Radiographs were taken using intraoral periapical (IOPA) radiographic films.

Results: Salivary alkaline phosphatase (S-ALP) activity in the MP3 G group was significantly higher than MP3 F group and MP3 I group. Total protein levels in MP3 F were significantly lower than in MP3 G. The mean value of S-ALP (33541.45 IU/L) and that of STP (2.77 mg/mL) was observed to be highest in the MP3 G group (G3) group.

Conclusion: Salivary total protein (STP) and S-ALP may be used as an additional diagnostic tool to assess skeletal maturation and optimize growth prediction during myofunctional orthodontic treatment.

Clinical significance: Skeletal maturity assessment plays a significant role in orthodontic diagnosis, treatment planning, and stability of orthodontic treatment. Radiographic parameters involve radiographic exposure; hence in this study noninvasive biomarkers such as S-ALP and STP have been evaluated for skeletal maturity assessment and growth prediction.

Keywords: Growth prediction, Middle phalanx of third finger radiographic stages, Pubertal growth spurt, Salivary alkaline phosphatase, Salivary total protein.

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Introduction

Skeletal maturity assessment and pubertal growth spurt play a significant role in growth modification orthodontic therapy.[1] Skeletal age has been used to determine the amount of remaining facial growth that impacts the decisions of orthodontic treatment onset and optimal orthodontic treatment.[2] In addition, this can also be used for making an informed decision of extraction vs nonextraction cases, maxillary expansion, modifying the growth of jawbones, determining the outcome of some orthognathic surgeries, and optimal timing for dental implant therapy in young growing patients.

Various craniofacial growth prediction methods have been suggested in the literature such as craniometry, anthropometry, cephalometry, vital staining, implant markers, natural markers, comparative anatomy, physiological age, dental maturation, lateral cephalometric analysis, and hand and wrist radiographic examination.[3] Hagg and Taranger explained that pubertal growth spurts are followed by the stages of ossifications of the middle phalanx of the third finger.[4] The MP3 stages represent the different stages of a pubertal growth spurt and are divided into five stages as follows. Stage1: MP3 F (epiphysis is as wide as metaphysis and it denotes the onset of a pubertal growth spurt), stage 2: MP3 FG (epiphysis is as wide as metaphysis and there is a distinct and/or lateral border of the epiphysis forming a line of demarcation at the right angle to the lateral border), stage 3: MP3 G (sides of the epiphysis has thickened and also capped its metaphysis forming a sharp edge distally at one or both), stage 4: MP3 H (fusion of epiphysis and metaphysis has begun. It is the deceleration period of a pubertal growth spurt), and stage 5: MP3 I (complete fusion of epiphysis and metaphysis; it marks the end of a pubertal growth spurt). Although there are disadvantages of radiographic techniques like repeated radiation exposure and long observation periods. Therefore, noninvasive parameters such as biomarkers are now used to assess skeletal maturity.[5] Studies have reported a rise in biomarkers of bone metabolism during a

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pubertal growth spurt.[6] These biomarkers have been detected in serum as well as saliva, that is insulin-like growth factor 1, growth hormon, creatinine, and ALP.[7]

A bone-specific ALP is associated with bone growth which hydrolyses inorganic pyrophosphate, which in turn affects osteoblast function and bone mineralization.[8] Like the serum, saliva is also a commonly used body fluid for diagnostic procedures. The salivary collection procedure is much easier, noninvasive, painless, and possible to collect multiple times. Changes in serum ALP levels are also reflected in saliva. Hence, the present study design investigates the correlation of salivary alkaline phosphatase (S-ALP) and salivary total protein (STP) levels with skeletal maturity assessment and a pubertal growth spurt.

Materials and Methods

This study includes 80 girls and 70 boys between the age of 8 and 15 years. The Institutional Ethical Committee approved the study protocol (No. IEC GDCH/PED.2/2021). As all subjects were under 18 years of age, written consent was taken from a legal guardian before the collection of saliva and recording MP3 radiograph. After the subject's agreement to participate in this study, demographic data, and medical history were obtained. Intraoral examination was done by the principal investigator using a mouth mirror and dental explorer under proper illumination.

Inclusion Criteria

- Healthy children of age between 8 and 15 years who either reported for a dental checkup or dental treatment (dmft/DMFT ≤ 4).
- Children exhibiting Frankel's behavior rating[9] grade 3–4.

Exclusion Criteria

- Children with systemic medical illness or on any medication that might affect growth and/or bone metabolism.
- Children with acute intraoral infection.
- Children whose parents were not willing to give their consent.

Methods

Subjects who were eligible to participate were scheduled for a subsequent visit. They were instructed regarding brushing and eating before sample collection to ensure high quality of salivary sample collection for analysis. During the second visit, 1–5 mL of unstimulated whole saliva was collected by a 2 mL syringe by suction method from the floor of the mouth. The salivary collection procedure was carried out between 09:00 am and 12:00 pm. Saliva was stored in a container labeled with subject ID (serial number) in a refrigerator at 2–8°C. Then, the samples were transported using a cold storage box to the laboratory of biochemistry, Indian Council of Medical Research–National Institute of Occupational Health (ICMR–NIOH), Ahmedabad, Gujarat, India for S-ALP and STP assay.

During the same visit, an X-ray was taken of the middle phalanx of the third finger of the left hand using intraoral periapical radiographic films.[7] The subject was asked to sit on a dental chair with hands putting on the hand-rest of the dental chair. Intraoral periapical (IOPA) film was placed below the third finger with an occlusal dot of film placed towards the distal side finger. IOPA was taken using an X-ray machine (Intraskan DC, Skanray Technologies Ltd, India) at settings of 70 kV, 8 mA, and 500 milliseconds. All MP3 radiographs were traced using an acetate sheet according to stages given by Hagg and Taranger.[10] Maturation of the middle phalanx of the third finger was evaluated by a single investigator to distribute the subjects into the following five groups according to the Hagg and Taranger method.

- Group I: Children with radiographic MP3 F stage.
- Group II: Children with radiographic MP3 FG stage.
- Group III: Children with radiographic MP3 G stage.
- Group IV: Children with radiographic MP3 H stage.
- Group V: Children with radiographic MP3 I stage.

Salivary alkaline phosphatase (S-ALP) assay and total protein assay were done at the biochemistry laboratory of ICMR–NIOH, Ahmedabad, Gujarat, India. S-ALP levels were determined by using a commercially available autozyme diagnostic kit by kinetic method (Accurex Biomedical Pvt Ltd, Mumbai, India) and the samples were assayed according to the kit's instructions.

Protein analysis was performed using Bio-Rad (Bradford) protein assay for all the subject's samples. Values of S-ALP and total protein concentration were entered in a Microsoft Excel worksheet (Office 2019) and all data was sent for statistical analysis.

Results

The subjective error was calculated using the Dahlberg formula by repeating tracing and was not statistically significant. Values of S-ALP and STP were statistically analyzed using OpenEpi software version 3.01. Results are presented as mean ± standard deviation (SD) (X ± SD).

Table 1 depicts the chronological age distribution within study groups. Mean S-ALP and STP in 8–15-year-old boys and girls were calculated. By applying the unpaired *t*-test, the mean difference between S-ALP of boys and girls is statistically not significant with a *p*-value of 0.77. By applying the unpaired *t*-test, the mean difference between the STP of boys and girls is statistically not significant with a *p*-value of 0.87.

Mean values of S-ALP concerning MP3 maturation stages are presented in Figure 1. On applying the one-way analysis of variance (ANOVA) F-test statistics come out to be 243.687 and *p*-value 0 showing a significant association between MP3 stages and S-ALP levels. The maximum mean S-ALP is shown in the MP3 G group with

Table 1: Age distribution (years) of the study sample

Study groups	*MP3 stages*	*Mean age*	*SD*	*Median*	*IQR*
Group I	MP3 F	8.43	0.68	8	1
Group II	MP3 FG	9.07	0.83	9	0
Group III	MP3 G	11.67	0.99	12	1
Group IV	MP3 H	13.83	1.05	14	0
Group V	MP3 I	14.5	0.51	14.5	0.5

Table 2: Mean S-ALP (IU/L) among MP3 stages

MP3 maturation stages	*Mean S-ALP (IU/L)*	*SD*	*p-value*
MP3 F	19,390	3821.81	0.001[a]
MP3 FG	29,439	3313.47	
MP3 G	43,772	5122.03	
MP3 H	26,917	2695.13	
MP3 I	16,373	2550.34	

One-way ANOVA test; [a], statistically significant

Table 3: Mean STP among MP3 stages

MP3 stage	*Mean STP (mg/mL)*	*SD*	*p-value*
MP3 F (G1)	1.62	0.48	0.001[a]
MP3 FG (G2)	2.06	0.66	
MP3 G (G3)	3.10	0.49	
MP3 H (G4)	2.58	0.54	
MP3 I (G5)	1.86	0.63	

One-way ANOVA test; [a],statistically significant

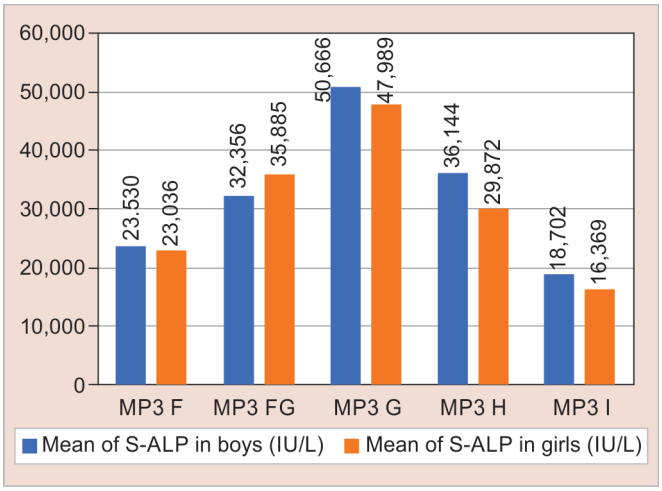

Fig. 1: Mean S-ALP (IU/L) in boys and girls

a maximum SD (Table 2). The mean ALP level increases from stage F (19390 IU/L) to stage G (43772 IU/L) and then decreases from stage G to stage I (16373 IU/L).

The mean value of S-ALP concerning MP3 maturation stages in boys and girls is highest in the MP3 G group (Fig. 1). Results of the one-way ANOVA test show a significant association between S-ALP levels and MP3 stages among boys and girls with a *p*-value of 0.001.

Table 3 shows mean values of total protein concentration related to MP3 maturation stages. On applying the one-way ANOVA test in Table 3, the F statistic comes out to be 33.36, with 0 *p*-value which shows a significant association between MP3 stages and total protein concentration levels.

Discussion

In the present study, the correlation of S-ALP and STP levels with skeletal age has been investigated. As they are associated with bone metabolism, their values may help in identifying the growth phase in children. In this study, salivary ALP instead of gingival crevicular fluid (GCF)—ALP and serum ALP was used, as it is much easier to collect saliva and is a far less invasive method as compared to other body fluids.[11] The suction method was used for the collection of unstimulated whole saliva as it is feasible.[12] The saliva of all subjects was collected between 09:00 am and 12:00 pm, as during this time saliva flow rate is at its peak, due to the circadian rhythm of the human body.[13] Subjects with dmft/DMFT 4 or less were taken in the study as levels of S-ALP activity were not related to the concentrations of salivary calcium and phosphate.[14]

The age range of 8–15 years with a mean age of 11.5 years ± 2.58 was taken in the present study as pubertal growth spurt is seen at the age of 8–13 years and 9–14 years in girls and boys respectively.[15] Unlike studies that used hand-wrist radiograph and cervical vertebral maturation (CVM) indicators, MP3 stages of the Hagg and Taranger method was used in this study to detect skeletal maturation, as the most standardized method for skeletal assessment is hand-wrist radiographs.[4] The radiograph of MP3 was taken using IOPA film due to its simplicity, reliability, and low patient radiation exposure.[16] STP was measured by Bio-Rad (Bradford) protein assay kit, as it is rapid and sensitive.[17] The salivary alkaline enzyme assay was done using an alkaline phosphatase assay kit (Accurex Biomedical Pvt Ltd, Mumbai, India) and the samples were assayed according to the kit's instructions as it is readily available, accurate, and easy to perform.[18]

The present data demonstrated STP among the MP3 stages with a statistically significant difference between MP3 FG and MP3 G (Table 3) and the highest level of protein in saliva was noted at the MP3 G stage. Cabras et al.[19] have reported elevated levels of proline-rich proteins in the whole saliva during the age of adolescence, which is in concordance with this study. The study showed a range of the STP between 1.47 mg/mL and 3.01 mg/mL. Perinetti et al.[7] reported total protein concentration in GCF, they found total protein concentration between 0.2 mg/mL and 2.4 mg/mL. Alhazmi et al.[20] measured total protein concentration along with salivary ALP activity and gave total protein concentration in a range of 0.79 mg/mL–2.45 mg/mL related to CVMs. Results of this study show a linear increase in STP with age until the MP3 G stage. This is in accordance with earlier studies by Nagler and Hershkovich, Deshpande et al., and Vibhakar et al.[21–24] A similar result was also found in a study by Kalipatnapu et al.[25] showing that protein content increase until middle age and remains constant in adults, further it decreases with advancing age. The present study also shows that children aged 11–12 years have more STP values than children aged 8–9 years because of developmental differences in the salivary gland.[26]

The present study showed no significant difference in salivary protein concentrations between girls and boys. This is similar to the results by Sivakumar et al.[27] and Vibhakar et al.[24] but in contrast with results by Dodds et al.[28] who stated that significant sex differences in salivary protein concentrations exist. This study shows the positive association between skeletal maturation and salivary protein concentration during the pubertal growth phase. Based on the findings of the present study, STP was found to be statistically significant in predicting pubertal growth spurt.

Table 2 depicts that there is a relationship between S-ALP levels in saliva and pubertal growth phases with the mean difference in each group significant at the *p*-value. The highest S-ALP levels were seen at the MP3 G stage (43772 IU/L), whereas the lowest S-ALP levels were seen at the MP3 I stage (16373 IU/L). The results of this study were analogous to a study conducted by Tarvade et al.,[29] which showed a correlation between S-ALP levels to maturation stages of the middle phalanx of the third finger (MP3). The findings of the present study were also similar to the study by Perinetti et al.,[7]

which showed dynamic levels of alkaline phosphatase levels in GCF in the maxilla during a pubertal growth spurt. The highest ALP levels occurred at the pubertal stage (78.8 IU/L), then the prepubertal stage (48.9 IU/L) and postpubertal stage (21.9 IU/L). According to their study, this difference in the levels of S-ALP is associated with changes in serum ALP levels due to the dynamic rate of bone mineralization during the growth spurt which increases at around 9 years of age and decreases after 12 years of age. A study was done by Irham et al.[30] also found the highest ALP levels at 10–11 years. Since ALP is a marker for osteoblastic activity, growing children have higher levels than fully-grown individuals.[31]

Alhazmi et al.[20] also reported change in S-ALP with growth stages but he has shown high S-ALP activity during the prepubertal growth stage while high S-ALP during the peak of the pubertal stage has been found in the present study. In addition, Tobiume et al.[32] and Christenson[31] stated that serum bone ALP activity peak occurred during infancy and puberty indicating high bone metabolism during these periods which is consistent with the present study.

This study can help clinicians to identify the pubertal growth spurt which is a critical phase for determining the optimal timing for orthodontic treatment by using noninvasive methods. The study also suggests that the combined use of S-ALP activity, STP, and MP3 stages can allow clinicians to make better predictions of the skeletal growth spurt. However, further studies require with greater sample size to draw a definite conclusion and baseline values.

Conclusion

Salivary alkaline phosphatase (S-ALP) activity and STP were lower at the early pubertal stage (MP3 F and MP3 FG), which rose at the MP3 G stage with a statistically significant difference and again declined in MP3 H and MP3 I. S-ALP activity was higher in males compared to females but it is not statistically significant. S-ALP activity and STP may be a promising, noninvasive diagnostic aid for the prediction of the pubertal growth phase.

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