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Repeatability of gingival crevicular fluid collection and quantification, as determined through its alkaline phosphatase activity: implications for diagnostic use

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Background and Objective: In spite of four decades of studies on gingival crevicular fluid, no data have been reported on the repeatability of gingival crevicular fluid collection and the subsequent quantification procedures. The present study reports, for the first time, on the repeatability and method error of gingival crevicular fluid collection and quantification, as determined through its alkaline phosphatase (ALP) activity. Diagnostic considerations are then explored.

Material and Methods: Twenty-seven healthy subjects (17 women and 10 men; mean age ± SD, 21.2 ± 4.8 years) with optimal periodontal status were enrolled according to a blind prospective design. The gingival crevicular fluid was collected at baseline, and after 1 d, 1 wk and 3 mo. At each clinical session, two consecutive rounds of gingival crevicular fluid collection were made from each of the four maxillary incisors, allowing the recovery of resting and flow gingival crevicular fluid. The total ALP activities were determined spectrophotometrically, and repeatability and method errors for the resting, flow and overall (resting + flow) gingival crevicular fluid ALP activities were calculated, relative to the corresponding baseline levels.

Results: No significant differences were seen over time, although the flow gingival crevicular fluid ALP activity was generally lower than that for the resting gingival crevicular fluid. The method errors ranged from 40 to 58%, with the flow and overall gingival crevicular fluid activities showing the highest and lowest errors, respectively.

Conclusion: Reliable use of the gingival crevicular fluid ALP collection and quantification, both in research and diagnosis on an individual basis, should take into account relevant errors, and variations are to be considered as true only above relevant thresholds.

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In spite of increasing numbers of investigations on gingival crevicular fluid over the last four decades (1), little data have been reported relating to repeatability errors during collection and quantification procedures. However, knowledge of these parameters is of crucial importance, especially in view of the potential clinical application of gingival crevicular fluid analysis (i.e. quantification of its constituents) in periodontology (2–5) and in orthodontics (3,6–9), the proposals for which are constantly on the increase (10).

Exact knowledge of the repeatability and method errors of gingival crevicular fluid collection and quantification procedures is also needed considering the large variations that have been seen in previous investigations, which have indicated large intersubject and intrasubject variations. Surprisingly, the little data available to date mainly relate to the amount of gingival crevicular fluid recovered using the different collection tools (11,12), the reliability of determination of the gingival crevicular fluid volume (13,14) and the effects of the sampling area (15), rather than to the reliability of the quantification of its molecular constituents. However, while the gingival crevicular fluid volume is subject to significant evaporation (16), the molecular constituents are not. On the basis of this difference, several gingival crevicular fluid constituents have been shown to be sensitive to orthodontic tooth movement, while the gingival crevicular fluid volume has not been shown to increase upon such tissue remodeling (17). A further aspect relates to the form of gingival crevicular fluid collected, which can be defined as the gingival crevicular fluid at rest (i.e. that present inside the sulcus/pocket) and as the gingival crevicular fluid flow (i.e. that newly formed after collection of the resting gingival crevicular fluid) (18,19).

Quantification of the gingival crevicular fluid constituents as biomarkers of tissue changes would have major diagnostic implications, especially in the treatment of periodontal disease (10). Among these biomarkers, alkaline phosphatase (ALP) was the first to be identified (20). This enzyme is sensitive to bone remodeling when released from osteoblasts (21), to inflammation when released from polymorphonuclear cells (4) and to periodontal regeneration when released from periodontal ligament fibroblasts (22). During chronic periodontitis, gingival crevicular fluid ALP activity has been shown to have predictive value, both alone (5) and in combination with other gingival crevicular fluid biomarkers (23), in terms of attachment loss and recurrent inflammation after sessions of scaling and root planing (24).

In spite of the diagnostic potential that has been proposed for gingival crevicular fluid ALP activity, both in periodontology (4,5,24) and in orthodontics (3,6,25), no previous studies have fully investigated the repeatability and method errors of gingival crevicular fluid collection and its subsequent ALP activity quantification. The present study was thus designed to evaluate the repeatability and method errors (systematic and random) of quantification of both the resting and flow gingival crevicular fluid ALP activities, according to a longitudinal design using healthy sites. The ultimate goal of this study was to identify a threshold value above which a variation in gingival crevicular fluid activity can indeed be considered as indicative of metabolic changes in periodontal tissues in individual subjects.

Material and methods

Subjects and study design

This study enrolled consecutive volunteer subjects recruited from the patients of the dental clinic who had never been orthodontically or periodontally treated before (excluding professional scaling). Signed informed consent was obtained from the subjects (or their parents, when the subjects were under 18 years of age) before entry into the study, and the protocol was reviewed and approved by the local Ethics Committee.

The following enrolment criteria were observed: (i) end of growth (as recorded though the cervical maturation method [stage 6] (26), or > 18 years of age); (ii) good general health, with absence of any nutritional problems; (iii) presence of the four maxillary incisors, with no restorations extending to the gingival margins; (iv) no use of anti-inflammatories or antibiotics in the month preceding entry to the study; (v) probing depth values not exceeding 4 mm for the whole dentition or 3 mm for the anterior maxillary sextant; (vi) no radiographic evidence of periodontal bone loss after panoramic X-ray examination; (vii) being a nonsmoker; and (viii) a full-mouth plaque score and a full-mouth bleeding score of ≤ 20%. The full-mouth plaque score and the full-mouth bleeding score were recorded as the percentage of tooth surfaces with supragingival plaque or showing bleeding within 15 s after probing with a 20 gf controlled-force probe (Vivacare TPS Probe; Vivadent, Schaan, Lichtenstein).

The subjects were scheduled for enrolment at their first clinical examination; subsequently, they underwent four consecutive clinical sessions: baseline, and 1 d, 1 wk and 3 mo later. At each clinical session, the clinical data were recorded and gingival crevicular fluid was collected. Moreover, 7–10 d before the baseline and the 3-mo clinical sessions, professional supragingival and subgingival scaling was carried out on each subject, and all subjects also received repeated oral-hygiene instructions. This procedure ensured optimal clinical conditions and allowed any possible mechanical injury to the tissue to be healed before sampling. Moreover, during the intervening period between each professional scaling and the subsequent clinical session(s), the subjects were asked to rinse their mouths out twice a day with 0.12% chlorhexidine mouthwash, and they were not allowed to take any anti-inflammatories or antibiotics.

A total of 30 subjects were screened, of which 27 were enrolled in the study. The study population comprised 17 female subjects and 10 male subjects (mean age ± standard deviation, 21.2 ± 4.8 years; age range, 14.1–27.6 years). A minimum of 25 subjects was required to avoid major bias in the calculation of methodological errors (27).

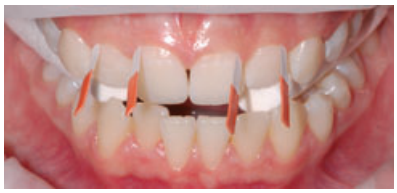

Fig. 1. Sampling sites for collection of gingival crevicular fluid.

Clinical measurements and gingival crevicular fluid collection

Clinical examinations were performed on each maxillary incisor, at four sites per tooth (mesio-buccal and disto-buccal; medio-buccal/palatal sites). The clinical examinations consisted of recording: (i) the presence of plaque (PL+), assessed dichotomously by visual criteria; (ii) the probing depth, measured from the gingival margin to the base of the pocket; and (iii) bleeding on probing (BOP+), as previously reported (7). The PL+ and BOP+ were recorded at each clinical session, while the probing depth was recorded at baseline and at 3 mo. The same operator (B.D.L.) always recorded the clinical data.

Four sampling sites – the disto-buccal aspects of each of the four maxillary incisors – were chosen for collection of gingival crevicular fluid (Figure 1). In each clinical session, two sequential rounds of collection were performed for each of the four gingival crevicular fluid collection sites. In more detail, each periodontal site included in the study was first isolated with cotton rolls. Before gingival crevicular fluid collection, if any supragingival plaque was present, it was removed with cotton pellets, and a gentle air stream was directed toward the tooth surface for 5 s, to dry the area. The gingival crevicular fluid was then collected using PerioPaper strips (Oraflow Inc., Smithtown, NY, USA), according to the method described by Offenbacher *et al.* (28). Briefly, the PerioPaper strips were inserted into the gingival crevice and left *in situ* for 60 s, thus collecting the resting gingival crevicular fluid (18). After removal of the PerioPaper strips and waiting for 60 s as the rest period, the second PerioPaper strips were inserted and left *in situ*, again for 60 s, thus collecting the flow gingival crevicular fluid (18). The samples of resting and flow gingival crevicular fluid were kept separately, although the four samples of the same type of gingival crevicular fluid (from each of the four collection sites) were pooled. Care was taken to avoid mechanical injury, and contamination of the gingival crevicular fluid samples was minimized by recording the PL+ before carefully cleaning the tooth with cotton pellets, collecting the gingival crevicular fluid from the isolated area and then recording the probing depth and BOP+, as previously described (7). Immediately after collection, the PerioPaper strips were transferred to plastic vials and stored under dry conditions at −80°C until analysis. All collections of gingival crevicular fluid were performed between 3 PM and 5 PM by the same operator (B.D.L.).

Enzymatic activity determination

The biochemical assays were performed by a single blinded operator (G.P.), as previously described (3). Briefly, the four samples from the four collection sites, for each of the resting and flow gingival crevicular fluid samples, were resuspended in 250 μL of buffer containing 200 mM Tris, 20 mM $MgCl_2$ (pH 9.8 ± 0.1) and 1 mg/mL of *p*-nitrophenol phosphate (N2770-5SET; Sigma Fast™; Sigma-Aldrich, St Louis, MO,USA). The samples were then incubated at 37°C (± fluctuations of < 0.1°C) for 3 h. During this incubation, the ALP in the samples hydrolyses the *p*-nitrophenyl phosphate to *p*-nitrophenol and inorganic phosphate. The reactions were then stopped by the addition of 5 μL of 3 M NaOH, and the absorbances were read using a spectrophotometer, at 405 nm (24). The relevant control for each analysis consisted of the reagent and the Tris buffer without the sample, and all of the samples were analyzed in a single session. 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 (2).

Data processing

The Statistical Package for Social Sciences program (SPSS® Inc., Chicago, IL, USA) was used to perform the data analysis. Parametric or non-parametric methods were chosen after testing the normality of the data using a Shapiro–Wilk test, and testing the equality of variance among the data sets using the Levene test. The percentage of tooth sites positive for plaque (%PL+) and for bleeding on probing (%BOP+) for each clinical session, and the mean probing depth at baseline and at 3 mo, were calculated for the maxillary incisors, considering them all together as the statistical unit. A Friedman test was used to evaluate the statistical significance of the differences of the %PL+ and %BOP+ over time. A paired Student's *t*-test was used to assess the significance of the differences in mean probing depth between readings taken at baseline and at 3 mo.

The resting and flow gingival crevicular fluid ALP activities were treated separately for the comparisons. Moreover, the overall gingival crevicular fluid ALP activities were defined as the (resting + flow) gingival crevicular fluid ALP activities, and these were also computed and analyzed statistically. For descriptive purposes, the mean, standard deviation, median, minimum, maximum and variability (standard deviation/mean, expressed as a percentage) were all calculated. The Friedman test was used to assess the significance of the difference for any of the gingival crevicular fluid ALP activities over time. Moreover, the Wilcoxon paired signed rank test was used to assess the significance of the differences between the resting and flow gingival crevicular fluid ALP activities within each clinical session.

For each gingival crevicular fluid ALP activity, the significance of the correlations between the values seen at the 1-d, 1-wk and 3-mo clinical sessions was also evaluated with regard to the corresponding baseline scores, according to the Spearman rho correlation coefficient. Moreover, intraclass correlation coefficients (ICCs) along the

95% confidence interval were calculated for each gingival crevicular fluid ALP activity at each 1-d, 1-wk and 3-mo clinical session, with regard to the corresponding baseline scores. An ICC of > 0.75 indicates 'excellent' reliability, an ICC of 0.40–0.75 represents 'fair to good' reliability and an ICC of < 0.40 is considered as 'poor' reliability (29). The ICC considers both the variability between individuals and between the test–retest recordings (e.g. at baseline and at 1 d).

With the aim of quantifying the full method error of the different recordings for each gingival crevicular fluid ALP activity, the method of moments (MME) variance estimator was used (27). Subsequently, for each gingival crevicular fluid parameter, the mean error and 95% confidence interval between the recordings at 1 d, 1 wk and 3 mo, with respect to those at baseline, were calculated using the MME variance estimator. The method errors were finally expressed as percentages. The MME variance estimator has the advantage of not being affected by any unknown bias (i.e. systematic errors) between pairs of measurements (27).

A $p < 0.05$ was used for rejection of the null hypothesis.

Results

All subjects were sampled at baseline, at 1 d and at 1 wk, but four of the subjects failed to present at the 3-mo clinical session.

The clinical conditions of the subjects were excellent throughout the study, with no significant differences at any time-point (data not shown). The overall mean ± standard deviation for %PL+ and %BOP+ were 8.0 ± 9.6 and 3.3 ± 5.5, respectively. Moreover, none of the specific sampling sites were BOP+ at any clinical session. The overall mean ± standard deviation for the probing depth was 1.6 ± 0.4 mm.

The gingival crevicular fluid ALP activities at each sampling time-point are given in Table 1. The resting gingival crevicular fluid ALP activity ranged from 13.69 ± 6.13 mU/sample at 1 d to 16.29 ± 8.72 mU/sample at 1 wk. The flow gingival crevicular fluid ALP activity yielded slightly lower values for each time point, which ranged from 9.97 ± 6.66 mU/sample at 1 d to 12.51 ± 4.88 mU/sample at 3 mo. The overall gingival crevicular fluid ALP activity also had a narrow range among the sampling time points, with values between 23.65 ± 6.95 mU/sample at 1 d and 26.88 ± 8.44 at 3 mo. No significant differences were seen over time for any of the resting, flow or overall gingival crevicular fluid samples. However, the flow gingival crevicular fluid ALP activity was significantly lower compared with the corresponding resting gingival crevicular fluid enzymatic activity, at 1 d and 1 wk time-points ($p < 0.001$). The variability was notably large, with values ranging from 29.4% (overall gingival crevicular fluid ALP activity at 1 d) to 59.3% (resting gingival crevicular fluid ALP activity at baseline).

The results of the reliability and method error analyses for the different gingival crevicular fluid ALP activities, compared with the corresponding baseline values, are given in Table 2. The Spearman correlation coefficients were generally low, and they did not reach statistical significance in any of the cases. In particular, these coefficients ranged from −0.13 (flow gingival crevicular fluid at 1 d) to 0.28 (overall gingival crevicular fluid at 1 wk). The ICCs were also generally low, with values of zero for the flow gingival crevicular fluid ALP activity seen at each 1 d, 1 wk and 3 mo sample; for the resting and overall gingival crevicular fluid, the ICCs were 0.07 at 1 d and 0.44 at 1 wk. Finally, the method errors

Table 1. Gingival crevicular fluid alkaline phosphatase (ALP) activities (as mU/sample) at the different sampling time-points according to the repeated samplings

Type of gingival crevicular fluid sample	Sampling time-point	Mean ± standard deviation	Median	Minimum	Maximum	Variability (%)
Resting	Baseline	14.16 ± 8.63	9.74	6.75	34.48	59.3
	1 d	13.69 ± 6.13	11.99	6.00	26.98	43.1
	1 wk	16.29 ± 8.72	13.49	3.75	38.20	51.0
	3 mo	14.36 ± 6.52	12.99	7.00	29.98	45.4
	Difference	$p = 0.218$; NS				
Flow	Baseline	11.49 ± 6.66	9.01	5.25	32.23	57.9
	1 d	9.97 ± 2.94*	8.99	6.75	17.99	29.6
	1 wk	10.82 ± 5.09*	10.49	4.50	30.73	41.1
	3 mo	12.51 ± 4.88	11.49	6.00	26.48	39.0
	Difference	$p = 0.162$; NS				
Overall	Baseline	25.65 ± 13.60	20.24	13.49	61.46	53.0
	1 d	23.65 ± 6.95	22.49	12.74	37.48	29.4
	1 wk	26.34 ± 10.44	23.99	10.25	50.22	39.7
	3 mo	26.88 ± 8.44	24.98	15.99	47.47	31.4
	Difference	$p = 0.620$; NS				

$n = 27$ for baseline, 1 d and 1 wk, and $n = 23$ for 3 mo.
*Significantly different from the corresponding first sampling, at $p < 0.01$.
NS, not significant.

Table 2. Reliability and method error analyses for the different gingival crevicular fluid alkaline phosphatase (ALP) activities, with respect to the baseline levels

Parameter	Type of gingival crevicular fluid sample	Sampling time-point		
		1 d	1 wk	3 mo
Rho correlation coefficient	Resting	−0.04 (−0.41–0.35)	0.30 (−0.09–0.61)	0.18 (−0.24–0.55)
	Flow	−0.13 (−0.49–0.26)	−0.07 (−0.44–0.32)	0.18 (−0.25–0.55)
	Overall	0.09 (−0.30–0.45)	0.28 (−0.11–0.59)	−0.05 (−0.45–0.37)
ICC	Resting	0.07 (0.00–0.57)	0.44 (0.00–0.75)	0.30 (0.00–0.70)
	Flow	–	–	–
	Overall	0.14 (0.00–0.61)	0.32 (0.00–0.69)	0.16 (0.00–0.64)
Method error	Resting	50.8% (25.7–75.9)	48.4% (24.4–72.3)	44.4% (22.4–66.4)
	Flow	47.9% (24.2–71.6)	58.0% (29.3–86.7)	54.4% (27.5–81.3)
	Overall	40.4% (20.4–60.5)	42.5% (21.5–63.6)	42.2% (21.3–63.1)

$n = 27$ for baseline, 1 d and 1 wk, and $n = 23$ for 3 mo.
ALL the data are presentes as mean (95% confidence interval).
ICC, intraclass correlation coefficient; –, ICC not derivable (negative value).

were generally greater for the flow gingival crevicular fluid and lower for the overall gingival crevicular fluid ALP activities. In particular, the mean errors for the resting gingival crevicular fluid ranged from 44.4% at 3 mo to 50.8% at 1 d. For the flow gingival crevicular fluid, the mean errors were between 47.9% for samples at 1 d and 58.0% for samples at 1 wk. The overall gingival crevicular fluid yielded mean method errors between 40.4% and 42.5%, again at 1 d and 1 wk.

Discussion

The present prospective longitudinal study initially reported on the repeatability and method error of gingival crevicular fluid collection and the subsequent quantification procedures to determine its ALP activity. Both resting and flow gingival crevicular fluid samples were collected. The data indicated a greater activity and slightly improved performance in terms of repeatability and method error for the resting gingival crevicular fluid compared with the flow gingival crevicular fluid, although the best performance was seen for the overall gingival crevicular fluid. However, while no systematic error was detected, the method error (i.e. intrasubject variability) ranged from 40 to 58%.

As the quality and quantity of gingival crevicular fluid changes during periodontal inflammation (18), local tissue health is necessary to exclude any possible unwanted bias. In the present study, all enrolled subjects were subjected to sessions of professional hygiene to avoid potential sources of bias, such as inflammation. The repeatability of the PL+, BOP+ and probing depth measurements were not determined. However, the periodontal status was optimal with minimal plaque accumulation and no bleeding at each session, and the operator was expert and well trained.

Sensitivity and specificity of the ALP assay were also not investigated because a highly sensitive kit was used under standardized protocols. However, the whole repeatability analysis performed herein would include any systematic or random error of this laboratory procedure. Moreover, ALP activity was expressed as total scores instead of being normalized by gingival crevicular fluid volume or gingival crevicular fluid total protein content, as this has been reported to be subject to less variability (2,30).

It is well known that traditional diagnostic procedures (i.e. probing depth or X-ray films) have inherent limitations, as they are sensitive to the periodontal disease history but have no predictive capabilities in terms of disease progression. For this reason, the use of gingival crevicular fluid biomarkers has been advocated for routine clinical practice (10). In spite of the evidence in favor of the use of gingival crevicular fluid ALP activity as a diagnostic aid across a wide range of periodontal (2,24) and orthodontic (3,25) treatments, the method errors associated with this gingival crevicular fluid enzyme remain unknown. In this regard, a previous study (2) analyzed gingival crevicular fluid ALP activity (expressed as total or concentration) and gingival crevicular fluid volume over time in a repeatability protocol. Unfortunately, this single investigation was limited to the analysis of systematic error (i.e. the significance of the differences in the mean values of activity recorded over the time points in a group of subjects). Indeed, this knowledge of the systematic error alone does not allow any important conclusions to be drawn about the repeatability of a method on an individual basis, which is necessary for diagnostic applications. Moreover, no distinction between resting and flow gingival crevicular fluid was reported (2).

The slightly lower gingival crevicular fluid activity seen here for the flow gingival crevicular fluid as compared with the resting gingival crevicular fluid is also consistent with previous findings (2), and this is to be expected, as most of the gingival crevicular fluid is recovered during the first (resting) collection (18). The lack of significant differences over time seen here supports the concept that the gingival crevicular fluid ALP activity, and presumably for all of the other gingival crevicular fluid constituents, is not subject to systematic variations, which would limit the diagnostic potential of these biomarkers. This conclusion is consistent with previous findings obtained for quantification of the gingival crevicular fluid ALP activity (2), and also for the the measurement of gingival crevicular fluid volume (13).

The lack of systematic error, however, does not exclude the presence of a certain degree of random error, as seen here when considering the results of the repeatability and method errors (Table 2). The ICC and correlation coefficients are useful indices for reliability analysis. While the ICC has not yet been used relative to gingival

crevicular fluid collection and quantification, the correlation coefficients have been used mainly to investigate the repeatability of the determination of gingival crevicular fluid volume (13), However, even when low, the ICC and the correlation coefficients still would not deny the diagnostic use of a given tool.

In this regard, the method error can provide a useful threshold to assess whether the differences recorded for individual subjects are real, irrespective of the statistical significance obtained through the analysis of a group of subjects. Moreover, by expressing the data as percentages, the method errors reported here are more widely applicable. The present study thus reports method errors for the resting and flow gingival crevicular fluid ALP activities of up to 50.8% and 58.0%, respectively (Table 2). As might be expected, lower errors, at up to 42.5%, are seen for the overall gingival crevicular fluid ALP activity (Table 2). These data do not deny the possible diagnostic use of gingival crevicular fluid measurements, but instead they set the threshold that will be needed to assume individual variations as reliable changes whenever dealing with healthy periodontal conditions. Indeed, in spite of the low ICCs and correlation coefficients, a difference of at least 50% in gingival crevicular fluid ALP activity can be considered as reliable in the diagnosis of any changes in the periodontal tissues (i.e. healing after treatment, or in the prediction of attachment loss).

The low repeatability and high method errors encountured in the present study might be a result of individual variations over time, although they might also relate to the collection procedures. Moreover, the amount of gingival crevicular fluid collected from a subject with good gingival health with a probing depth of about 1 mm would be expected to be very small. On the contrary, during gingivitis (2) or periodontitis (24), such as in the case of monitoring the responsiveness or recurrent inflammation of a deep periodontal pocket to treatment, the expected larger amounts of gingival crevicular fluid collected would be consistent with smaller collection errors.

In terms of reliability, the use of the resting gingival crevicular fluid appears better when compared with the flow gingival crevicular fluid, even though a combination of both (the overall gingival crevicular fluid) can give a better performance. Therefore, collection of a second (or third) gingival crevicular fluid sample according to the method of Offembacher (28) is preferable, as long as this is pooled with the first (resting) sample.

A previous study (5) showed good prediction capabilities of total gingival crevicular fluid ALP activity with regard to attachment, with up to 64% accuracy. Even though this previous study did not include any method error analysis, the mean enzymatic activities for the active sites were about 50% higher than those for the control sites. Moreover, another study (24) showed that total gingival crevicular fluid ALP activity reflects the healing and recurrent inflammation phases of chronic periodontitis, whereby gingival crevicular fluid ALP activity was reduced by about 40% and 50% in 4–6 mm and > 6 mm pockets, respectively, after a session of scaling and root planing. These previous data show that changes of up to 50% in gingival crevicular fluid ALP activity can be detected, and hence the diagnostic capabilities of this gingival crevicular fluid parameter can indeed be proposed. Further confirmation derives from a study (3) that showed a two-fold peak increase in gingival crevicular fluid ALP activity in pubertal subjects, as compared with prepubertal subjects, thus proposing this parameter as a diagnostic aid in the assessment of the growth phase in individual subjects, to establish correct treatment timing. However, the differences in the protocols used to collect the gingival crevicular fluid among these different studies also need to be taken into account.

Conclusions

Reliable use of the gingival crevicular fluid collection and quantification, both in research and diagnosis on an individual basis, should take into account relevant errors. For the ALP activity, variations are to be considered as genuine only above relevant thresholds, which are at least 40%.

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Conflict of interest

The authors declare that they have no competing financial interests.

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