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Gingival crevicular fluid phosphatase activity as a non-invasive biomarker of skeletal maturation

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Structured Abstract

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Objectives – To evaluate the gingival crevicular fluid (GCF) alkaline phosphatase (ALP) activity in growing subjects in relation to the stages of individual skeletal maturation.

Setting and Sample Population – The Department of Biomedicine, University of Trieste. Seventy-two healthy growing subjects (45 women and 27 men; range, 7.8–17.7 years).

Materials and Methods – Double-blind, prospective, cross-sectional design. Samples of GCF were collected from each subject at the mesial and distal sites of both of the central incisors, in the maxilla and mandible. Skeletal maturation phase was assessed through the cervical vertebral maturation (CVM) method. Enzymatic activity was determined spectrophotometrically.

Results – The relationship between GCF ALP activity and CVM stages was significant. In particular, a twofold peak in enzyme activity was seen at the CS3 and CS4 pubertal stages, compared to the pre-pubertal stages (CS1 and CS2) and post-pubertal stages (CS5 and CS6), at both the maxillary and mandibular sites. No differences were seen between the maxillary and mandibular sites, or between the sexes.

Conclusions – As an adjunct to standard methods based upon radiographic parameters, the GCF ALP may be a candidate as a non-invasive clinical biomarker for the identification of the pubertal growth spurt in periodontally healthy subjects scheduled for orthodontic treatment.

Key words: alkaline phosphatase; diagnosis; gingival crevicular fluid; orthodontics; skeletal age measurement

Introduction

Treatment timing has a significant role in the outcomes of orthopaedic treatment of dentoskeletal disharmonies in growing patients (1–4). In class II growing subjects, the amount of supplementary mandibular growth induced by functional appliances appears to be significantly greater when the functional treatment is performed during the pubertal growth spurt (2–5). Orthopaedic treatment of patients with class III malocclusion (6) and rapid maxillary expansion (7) also benefits from the identification of the optimal timing to achieve maximum efficacy, which

in these cases is at a pre-pubertal growth phase. Therefore, the correct identification of the different phases of skeletal maturation represents a crucial issue in orthodontic diagnosis and treatment planning. Several maturational indices have been proposed to evaluate skeletal maturity in the growing patient. Among these indices, the most common are the radiography-based, hand-wrist analysis (8) and the cervical vertebral maturation (CVM) method (1). These methods are mainly morphological, while new possibilities might be offered by biochemical markers. Biomarkers avoid radiographic exposure, and they represent agents that are involved directly in bone growth and remodelling.

Gingival crevicular fluid (GCF) is a transudate, and its molecular constituents derive mainly from serum, although its composition also depends on the local periodontal environment (9). In recent years, a number of GCF constituents have been proposed as diagnostic indicators of periodontal status, including host enzymes (10). Among these enzymes, alkaline phosphatase (ALP) is essential for bone mineralization (11), and it was one of the first enzymes to be identified in GCF (12). More recently, GCF ALP activity has been proposed as a diagnostic aid in periodontology (10, 13) and orthodontics (14, 15).

Considering serum ALP increases (16) and the mandibular growth spurt (1) during puberty, it can be hypothesized that increases in GCF ALP activity occur in relation to the pubertal spurt. Therefore, the present prospective, double-blind study was aimed at evaluating the GCF ALP activity in growing subjects in relation to their stages of individual skeletal growth, as recorded through the CVM method (1) to ultimately candidate the GCF ALP activity as a non-invasive biomarker of individual skeletal maturation in orthodontic patients.

Materials and methods
Study population and design

This study enrolled consecutive subjects seeking orthodontic treatment who had never been treated before. A signed informed consent was obtained from the parents of the subjects prior to entry into the study, and the protocol was reviewed and approved by the Ethical Committee of the University of Trieste, Italy. The following enrolment criteria were observed: 1) age between 7 and 18 years; 2) intermediate or late mixed, or permanent phases of dentition; 3) good general health with absence of any nutritional problems; 4) no use of anti-inflammatories or antibiotics in the month preceding entry to the study; 5) probing depth (PD) values not exceeding 4 mm for the whole dentition and 3 mm for the anterior sextants; and 6) full-mouth plaque score and full-mouth bleeding score ≤25%.

The subjects were scheduled for enrolment at their first clinical examination; subsequently, during a second visit 7–10 days prior to GCF collection, they underwent a session of professional supragingival and subgingival scaling and also received repeated oral hygiene instructions. Moreover, over the days between professional scaling and GCF collection, the subjects were asked to rinse their mouths out twice a day with 0.012% chlorhexidine mouthwash and were not allowed to take any anti-inflammatories or antibiotics. At the last clinical session, when GCF was collected for ALP activity determination, their clinical parameters were recorded and dental panoramic radiographs and lateral cephalograms were taken immediately after GCF collection.

A total of 84 subjects were screened, out of which 72 were enrolled in the study: 45 women and 27 men (mean age, 12.1 ± 2.3; range, 7.8–17.7 years).

Appraisal of individual skeletal maturity

Appraisal of skeletal maturity was carried out through the CVM method on lateral cephalograms. This method comprises six stages (CS1–6) for CVM (1). Briefly, these are defined as follows:

CS1: when the lower borders of the second, third and fourth vertebrae (C2, C3 and C4) are flat and the bodies of C3 and C4 are trapezoid in shape. CS1 occurs at least 2 years before the pubertal growth spurt.

CS2: when only the lower border of C2 is concave and the bodies of C3 and C4 are trapezoid. CS2 occurs 1 year before the growth spurt.

CS3: when the lower borders of both C2 and C3 have concavities and the bodies of C3 and C4 are either trapezoid or rectangular horizontal in shape. CS3 marks the ascending portion of the growth spurt.

CS4: when the lower borders of C2–C4 have concavities, and the bodies of both C3 and C4 are rectangular horizontal. CS4 marks the descending portion of the growth spurt.

CS5: when the lower borders of C2–C4 have concavities and at least one of the bodies of C3 and C4 is square. CS5 occurs 1 year after the growth spurt.

CS6: when the lower borders of C2–C4 have concavities and at least one of the bodies of C3 and C4 is rectangular vertical. CS6 occurs at least 2 years after the growth spurt.

An experienced orthodontist (TB) who was blinded to the GCF ALP activities assessed the skeletal maturity of the subjects.

Clinical monitoring and GCF collection procedures

The intra-oral clinical examination was performed by a single operator (GP) on four sites per maxillary and mandibular central incisor (mesial, distal, medio-buccal and medio-palatal/lingual), as previously described (15). Briefly, the presence of supragingival plaque (PL+), the gingival bleeding within 15 s after probing (BOP+) and the PD were recorded. A single expert examiner (LC) collected the clinical data in all subjects. Contamination of the GCF was minimized by recording the PL+ before carefully cleaning the tooth with a sterile curette, then by collecting GCF and subgingival plaque from the isolated area and finally by recording the PD and BOP+. The GCF collection was performed at both the mesial and distal sites on each of the central upper and lower incisors as previously described (17). Briefly, #25 standardized sterile paper strips (Inline, Torino, Italy) were inserted 1 mm into the gingival crevice and left *in situ* for 60 s. The four samples from the same dental arch were pooled, as the maxillary and mandibular samples. The GCF samples were transferred to plastic vials and immediately stored at −80°C, until analysed.

Enzymatic activity determination

The biochemical assays were performed by a single operator (GP) who was blinded to the CVM stages. The four GCF samples from both the maxillary and mandibular sites were resuspended in 200 μl buffer containing 100 mM Tris and 20 mM $MgCl_2$ (pH 9.8 ± 0.1) and 6 mM p-nitrophenol phosphate. The samples were then incubated at 37°C (±<0.1°C fluctuations) for 2 h, whereby the ALP in the samples hydrolyses the p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate. The reactions were then stopped by adding 5 μl 3 M NaOH, and the rates of increase in absorbance were read with a spectrophotometer at 405 nm (13). The relevant control for each analysis consisted of the reagent and the Tris buffer without the sample. Using 18.45 as the p-nitrophenol mM absorptivity, the absorbance was converted into enzyme activity units (1 unit = 1 mmol of p-nitrophenol released per minute at 37°C) and expressed as total activity in mU/sample (18).

Data analysis

SPSS software, version 13.0 (SPSS® Inc., Chicago, IL, USA) was used to perform the statistical analyses. Parametric methods were used only for those continuous data sets that met the required assumptions of normality of the distributions and equality of variances, tested by the Shapiro–Wilk and Levene's tests, respectively.

The balancing of experimental groups (clustered according to the CVM stages) by sex was tested by chi-squared analysis. The following analyses were carried out considering the maxillary and mandibular sites of each patient as the statistical unit. Among the different CVM stages, the significance of the differences in the %PL+ and %BOP+ was assessed by a Kruskal–Wallis test, while the significance of the difference in the mean PD was assessed by a one-way analysis of variance. Between the maxillary and mandibular sites, within each CVM stage, the significance of the differences in the %PL+ and %BOP+ was assessed by a Wilcoxon rank sum test, while the significance of the differences in the mean PD was assessed by a paired *t*-test. The Kruskal–Wallis test, followed by a Tukey test, assessed the significance of the differences in the enzyme activities between the different CVM stages.

Finally, the subjects, considered as the statistical units, were also clustered into three groups according to their growth phases, as pre-pubertal (CS1 and CS2), pubertal (CS3 and CS4) and post-pubertal (CS5 and CS6). This clustering was used for the calculation of the effect size (ES) coefficients (19). The ES coefficient is the ratio of the difference between the recordings of two different groups, i.e. GCF ALP activity of the pre-pubertal and pubertal groups, divided by the within-subject standard deviation (SD). Sample size of at least 20 subjects for each of these three pre-pubertal, pubertal and post-pubertal groups was set to detect a

minimum ES coefficient of 1.0 among them, with an alpha set at 0.05 and a power of 0.8 (19). Moreover, the actual ES coefficients retrieved have also been used as indexes of potential diagnostic accuracy of the GCF ALP activity as previously described (20). In this regard, a threshold of 1.0 was used to assess a potential good diagnostic accuracy (20). The Kruskal–Wallis test followed by the Tukey test assessed the significance of the differences in the enzyme activities between the growth phases.

Kappa statistics testing the intra-examiner agreement of the CVM appraisal showed a value above 0.90. A *p* value <0.05 was used for rejection of the null hypothesis.

Results

The age of the subjects clustered according to the CVM stages, as shown in Table 1. Moreover, the distribution of the sexes was similar among the groups compared (not shown).

The pooled maxillary and mandibular %PL+ and %BOP+ were, as medians (25th–75th percentile): 12.5 (0;25.0) and 6.25 (0;12.5), respectively. The pooled maxillary and mandibular PD as the mean ± SD was 1.7 ± 0.3. No significant differences were seen between the CVM stages (not shown).

The GCF ALP activities seen in the subjects clustered according to CVM stage are shown in Table 2. The GCF ALP activities were significantly different across the CVM stages, for both the maxillary and mandibular sites. About a twofold peak in GCF ALP activity was recorded

Table 1. Ages of the subjects in the different groups according to cervical vertebral maturation (CVM) stages (n = 72)

CVM stage	N	Age (years) Mean ± SD	Min–max
CS1	12	9.6 ± 1.0	7.8–10.8
CS2	14	11.0 ± 1.1	8.6–13.0
CS3	11	11.0 ± 1.5	8.3–13.6
CS4	12	13.0 ± 1.7	10.8–16.5
CS5	11	13.3 ± 1.5	9.5–15.2
CS6	12	15.1 ± 1.9	11.8–17.7

N, number of subjects in each group; min, minimum age; max, maximum age.

Table 2. Gingival crevicular fluid (GCF) alkaline phosphatase (ALP) activities from the maxillary and mandibular sites according to cervical vertebral maturation (CVM) stage (n = 72)

	GCF ALP activity (mU/sample)			
	Maxillary sites		Mandibular sites	
CVM stage	Median (25th–75th percentile)	Mean	Median (25th–75th percentile)	Mean
CS1	52.1 (31.5–77.4)	54.4	41.4 (33.1–73.4)	49.5
CS2	32.5 (24.4–70.9)	48.9	41.5 (25.6–60.9)	44.7
CS3	61.9 (48.9–157.8)	98.4	73.1 (57.4–99.0)*	87.7
CS4	97.3 (58.6–128.2)*	97.6	78.9 (47.3–92.9)	74.2
CS5	66.6 (21.9–72.0)	58.3	38.2 (19.1–49.3)	45.9
CS6	31.5 (19.1–100.8)	50.6	39.7 (18.2–66.4)	49.8
Diff.	*p* < 0.05	*p* < 0.05		

Statistically significant differences at the pairwise comparisons: *, with the corresponding CS2 (*p* < 0.05). No significant differences were seen between the maxillary and mandibular sites within each CVM stage. Diff., significance of the differences between the CVM stages.

for the pubertal phases (CS3 and CS4), compared to those of the pre-pubertal (CS1 and CS2) and post-pubertal (CS5 and CS6) phases. At the pairwise comparisons, the enzymatic activities recorded for the CS4 maxillary sites and the CS3 mandibular sites were significantly greater when compared to the corresponding activities of CS2 (*p* < 0.05). Moreover, the GCF ALP activities showed no significant differences between the maxillary and mandibular sites within each of the CVM stages or between the sexes (not shown).

The maxillary and mandibular pooled GCF ALP activities clustered according the growth phases (pre-pubertal, pubertal and post-pubertal) are shown in Fig. 1. Statistically significant differences between the groups were seen for all of the comparisons (*p* = 0.001). The ES coefficients between the pubertal group with respect to the pre-pubertal and post-pubertal groups were 1.80 and 1.03, respectively.

Discussion

The present study investigated the possible relationships between GCF ALP activity and skeletal maturation, and it demonstrates that the pubertal growth spurt can be detected at the level of the GCF. Therefore, a diagnostic potential of GCF as a non-invasive tool for
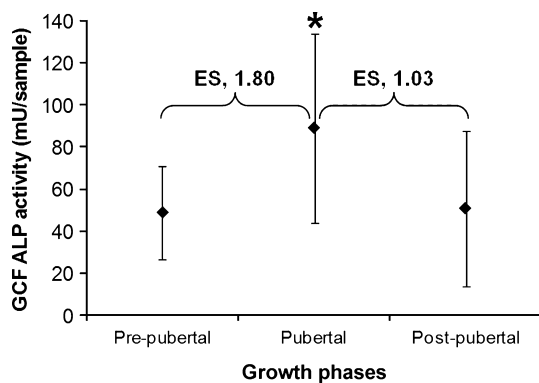
Fig. 1. Pooled maxillary and mandibular gingival crevicular fluid alkaline phosphatase activities according to growth phases and the corresponding ES (n = 72). Data are presented as means ± SD. ES, effect size coefficients, as indicated. Statistically significant differences at the pairwise comparisons: *, with the pre-pubertal and post-pubertal growth phases ($p = 0.001$). Pre-pubertal n = 26, pubertal n = 23, post-pubertal n = 23.

the identification of individual skeletal maturation stages is initially uncovered here.

As GCF ALP activity increases during periodontal inflammation (12, 13), periodontal health is necessary to exclude any possible unwanted sources of this enzyme. Herein, all of the subjects received a session of professional oral hygiene and showed optimal periodontal conditions, with very low %PL+ and %BOP+ scores and with mean PD below 2 mm. The clinical conditions were thus similar among the subjects at the different CVM stages, and between the maxillary and mandibular sites within each group (not shown).

Once local tissue inflammation is excluded, and by considering the GCF formation modalities (21), two potential sources might be responsible for these variations in GCF ALP activity: 1) serum ALP (as a systemic factor) and 2) maxillary/mandibular growth (as a local skeletal factor). In more detail, the serum ALP activity, which is the most used biochemical marker for bone turnover, has been reported to increase at puberty and to decrease in adulthood (16).

When the GCF ALP activities were assessed in relation to the different CVM stages, increased activities were seen for both the maxilla and mandible at CS3 and CS4, compared to all of the other CVM stages. These differences were statistically significant within the maxillary and mandibular sites. However, no significant differences were seen between the maxillary and mandibular sites within each of the CVM stages (Table 2). In this regard, the similar behaviours of the GCF ALP activities of the maxillary and mandibular

sites indicate that the differential rates and timing of the local basal bones have little influence on the GCF enzymatic activities, which would thus be more responsive to systemic factors. As growth areas of the maxillary and mandibular bones are not located in close proximity to the sampling sites, this might explain these data. Therefore, influence from the serum ALP can be hypothesized, even though no previous studies have specifically correlated serum ALP activities with CVM stages.

Interestingly, the pairwise comparisons showed significant differences in the GCF ALP activities of the pubertal stages (CS3 and CS4) with CS2, which, in turn, were slightly lower and less variable when compared to CS1 (Table 2). Although the differences between CS1 and CS2 in terms of enzymatic activities were minimal and not significant, these data are consistent with previous evidence relating to a minimal pre-pubertal growth phase that precedes the growth spurt (4). The GCF ALP activities in the post-pubertal stages (CS5 and CS6) then decreased to levels comparable to those of the pre-pubertal stages (CS1 and CS2). This decrease can be explained by reduction in serum bone ALP activity (16) during the post-pubertal periods. Therefore, this tends to confirm that systemic growth has an impact on GCF composition, while sex appears to have no effects on its ALP activity.

Diagnostic potential and accuracy of GCF ALP activity as a biomarker of the growth spurt

The maxillary and mandibular pooled GCF ALP activities showed similar levels between the two pre-pubertal (CS1 and CS2) and the two post-pubertal (CS5 and CS6) stages (Table 2); therefore, it appears that any diagnostic use of GCF ALP activity would be addressed to the identification of the three main growth phases, pre-pubertal, pubertal and post-pubertal, irrespective of the maxillary or mandibular sampling sites (Fig. 1).

The clinical usefulness of a biomarker for the identification of the pubertal growth spurt is critically dependent upon the accuracy that such a diagnostic tool has. Therefore, a critical approach to assess the potential of GCF ALP activity as a diagnostic aid in orthodontics has to rely on the concept that to have high accuracy, the measurement outcomes recorded in two groups of subjects (i.e. pubertal vs. pre-pubertal or post-pubertal) have to show large enough differences

between the groups, compared to their corresponding variances. A statistical approach to quantify this ratio (taking into account the size of the study populations) is provided by the calculation of the ES coefficient (19). A theoretical prerequisite for a diagnostic tool to be accurate is to show an ES coefficient at least equal to 1.0 (20). In the present study, the ES retrieved between the pubertal (CS3 and CS4) and pre-pubertal (CS1 and CS2) or post-pubertal (CS5 and CS6) subjects was 1.80 and 1.03, respectively (Fig. 1). In this regard, the inclusion of multiple collection sites in each of the maxillary and mandibular sites probably contributed to the reduction in inter-subject variability that has been encountered when using single GCF samples (14, 15). These data thus warrant further longitudinal studies to define diagnostic ranges and individual curves for the GCF ALP activity according to the skeletal maturation phases.

Clinical implications

As shown in Table 2, the enzyme activity peak seen for CS3 and CS4 would overlap with the mandibular growth spurt, which has been shown mainly in skeletal class I subjects (1, 22). In this regard, the present sample included consecutive patients with all of the three skeletal class patterns, which were equally distributed among the different CVM stages (not shown). However, given the lack of differences between the maxillary and mandibular sites, the type of sagittal skeletal malocclusion *per se* is not expected to influence the GCF ALP activity. Therefore, given the temporal overlap of the stature and mandibular growth spurts (23), the GCF ALP activity would be a candidate as a biomarker of the onset of the mandibular growth spurt, which would have relevant diagnostic purposes in orthopaedic treatment of class II subjects.

Finally, GCF analysis offers several advantages from a clinical standpoint. Its sampling involves a very simple, rapid and non-invasive procedure that can be performed in a clinical setting, even in the case of multiple GCF collections. Moreover, the ALP activity can be determined through routine and cheap laboratory analyses that are already available.

Conclusions

This study is the first that demonstrates an influence of skeletal maturation on GCF composition. As an adjunct to standard methods based upon radiographic parameters, GCF ALP appears to be a valid candidate as a non-invasive biomarker of the pubertal growth spurt in periodontally healthy subjects scheduled for orthodontic treatment.

Clinical relevance

The identification of the individual skeletal maturation stages is crucial for the enhancement of the efficiency of dentofacial orthopaedic treatment. GCF ALP activity is a candidate as a non-invasive biomarker of individual skeletal maturation and has diagnostic potential for the identification of the optimal timing for facial orthopaedic treatment of different dentoskeletal disharmonies in growing patients.

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