Original Article

Gingival crevicular fluid protein content and alkaline phosphatase activity in relation to pubertal growth phase

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ABSTRACT

Objective: To evaluate gingival crevicular fluid (GCF) protein content and alkaline phosphatase (ALP) activity in growing subjects in relation to stages of skeletal maturation, ie, the growth phase, as prepubertal, pubertal, and postpubertal.

Subjects and Methods: Fifty healthy growing subjects (31 girls and 19 boys; age range, 7.8–17.7 years) were enrolled in this study that followed a double-blind, prospective, cross-sectional design. Collection of GCF was performed at the mesial and distal sites of both central incisors, for the maxilla and mandible. Growth phase was assessed through the cervical vertebral maturation method. GCF parameters were expressed as total protein content, total ALP activity, and normalized ALP activity.

Results: The total GCF protein content was similar between the different growth phases. On the contrary, the total ALP activity showed a peak for the pubertal growth phase. The normalized GCF ALP activity was only poorly associated with growth phase. No differences were seen between the maxillary and mandibular sites, or between the sexes, for any GCF parameter.

Conclusions: The total GCF protein content is not sensitive to the growth phase. However, GCF ALP activity has potential as a diagnostic aid for identification of the pubertal growth phase in individual subjects when expressed as total, but not normalized, values. (*Angle Orthod.* 2012;82:1047–1052.)

KEY WORDS: Gingival crevicular fluid; Protein content; Alkaline phosphatase; Growth phase; Diagnosis; Orthodontics

INTRODUCTION

Identification of skeletal maturity, ie, the growth phase, with particular regard to the onset of the pubertal growth phase, has major clinical implications when dealing with orthodontic treatment in growing subjects, especially when there are skeletal disharmonies.[1,2] Several indices have been proposed to identify the skeletal maturation phases, with the most common being the radiography-based, hand-wrist analysis[3] and the cervical vertebral maturation (CVM) method.[1] However, new possibilities might be provided by biochemical markers, ie, biomarkers that avoid invasive X-ray exposure and represent agents that are directly involved in bone growth and remodeling. Gingival crevicular fluid (GCF) is a potential source of biomarkers, with molecular constituents that derive mainly from serum, and also from the interstitial fluids of periodontal tissues.[4] Under healthy conditions, with protein concentrations of the serum and tissues constant, the total protein content of the GCF can be considered as an index of the amount of GCF, its volume. In particular, both the volume[5] and total protein content[6] of the GCF have been used extensively to calculate the concentrations of the different GCF constituents, for their normalization. The total protein content of the GCF has been used extensively, as determination of the GCF volume is not fully reliable due to evaporation.[7]

Of interest, GCF formation has been shown to be correlated with serum steroid sex hormone changes

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during pubertal growth,[8] and the total protein content of the GCF has been reported to be greater in growing subjects as compared with an older group.[9] More recently, total GCF alkaline phosphatase (ALP) activity has been shown to be related to the pubertal growth spurt and has thus been proposed as a noninvasive diagnostic aid for the determination of optimal treatment timing in functional jaw orthopedics.[10] However, no studies have investigated whether the total GCF protein content is sensitive to the different growth phases, nor which of the total or normalized GCF ALP activities might be better suited as a biomarker for the pubertal growth phase.

Therefore, the aim of the present prospective, double-blind study was twofold: (1) evaluation of total GCF protein content in relation to stages of individual growth phase, as recorded through the CVM method,[1] to determine whether the total GCF protein content represents a noninvasive biomarker of individual skeletal maturation in growing orthodontic patients; and (2) comparison of the sensitivities of the total and normalized GCF ALP activities with respect to the growth phases.

MATERIALS AND METHODS

Study Population and Design

This study enrolled subjects who were seeking orthodontic treatment and had never been treated previously. 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 local ethical committee. The following enrollment criteria were observed: (1) aged between 7 and 18 years; (2) intermediate or late mixed, or early permanent phases of dentition; (3) good general health with absence of any nutritional problems; (4) no use of anti-inflammatory agents or antibiotics in the month preceding entry to the study; (5) probing depth (PD) 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 $\leq$ 25%.

The subjects were scheduled for enrollment at their first clinical examination; subsequently, during a second visit 7 to 10 days prior to GCF collection, they underwent a session of professional supragingival and subgingival scaling and also received repeated oral hygiene instructions. Moreover, between the professional scaling and the 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-inflammatory agents or antibiotics. At the last clinical session, when the GCF was collected for total protein content and ALP activity determinations, their clinical parameters were recorded, and lateral cephalograms were recorded immediately after GCF collection.

A total of 54 consecutive subjects were screened, of which 50 were enrolled in the study: 31 girls and 19 boys (mean age, 11.6 $\pm$ 2.3 years; range, 7.8–17.7 years). In particular, 28 of these subjects constituted a subset of a different study.[10]

Clinical Assessment and GCF Collection Procedures

Assessment of skeletal maturity was carried out through the CVM method on lateral cephalograms. This method comprises six stages (CS1 to CS6) for cervical vertebral maturation.[1] An experienced orthodontist who was blinded to the GCF ALP activities assessed the skeletal maturity of the subjects. Finally, the subjects were clustered into three groups according to their growth phases, as prepubertal (CS1 and CS2), pubertal (CS3 and CS4), and postpubertal (CS5 and CS6).

The intraoral clinical examination was performed by a single operator (Dr Perinetti) on four sites per each maxillary and mandibular central incisor (mesial, distal, medio-buccal, and medio-palatal/ lingual). The presence of supragingival plaque (PL+), gingival bleeding within 15 seconds of probing (BOP+), and PD were recorded as previously reported.[11] GCF collection was performed at two sites on each maxillary and mandibular central incisor, as the mesial and distal aspects, using #25 standardized sterile paper strips (Inline, Torino, Italy), which were inserted 1 mm into the gingival crevice and left in situ for 60 seconds.[12] The four samples from the same dental arch, as either maxillary or mandibular, were pooled and immediately stored at -80°C, until analysis.

Biochemical Assays

The biochemical assays were performed by a single blinded operator.

The four GCF samples from both the maxillary and mandibular sites were resuspended in 250 μL buffer containing 100 mM Tris and 1 mM $MgCl_2$ (pH 9.8 $\pm$ 0.1). The supernatant was recovered, and 180 μL was used for the total enzymatic activity determination, with 50 μL used for total protein content determination.

The ALP activity was monitored by adding p-nitrophenol phosphate to a final concentration of 6 mM, with a total sample volume of 200 μL. The samples were incubated at 37°C for 120 minutes, and the rate of increase in absorbance was read with an ultraviolet-visible spectrophotometer, at 405 nm.[10] For each analysis, a control was used that consisted of the reagent and the Tris buffer without any sample. By 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 per sample.[5]

The total protein content was determined using the bicinchoninic acid (BCA, Sigma-Aldrich Inc, St Louis, Mo) and copper (II) sulphate pentahydrate (Sigma-Aldrich) protein assay reagents, according to the manufacturer instructions. Briefly, the BCA working reagent was prepared by mixing 50 parts BCA reagent with 1 part of copper (II) sulphate reagent, and mixing until this was light green in color. Then, 160 μL BCA working reagent was added to 40 μL of the remaining samples after the enzymatic activity assay, and incubated at 37°C for 60 minutes. Protein standard curves were obtained against samples of bovine serum albumin.

Finally, the normalized GCF ALP activity was expressed as total activity divided by total protein content (mU/μg proteins).

Data Analysis

The balancing of the experimental groups (clustered as growth phases) by sex was tested by chi-square analysis.

The following analyses were carried out considering the maxillary and mandibular sites of each patient as the statistical units. A Kruskal-Wallis test and a one-way analysis of variance (ANOVA) were used to assess the significances of the differences in the number of PL+ and BOP+ sites and the mean PD, respectively, among the different growth phases. Between the maxillary and mandibular sites, within each growth phase, the significances of the differences of the percent PL+ and percent BOP+ were assessed by Wilcoxon rank sum tests, while the significances of the differences in the mean PD were assessed by paired *t*-tests. The Kruskal-Wallis test, followed by a Bonferroni-corrected Mann-Whitney *U*-test as for pairwise comparisons, were used to assess the significances of the differences in total GCF protein content and ALP activity (either total or normalized) among the growth phases. Wilcoxon rank sum tests were used to assess the significances of the differences between the maxillary and mandibular sites, within each growth phase, for all three of the GCF parameters.

The rest of the analyses were carried out by pooling the maxillary and mandibular sites, thus considering the subject as the statistical unit. For the three GCF parameters, the effects size (ES) coefficients[13] were calculated as an index of potential diagnostic accuracy in the identification of pubertal growth phase. The ES coefficient of a given parameter is the ratio of the difference between the recordings of two different groups, ie, the prepubertal and pubertal groups, divided by the within-group standard deviation (SD). Here, a threshold of 1.0 was used to assess potential good diagnostic accuracy in individual subjects.[14]

A *P* value less than .05 was used for rejection of the null hypothesis.

Table 1. Ages of the Subjects According to Growth Phase[a]

		Age, y	
CVM Stage	n	Mean ± SD	Min–Max
Prepubertal	21	10.2 ± 1.3	7.8–13.0
Pubertal	18	11.4 ± 1.4	8.3–13.7
Postpubertal	11	14.8 ± 1.8	12.6–17.7

[a] CVM indicates cervical vertebral maturation; n, number of subjects in each group; Min, minimum age; and Max, maximum age.

RESULTS

The ages of the subjects clustered according to the growth phases are shown in Table 1. The distribution of the sexes was similar among the groups compared ($P > .9$; not shown).

The pooled maxillary and mandibular percent PL+, percent BOP+ as medians (25th; 75th percentiles) were 12.5 (0; 21.9) and 6.3 (0; 12.5), respectively. The mean ± SD pooled maxillary and mandibular PD was 1.7 ± 0.4 mm. No significant differences were seen among the growth phases or between the maxillary and mandibular sites within each growth phase (not shown).

The GCF parameter scores are shown in Table 2. Within each growth phase, no differences were seen between the maxillary and mandibular sites for each of the parameters. The maxillary and mandibular total GCF protein contents showed no significant differences among the growth phases, although at the maxillary sites the total GCF protein content was slightly greater for the pubertal growth phase, as compared with the prepubertal and postpubertal growth phases. The total GCF ALP activity was significantly greater in the pubertal growth phase as compared with the prepubertal and postpubertal growth phases for both the maxillary and mandibular sites. Moreover, lower total GCF ALP activities were seen for the postpubertal growth phase, as compared with the corresponding prepubertal activities, although this only reached statistical significance for the mandibular sites.

Figure 1 shows the pooled maxillary and mandibular GCF parameters in the subjects clustered according to the growth phases, with the corresponding ES coefficients between the growth phases. Low ES coefficients were seen for total GCF protein, which reached 0.6. On the contrary, the highest ES coefficients were those related to the total GCF ALP activity, especially when considering the pubertal and prepubertal growth phases, which reached 2.0. Intermediate ES coefficients were seen for the normalized GCF ALP

Table 2. The Different Gingival Crevicular Fluid (GCF) Parameters From the Maxillary and Mandibular Sites According to Growth Phase (n = 50)*

Parameter	Growth Phase	Maxillary Sites	Mandibular Sites
Total GCF proteins (μg/sample)	Prepubertal	1.0 (0.6; 2.4)	1.2 (0.6; 1.7)
	Pubertal	1.7 (0.8; 2.1)	1.2 (0.6; 1.5)
	Postpubertal	1.0 (0.2; 1.9)	1.1 (0.5; 1.4)
	Diff	NS	NS
Total GCF ALP activity (mU/sample)	Prepubertal	48.9 (25.6; 66.9)	42.0 (34.2; 47.9)
	Pubertal	73.8 (46.5; 122.7)a	78.9 (55.7; 92.1)a
	Postpubertal	21.9 (12.4; 67.5)b	34.0 (14.2; 37.1)a,b
	Diff	$P < .01$	$P < .001$
Normalized GCF ALP activity (mU/μg proteins)	Prepubertal	38.6 (29.9; 45.3)	34.1 (27.5; 58.3)
	Pubertal	50.2 (32.5; 89.9)	69.5 (36.2; 121.6)a
	Postpubertal	46.5 (19.6; 59.4)	36.0 (13.5; 66.7)
	Diff	NS	$P < .05$

* Data are presented as medians (25th;75th percentiles). Diff indicates significance of the differences among the growth phases; ALP, alkaline phosphatase. Statistically significant differences at the pairwise comparisons: a, with the corresponding prepubertal phase; b, with the corresponding pubertal phase. NS indicates no statistically significant difference. No significant differences were seen between the maxillary and mandibular sites within each growth phase for any GCF parameter.

activity, as between 1.3 and 1.4. However, the variability for the pubertal growth phase of this GCF parameter was also notably large.

DISCUSSION

The present study has initially shown that total GCF protein content is not a reliable indicator of the different growth phases. While confirming that total GCF ALP activity would be a reliable biologic indicator of skeletal maturation,[10] it firstly showed that this GCF ALP activity would have diagnostic potential when expressed as total but not normalized values.

As the quality and quantity of GCF changes during periodontal inflammation,[7] local tissue health is necessary to exclude any possible unwanted bias. In the present study, all of the enrolled subjects showed optimal periodontal conditions. Moreover, by sampling the GCF at the central incisors, which like the lateral incisors, were fully erupted, the present data and previous[12] data show that neither the total GCF protein content nor the total GCF ALP activity are influenced by the different dentition stages. Moreover, to reduce intersubject variability, multiple collection sites in each of the maxilla and mandible sites were used. According to previous evidence,[10] no significant differences were seen for these GCF parameters when comparing maxillary and mandibular scores within each dentition phase (recorded as intermediate and late mixed and permanent; not shown).

A number of GCF constituents have been proposed as diagnostic indicators of periodontal status during periodontitis[15,16] and orthodontic tooth movement.[17] However, although the total GCF protein content was initially evaluated some 40 years ago[18] and has been shown to be lower when compared to that of serum under healthy conditions,[7] no investigations have evaluated the possibility of using this GCF parameter as a clinical diagnostic aid in dentistry. The rationale for using total GCF protein content derives from the overall greater amounts in terms of the full protein content, as compared to a single constituent, whereby the quantification is easier and more reliable, especially when considering the setting up of a chairside kit for routine clinical determination.

Although not using any method for precise assessment of the individual growth phases, a previous study[9] reported greater gingival total protein content in growing subjects as compared with an older group. In contrast, no differences in total GCF protein content were seen.[9] The lack of significant changes in total GCF protein content is consistent with the hypothesis that the amount of GCF, ie, the flow rate, is not sensitive to the different growth phases. Therefore, the GCF volume would also not be expected to be influenced by the pubertal growth phase.

ALP is an enzyme that is necessary for bone mineralization,[19] with its activity shown to be correlated with local tissue remodeling during orthodontic tooth movement[20] and periodontal inflammation.[7,16] Previous findings have shown a two-fold peak increase in total GCF ALP activity during the pubertal growth phase[10]; however, the present study initially shows that the GCF ALP activity is more sensitive to the growth phases when expressed as the total GCF ALP activity rather than the normalized GCF ALP activity.

A further goal of the present study was to estimate the diagnostic accuracy of the GCF parameters as indicators of growth phase in individual subjects. In this regard, a difficulty resides in intragroup variability and the number of subjects examined, which also determines the variability of the data. A statistical approach to quantify this ratio (taking into account the sizes of the study populations) is provided by the calculation of

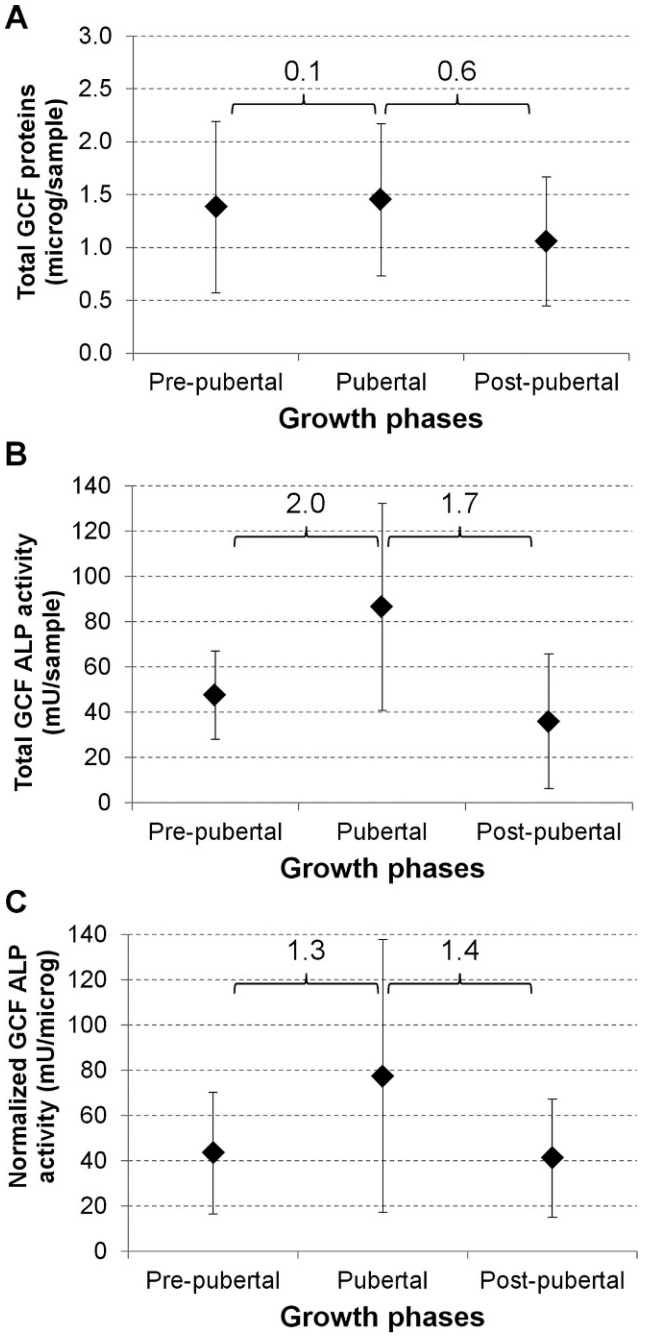

Figure 1. Pooled maxillary and mandibular GCF parameters according to growth phase, and the corresponding ES coefficients (n = 50). Data are presented as mean ± SD. (A) Total GCF protein content. (B) Total GCF ALP activity. (C) Normalized GCF ALP activity. The effects size coefficients between the growth phases are as indicated.

the ES coefficient,[13] as has been used previously for this purpose for different parameters.[10,21,22]

In the present study, the ES coefficients show that the total GCF protein content has no clinically relevant association with the different growth phases, while the total GCF ALP activity shows notable changes when comparing the prepubertal or postpubertal growth phases with the pubertal growth phase (Figure 1). In particular, the ES coefficients between the pubertal and prepubertal, and the pubertal and postpubertal growth phases are 2.0 and 1.7, respectively. Therefore, the total GCF ALP activity has potential as a diagnostic aid in the identification of the pubertal growth phase in individual subjects, as has also been reported previously.[10] In the present study, a first comparison of the diagnostic potential of the GCF ALP, expressed as total or normalized activities, was made, which shows that the use of the total GCF ALP activity is preferable both for research and for future clinical practice. Indeed, although they are above the threshold of 1.0, the ES coefficients for the normalized GCF ALP activities are not greater than 1.4 (Figure 1). The notably large variability of the normalized GCF ALP activities and especially for the pubertal growth phase (Table 2; Figure 1C) might be responsible for this summatory variability effect of the quantifications of total protein content and enzyme activity. This has major implications in the development of future chairside kits for the identification of growth phases in individual subjects for routine use in clinical practice. Moreover, future research will address whether reference values for ALP activity within each growth phase have satisfactory diagnostic performances.

CONCLUSIONS

- Total GCF protein content is not sensitive to the growth phases.
- GCF ALP activity is a promising diagnostic tool for identification of the growth phases in individual subjects when expressed as the total, rather than the normalized, values.

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