DETECTION OF TYPE I COLLAGEN FRAGMENTS IN THE GINGIVAL CREVICAL FLUID AND SALIVA DURING ORTHODONTIC TOOTH MOVEMENT

Authors list

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ABSTRACT

Introduction: The purpose of this study was to investigate the levels of Type I Collagen fragments ICTP (cross-linked carboxyterminal telopeptide of type I collagen) and CTX (C-terminal cross-linked telopeptide of type I collagen) in gingival crevicular fluid (GCF) and saliva in the leveling and alignment phase of orthodontic tooth movement.

Materials and methods: Fourteen adolescent patients [5 males, 9 females; mean age = 15.5 yrs (range = 13.1 - 17.6)] with moderate mandibular incisor crowding were fitted with preadjusted edgewise brackets and 0.014 nickel-titanium (NiTi) wires. The main outcome measure was the levels of CTX and ICTP in the GCF and saliva. Samples were obtained from lower anterior teeth at 0, 1, 3, 7, 14, and 21 days after appliance placement. Enzyme-linked immunosorbent assay analysis (ELISA) was used to measure the levels of ICTP and CTX in the collected samples.

Results: GCF and saliva ICTP and CTX levels did not significantly change between the time points (GCF ICTP P= 0.529, GCF CTX P= 0.581, saliva ICTP P= 0.023, saliva CTX P= 0.390). A statistically significant positive correlation and agreement was found between the levels of ICTP and CTX in saliva (P= 0.0001). However, there was no statistically significant correlation between the GCF and salivary levels of either ICTP or CTX or between ICTP and CTX levels in GCF. The CTX saliva level was not a statistically significant predictor of ICTP in saliva (p = 0.986).

Conclusion: Changes in ICTP and CTX levels in human GCF and saliva cannot be used as biological biomarkers during the initial stage of orthodontic tooth movement in adolescent patients.

Clinical trials registration number: NCT05020431.

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Keywords: Orthodontic tooth movement, Type I collagen fragments, ICTP, CTX, Gingival crevicular fluid (GCF), Saliva.

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We confirm that:
1. This manuscript has not been sent for publication, nor has it been published in whole or in part elsewhere.
2. I attest that all the authors have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to the Egyptian Orthodontic Journal.
INTRODUCTION

Tooth movement occurs as a result of controlled mechanical forces applied to the teeth and the biological response to these forces. The mechanical forces applied to teeth induce strains that alter the periodontal ligaments (PDL) blood flow resulting in the release of various substances such as inflammatory mediators, cytokines, growth factors, enzymes, and metabolic products. These substances or biomarkers can provide a favorable micro-environment for bone resorption or apposition by evoking osteoblastic and osteoclastic activity [1].

A biomarker or a biological marker is defined as a substance that can be objectively measured to assess normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [2]. Biological biomarkers can modulate bone resorption by stimulation of osteoclastogenesis, activation of osteoclasts via the release of the receptor activator of the nuclear factor kappa-B ligand, inhibition of osteoprotegerin and deformation of the extracellular matrix resulting in bone resorption [3].

The GCF is a biological exudate that is released into the gingival sulcus, which offers a great source of biomarkers associated with changes in the underlying periodontium due to orthodontic force application [4]. Quantification of its constituents is a classical way to identify specific biomarkers associated with bone resorption during orthodontic tooth movement [5]. On the other hand, salivary analysis is a simple, non-invasive, and safe collection method that can be used instead of GCF for measuring biomarkers associated with orthodontic force application [6,7]. The salivary biomarkers of inflammation and bone turnover are derived from both the GCF and the mucosal transudate [8].

Bone destruction and collagen breakdown are correlated with molecules released in saliva and GCF [9]. After bone resorption, type I collagen is degraded by proteolytic enzymes such as matrix metalloproteinases (MMPs) and cathepsin K which lead to the release of stable fragments such as cross-linked carboxyterminal telopeptide of type I collagen (ICTP) and C-terminal cross-linked telopeptide of type I collagen (CTX). The generation of these markers depends on different enzymatic pathways which allow the assay of these two biomarkers to discriminate between different bone pathologies [10].

ICTP and CTX have been shown to be useful for the evaluation of the rates of bone remodeling in metabolic bone diseases such as hyperparathyroidism [11], post-menopausal osteoporosis [12] and rheumatoid arthritis [13]. Moreover, ICTP and CTX represent a potentially valuable diagnostic aid that may be useful in the differentiation of gingivitis from periodontitis [14] and peri-implant bone destruction [15].

ICTP has been found to be expressed in both GCF and saliva. The levels of ICTP have been shown to be elevated in the GCF during periodontal disease progression [16]. Additionally, there was an increase in the salivary concentration of ICTP in chronic
periodontitis patients than in gingivitis and healthy patients \cite{17}. Similarly, GCF levels of CTX were found to be elevated in periodontitis patients and its levels subsequently reduced after periodontal therapy \cite{18}. Salivary CTX has shown a statistically significant difference between periodontally healthy and diseased groups, where the concentrations of salivary CTX were higher in subjects with periodontitis than in controls \cite{19}.

To the best of our knowledge, there is a paucity of studies that measured the GCF and salivary levels of ICTP and CTX during orthodontic tooth movement. Since the two biomarkers, both products of degradation of Type I Collagen are produced by distinct enzymatic pathways (cathepsin K vs. matrix metalloproteinase MMP), \cite{10} we were interested to find out which of the two collagen breakdown products predominates. The knowledge of the enzymatic pathway will help our understanding of the biological processes underlying orthodontic tooth movement. Possible applications include relating the velocity of orthodontic tooth movement to genetic variations between individuals expressing different levels of the aforementioned enzymes. Another possible application is pharmacologically targeting these pathways. Expression of these biomarkers in saliva will offer a more convenient method for monitoring tooth movement.

Hence, the objective of this study is to measure the change in the levels of ICTP and CTX biomarkers in the GCF and saliva during initial leveling and alignment as a model of orthodontic tooth movement in adolescent subjects. The null hypothesis is that there is no significant change in the GCF and salivary level of ICTP and CTX during initial leveling and alignment in a sample of adolescent patients treated with fixed orthodontic appliances.

**Materials and methods**

**Subjects:**

Fourteen healthy adolescent patients (mean age, 15.15 years [range, 13.0–17.6]); five boys and nine girls from the Department of Orthodontics, Faculty of Dentistry, Alexandria University, Alexandria, Egypt were recruited for this study. All subjects required orthodontic treatment for moderate mandibular incisor crowding. The amount of mandibular anterior crowding was measured using Little’s Irregularity Index \cite{20} before and 21 days after appliance placement using dental casts poured from alginate impressions. **Table 1**

All subjects were screened by the principal investigator O.T. to meet the following **inclusion criteria:** 1. Permanent dentition; 2. No previous orthodontic treatment; 3. Good oral hygiene [Plaque index = 0 – 1; Gingival index = 0 – 1] \cite{21}; 4. Regular salivary flow (0.3-0.4 ml/min) \cite{22}. While **exclusion criteria** included: 1. History of orthodontic or periodontal treatment other than routine prophylaxis; 2. Systemic diseases or metabolic bone diseases affecting bone metabolism or periodontal supporting tissues; \cite{23} 3. Smokers; and 4. Recent history of antibiotics or anti-inflammatory drug administration.
A minimum of 14 subjects were needed to detect a standardized effect size of 0.674 in the change of the levels of ICTP and CTX at a power of 80% and level of significance of 95% ($\alpha=0.05$) based on the findings of Nunes et al [24]. The power analysis was conducted using GPower version 3.1.9.2 [25].

This study was performed in accordance with the guidelines of the Declaration of Helsinki. The study protocol was approved by the Ethical Committee at the Faculty of Dentistry, Alexandria University (IRB NO: 00010556 – IORG 0008839). The study was registered on Clinical Trials.gov (NCT05020431). No changes to the study protocol were made throughout the trial. Signed informed consent was obtained from the patients’ parents or guardians before the onset of the study.

**Treatment protocol**

Preadjusted edgewise brackets 0.022 x 0.028-inch slot size (Forestadent, Pforzheim, Germany) were bonded to all mandibular teeth using a light-cured orthodontic composite (Enlight, Ormco, California, USA) system. A 0.014 round NiTi archwire (Ormco, California, USA) was ligated to the brackets with elastomeric ligatures.

**Sample collection**

The GCF and salivary samples were collected in the morning from 9:00 to 11:00 am [26]. Samples were obtained from lower incisors and canine teeth at 6-time points: 0, 1, 3, 7, 14, and 21 days after appliance placement. The patient was informed not to eat or drink one hour prior to sample collection [27].

GCF was collected from mesiobuccal and distobuccal aspects of the lower six anterior teeth with sterile paper strips (Whatman International Ltd, Maidstone, UK), cut into a standardized size (2 x 10 mm) [28]. Before GCF collection, the teeth were isolated with cotton rolls, cleaned of plaque deposits and dried gently with air before paper strips were carefully inserted into the gingival crevice until a slight resistance was felt. Each GCF strip remained in position for a total of 60 s [29]. (Figure 1) Lamster et al [30] recommended a GCF collection time of 30 s with filter paper strips. GCF collection times have been found to vary greatly in the literature ranging from as little as 30 seconds up to 3 minutes due to the high variability of GCF fluid flow which was found to be slower in healthy hosts [29]. However, the collection time used in this study was increased to 60 s since we have not been able to collect sufficient GCF volume for testing.

Papers with visible blood contamination were discarded. After the collection of GCF, filter paper strips were immediately placed in Eppendorf tubes containing 200 µL of phosphate-buffered saline. The tubes were then centrifuged at 4000 RPM for 5 min and stored at −80 °C for further study.

Salivary samples were collected according to Navazesh and Kumar collection procedure [22]. 5 ml of the unstimulated saliva was collected in graduated sterile plastic tubes. After centrifugation at 4000 RPM for 5 minutes to remove debris, the supernatant fraction was kept at −80 °C for further study.
Analysis of biomarker levels

In GCF and saliva samples, ICTP and CTX analyses were made with the Enzyme-linked immunosorbent assay analysis (ELISA) method (Bioassay Technology Laboratory, Zhejiang, China) (Cat.NO: E1334Hu, E1349Hu), following the package inserts. ELISA kits in this study were devised to detect the 0.1–30 ng/mL range with a sensitivity of 0.051 ng/ml for ICTP and 7–1500 ng/ml range with a sensitivity of 4.21ng/ml for CTX. The optical density value of each well was immediately determined using a microplate reader set to 450 nm within minutes after adding the stop solution. The concentration of ICTP and CTX were determined from the standard curves where the levels in GCF were expressed as ng/min and the levels in saliva were divided by the collected volume (5 ml) expressed as ng/ml.

Considering the small volume of the collected GCF, the authors reported the biomarker levels as ng/min sample instead of correcting for the volume of GCF. Wassal and Preshaw [31] recommended this approach for small volume samples to avoid errors associated with GCF fluid volume measurement using Periotron, where the small volume may fail to be registered or fall below the calibration curve’s lower limit. It has been reported that volume determination represents a source of error in GCF marker level concentration [32].

Statistical analysis

Normality of quantitative data was checked using Shapiro Wilk test, box plots and descriptives. Data was not normally distributed and presented using mainly Median and 1st and 3rd Quartiles.

Age was presented using mean and standard deviation and gender was presented using frequency and percent. Age was compared using independent t test and gender was compared using Fisher’s Exact test. Changes across time intervals within each group were assessed using Friedman test followed by post hoc test when results were significant. Correlation between ICTP and CTX levels in GCF and saliva was estimated using Spearman's rank correlation coefficient. Curve estimation regression was performed to identify models to be used then linear predictive models were performed using salivary parameters such as ICTP and CTX as independent variables. Age and gender were adjusted for all models. All tests were two tailed and the significance level was set at P value of 0.05. All tests were two tailed.

Data were analyzed using IBM SPSS for windows version 23, Armonk, NY, USA. All Figures were depicted using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA.
Results

I. Demographic data
Fourteen adolescent patients with a mean age of 15.15 (range = 13.1 - 17.6 yrs). 5 males (35.7%), 7 females (64.3%) participated in the study. Table 1.

The mean pre-irregularities score was 5.43 mm, SD=2.59, while the mean post-irregularities score was 4.51 mm, SD=2.59. There was a statistically significant decrease in Little's irregularity index at 21 days [95% C.I. 3.02, 6.01; p = 0.001]. Table 1.

II. Changes in ICTP and CTX biomarker levels
A total of 84 GCF and 84 saliva samples were collected at the specified time points; none of the recruited subjects were lost to follow-up. The changes in ICTP and CTX levels in GCF and saliva in every subject are presented in Figure 2.

The median (Q1 – Q3) of ICTP and CTX levels in GCF and saliva are shown in Table 2. There was no statistically significant difference in ICTP levels in GCF or saliva between the different time points. Similarly, CTX levels showed no statistically significant difference between time points in either GCF or saliva.

Box plots of the levels of ICTP and CTX in GCF and saliva are shown in Figure 3.

III. Relationship between ICTP and CTX GCF levels to their respective levels in saliva
The Spearman's rank correlation between pooled ICTP levels in saliva and GCF was not statistically significant (rho = -0.021, p = 0.847). The correlation between CTX levels in saliva and GCF was also not statistically significant (rho = -0.033, p = 0.763). The correlation between CTX and ICTP levels in GCF was also not statistically significant (rho = 0.144, p = 0.191). However, a statistically significant positive correlation between CTX and ICTP levels in saliva (rho = 0.832, p < 0.0001). The correlation between pooled ICTP and CTX levels in saliva and GCF is shown in Table 3.

IV. Relationship between the levels of ICTP and CTX in GCF and saliva
The intraclass correlation coefficient showed non statistically significant agreement between ICTP levels in GCF and saliva as well as CTX levels in GCF and saliva [ICC = -0.034, p = 0.621; ICC = 0.019, p = 0.430, respectively].

The intraclass correlation coefficient showed a statistically significant strong agreement for the levels of ICTP and CTX in saliva (ICC= 0.776, p = 0.0001). Otherwise, all the tested agreements were not statistically significant. The intraclass correlation between CTX and ICTP levels in GCF and saliva is shown in Table 4.

However, the linear regression model of ICTP and CTX saliva levels CTX was not statistically significant [B = -0.003, C.I. =-0.299, 0.294, p =0.986]. The quadratic models showed non statistically significant results as well. Hence, CTX saliva level cannot be used as a predictor for ICTP saliva level. The regression models predicting is shown in Table5.
Discussion

When orthodontic appliances exert forces on teeth, eventually movement occurs. Alveolar bone remodeling, tissue inflammation, and root resorption are the main phenomena that occur before, during and following tooth movement. Each of these events can potentially be detected using appropriate biomarkers [33]. A good biomarker should be able to provide information about the biological status of periodontal tissue changes. Evaluation of these biological mechanisms can be done by assessing the rate and amount of biomarker synthesis in the periodontal tissue [34].

Understanding the modeling and remodeling of periodontal tissues during orthodontic treatment may be a clinically useful procedure allowing the proper selection of optimum force magnitude that minimizes unnecessary tissue damage, and hence shortens the course of treatment [35]. Bone resorption is caused by proteolytic enzymes which breakdown type I collagen into crosslinked C-terminal telopeptides such as ICTP and CTX which are released into the circulation as stable fragments [10]. CTX and ICTP release has been shown to be mediated by different enzymatic pathways [10]. Finding out which collagen breakdown product is the more predominant during orthodontic force application may provide pharmaceutical targets for the control of orthodontic tooth movement either by increasing its rate during active treatment or reducing it during the retention phase.

GCF and saliva are particularly promising because they can be obtained non-invasively with minimal patient pain and they contain both locally synthesized and systemically obtained molecules. Examining various physiologically specific proteins or markers in oral fluids using immunologic or biochemical techniques may offer information on what is happening in the periodontal microenvironment [36]. Notwithstanding, contamination of GCF strips with blood, saliva, or plaque is also a potential problem with GCF collection. Compared to GCF, saliva collection is easier, noninvasive, does not require special equipment and can also provide abundant information about the periodontium due to the open communication between GCF and saliva. Several biomarkers were discovered in saliva during orthodontic treatment [37,38].

In this study, it was found that there was no statistically significant difference between the time points for the levels of ICTP or CTX in GCF or saliva during initial orthodontic tooth movement. Similarly, Kloukos et al [39] reported no statistically significant changes in CTX levels in the GCF collected before, 5 and 14 days of orthodontic tooth movement. The authors postulated that these biomarkers may not reflect a part of the biological activity in the periodontium during the early phases of orthodontic treatment. Alternatively, it was suggested that the specific biomarkers in the GCF and serum of the lower anterior teeth may not be representative of the local bone microenvironment [39].

On the other hand, Huang et al [40] reported a significant increase in GCF CTX in the fourth week following the application of 100g of force to the premolar; it may be argued that collagen breakdown products are only
expressed in response to heavy forces. However, the force exerted by 0.014" NiTi wire has been estimated to be between 40 – 60g [41,42]. This may explain the ability to detect GCF CTX in response to 100g of force applied in the study by Huang et al [40]. Cesur et al [43] found a transient increase in CTX one week after loading orthodontic miniscrews with 75 or 150 g. It may be postulated that CTX can be more readily detected in tissue fluids in direct contact with the local bone environment where no periodontal ligament intervenes. Therefore, translation of findings from peri-implant sites to GCF should be done with caution.

To date, there has not been published data on the salivary concentration of CTX and ICTP during orthodontic tooth movement. Some studies have reported that salivary ICTP levels were below the detection level in subjects of periodontitis [44,45], whereas others found significantly higher levels in the periodontitis group compared to the control group [17,46]. In the same manner, Al-Sabbagh et al [47] found the saliva levels of β-CTX were at or below the level of detection in all samples. However, Kumar et al [48] reported there was a statistically significant increase in salivary CTX levels in chronic periodontitis when compared to healthy controls, and these levels reduced significantly after treatment. Similarly, Akram et al [49] demonstrated that the GCF CTX values were significantly correlated with the probing depth and clinical attachment loss for periodontal disease patients; however, CTX was hardly detected at baseline for healthy sites. It appears that ICTP and CTX GCF levels are readily detected in cases with significant bone destruction, whereas the initial stage of orthodontic tooth movement with light forces results in physiologic bone modeling and remodeling.

In the present study, a consistent correlation of moderate strength has been found between ICTP and CTX salivary levels. Previous studies have shown a significant correlation between saliva, and GCF levels of bone turnover biomarkers [50,51]. For example, Behfarnia et al [51] reported that the RANKL/OPG ratio was positively correlated between GCF and saliva. Others found there was no correlation between GCF and saliva levels of osteocalcin regardless of periodontitis status [52]. No strong assumptions can be based on the correlation found in the current study, since the levels of the biomarkers were invariably constant between the time points.

One of the limitations of this study may be the short sampling period. In routine practice, a small diameter archwire, 0.014-inch NiTi is applied to the teeth for 3 weeks (21 days). As tooth movement progresses, a larger diameter archwire is inserted and a new orthodontic stimulus is initiated [53]. The sampling period was limited to 21 days to avoid the change in force magnitude brought about by changing the archwire. Moreover, GCF flow rate and volume have been found to be the highest at 14 and 21 days following orthodontic force application to reach minimal volume at 34 days [54]. Furthermore, orthodontically associated tissue modeling starts with the resorption phase where the life span of osteoclast is short in the basic multicellular unit (3 weeks) [55]. Since ICTP and CTX are markers of tissue breakdown, the greatest variations in
Biomarker levels are expected in the early stages of orthodontic tooth movement [39].

Another limitation of the study is the use of one technique to detect ICTP and CTX in saliva and GCF. Changes in the levels of both biomarkers in either GCF or saliva have not been extensively studied. Since physiologic force levels are recommended for orthodontic tooth movement, the resultant tissue modeling is expected to be subtle, hence the small magnitude of the biomarker detected in GCF and saliva [39]. This holds true when the levels of the biomarkers are compared to those associated with surgical interventions [56] and periodontal diseases/therapy [48,56]. It has been suggested that monoclonal antibody ELISA may be a more suitable approach especially when low biomarker concentrations are present [57].

The results of this study can only be generalized to the adolescent patient population when gentle orthodontic forces are applied in the initial stages where small diameter resilient archwires are used.

Conclusion

1. ICTP and CTX does not considerably change during initial orthodontic tooth movement. Neither assay can be used as a biomarker for orthodontic tooth movement, either in GCF or Saliva.

2. No relationship could be demonstrated between GCF and saliva level of ICTP or CTX.

3. Saliva levels of ICTP and CTX showed good agreement. However, it has not been possible to statistically model this relationship.

Further research is required for later stages of orthodontic tooth movement involving a higher level of force and a longer duration of treatment.

Declarations:

Ethics approval and consent to participate

This study was performed in accordance with the guidelines of the Declaration of Helsinki. The study protocol was approved by the Ethical Committee at the Faculty of Dentistry, Alexandria University (IRB NO: 00010556 – IORG 0008839). A signed informed consent was obtained from all participants before the onset of the study. Participants under the age of 16 have obtained informed consent from their parents or legal guardians.

Availability of supporting data

All data generated or analyzed during this study are included in this published article in the form of tables and figures.

Authors’ contribution:

O.T: Protocol writing, Carrying out experimental procedures, Data collection, manuscript preparation. N.T: Supervised the laboratory procedures, analysis of results, and revision of the manuscript. N.E: Interpreted results and revised the manuscript. H.K: Conceptualization, Protocol preparation, supervised the clinical procedures, data analysis, and preparation of the manuscript.

All authors reviewed the manuscript.

Acknowledgement

Not applicable.
REFERENCES


25. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and


37. Flórez-Moreno GA, Isaza-Guzmán DM, Tobón-Arroyave SI. Time-related changes in salivary levels of the osteotropic factors sRANKL and OPG through orthodontic


48. Kumar SA. S. Aravind Kumar. Effect of phase I therapy on salivary carboxy terminal telopeptide of type I collagen in chronic periodontitis patients Effect of phase I therapy on salivary carboxy terminal telopeptide of


Table 1: Demographic of the participants

<table>
<thead>
<tr>
<th>Age (yrs): Mean (SD)</th>
<th>15.15 (1.71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender: n (%)</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>5 (35.7%)</td>
</tr>
<tr>
<td>Females</td>
<td>9 (64.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>14 (100%)</td>
</tr>
<tr>
<td>Pre-treatment Little's irregularity index (mm) n=14</td>
<td>Mean (SD) 5.43 (2.59)</td>
</tr>
<tr>
<td></td>
<td>95% CI 3.94, 6.92</td>
</tr>
<tr>
<td>Post-treatment Little's irregularity index (mm) n=14</td>
<td>Mean (SD) 4.51 (2.59)</td>
</tr>
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<td>95% CI 3.02, 6.01</td>
</tr>
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</table>

*Statistically significant at \( p \leq 0.05 \)

Table 2. ICTP and CTX levels in GCF and saliva at 0, 1, 3, 7, 14, and 21 days after appliance placement.

<table>
<thead>
<tr>
<th>Time points</th>
<th>ICTP (n=14)</th>
<th>CTX (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GCF (ng/min)</td>
<td>Saliva (ng/ml)</td>
</tr>
<tr>
<td></td>
<td>Median (Q1 – Q3)</td>
<td>Median (Q1 – Q3)</td>
</tr>
<tr>
<td>0 day</td>
<td>97.55 (82.52 – 103.13)</td>
<td>0.65 (0.44 – 0.83)</td>
</tr>
<tr>
<td>1 day</td>
<td>94.00 (82.05 – 98.03)</td>
<td>0.53 (0.45 – 0.80)</td>
</tr>
<tr>
<td>3 days</td>
<td>94.30 (88.95 – 102.98)</td>
<td>0.59 (0.45 – 0.82)</td>
</tr>
<tr>
<td>7 days</td>
<td>87.65 (80.75 – 101.12)</td>
<td>0.75 (0.46 – 0.83)</td>
</tr>
<tr>
<td>14 days</td>
<td>95.35 (75.48 – 106.43)</td>
<td>0.61 (0.50 – 0.77)</td>
</tr>
<tr>
<td>21 days</td>
<td>99.20 (91.75 – 107.18)</td>
<td>0.64 (0.54 – 0.86)</td>
</tr>
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| Test (p value) | 3.098 (0.685) | 3.429 (0.634) | 5.020 (0.413) | 4.939 (0.423) |

ICTP, cross-linked carboxyterminal telopeptide of type 1 collagen; CTX, C-terminal telopeptide of type I collagen; GCF, gingival crevicular fluid; Q1 – Q3, 1st and 3rd Quartiles.

*Statistically significant difference at \( p \leq 0.05 \),
Table 3: Correlation between ICTP and CTX levels in GCF and saliva.

<table>
<thead>
<tr>
<th></th>
<th>ICTP (n=14)</th>
<th>GCF</th>
<th>Saliva</th>
<th>CTX (n=14)</th>
<th>GCF</th>
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<tr>
<td></td>
<td>rho</td>
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<tr>
<td>ICTP</td>
<td>Saliva</td>
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<tr>
<td>GCF</td>
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<td>0.654</td>
<td>0.832</td>
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<tr>
<td>CTX</td>
<td>Saliva</td>
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<td>0.832</td>
<td>&lt;0.0001*</td>
<td>0.033</td>
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<tr>
<td>GCF</td>
<td></td>
<td>0.144</td>
<td>0.191</td>
<td>0.050</td>
<td>0.654</td>
<td>1.00</td>
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</table>

ICTP: cross-linked carboxyterminal telopeptide of type 1 collagen; CTX: C-terminal telopeptide of type I collagen; GCF: gingival crevicular fluid; rho: Spearman correlation coefficient.

*Statistically significant at p ≤ 0.05.

Table 4. Intraclass correlation coefficients between overall ICTP and CTX in GCF and saliva.

<table>
<thead>
<tr>
<th></th>
<th>ICTP (n=14)</th>
<th>GCF</th>
<th>Saliva</th>
<th>CTX (n=14)</th>
<th>GCF</th>
<th>Saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICC</td>
<td>P value</td>
<td>ICC</td>
<td>P value</td>
<td>ICC</td>
<td>P value</td>
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<tr>
<td>ICTP</td>
<td>Saliva</td>
<td>-0.034</td>
<td>0.621</td>
<td>-0.034</td>
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<tr>
<td>GCF</td>
<td></td>
<td>-0.034</td>
<td>0.621</td>
<td>-0.034</td>
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<tr>
<td>CTX</td>
<td>Saliva</td>
<td>-0.083</td>
<td>0.776</td>
<td>0.776</td>
<td>&lt;0.0001*</td>
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<tr>
<td>GCF</td>
<td></td>
<td>0.061</td>
<td>0.289</td>
<td>0.056</td>
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</table>

ICTP: cross-linked carboxyterminal telopeptide of type 1 collagen; CTX: C-terminal telopeptide of type I collagen; GCF: gingival crevicular fluid; ICC: intraclass correlation coefficients.

*Statistically significant at p ≤ 0.05

Table 5. Regression models predicting.

<table>
<thead>
<tr>
<th>Model Type</th>
<th>Constant</th>
<th>Parameter estimate</th>
<th>95% CI</th>
<th>Adj R²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>41.866</td>
<td>-0.003</td>
<td>-0.299, 0.294</td>
<td>0.036</td>
<td>0.986</td>
</tr>
<tr>
<td>Quadratic</td>
<td>49.443</td>
<td>-0.454</td>
<td>-1.279, 0.371</td>
<td>0.040</td>
<td>0.277</td>
</tr>
<tr>
<td>Quadratic</td>
<td>56.705</td>
<td>0.009</td>
<td>-1.296, 1.314</td>
<td>-0.003</td>
<td>0.989</td>
</tr>
<tr>
<td>Quadratic</td>
<td>59.360</td>
<td>-0.659</td>
<td>-2.488, 1.171</td>
<td>0.030</td>
<td>0.476</td>
</tr>
<tr>
<td>Quadratic</td>
<td>59.360</td>
<td>0.005</td>
<td>-0.008, 0.018</td>
<td>0.050</td>
<td>0.472</td>
</tr>
</tbody>
</table>

Adj R²: Adjusted R squared, CI: Confidence Interval

*Statistically significant difference at p ≤ 0.05.
Figure 1. Collection of GCF from the mandibular incisors and canines using standardized paper strips. Before appliance placement; 1 day after appliance placement
Figure 2. ICTP and CTX levels in GCF and saliva at 0, 1, 3, 7, 14, and 21 days after appliance placement.
Figure 3. ICTP and CTX levels in GCF and saliva at 0, 1, 3, 7, 14, and 21 days.