Research Article

Diagnostic biomarkers for periodontitis (observational case-control study)

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Abstract: Background: Early detection of periodontal tissue loss prevents further development and halts additional damage. The purpose of this study was to investigate the diagnostic ability of salivary Pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen (ICTP) and deoxypyridinoline (DPD) in differentiating periodontitis from periodontal health. Material and method: A case-control included 80 participants who were divided into two groups: 40 periodontitis patients and 40 subjects with a healthy periodontium. Salivary samples were collected from each patient, followed by a clinical examination. The collected saliva samples were centrifuged and frozen at -80°C until analysis using Enzyme-Linked Immunosorbent Assay. Results: the results indicated that both biomarkers were effective in diagnosis periodontitis. The area under the curve (AUC) for ICTP was 0.99 and the proposed cut-off point was 6.6 ng/ml, while for DPD the AUC was 0.95 and the proposed cut-off point was 211.5 nmol/L. Conclusion: Salivary ICTP and DPD demonstrated diagnostic ability in distinguishing periodontitis from a healthy periodontium, making them valuable tools in early detection and management of periodontal diseases.

Keywords: periodontitis, ICTP, deoxypyridinoline, diagnosis.

Introduction

Periodontitis is an inflammatory periodontal disease brought on by a variety of pathogenic microorganisms that result in loss of attachment and resorption of alveolar bone (1). Additionally, it is a complex, chronic disease characterized by host-mediated inflammation of the periodontal tissue in in response to plaque biofilms, which results in the progressive destruction of the tooth-supporting apparatus and loss of periodontal attachment (2). It is distinguished by the induction of osteoclastogenesis and the subsequent permanent breakdown of alveolar bone, which causes loss of tooth support (3).

Improvements have been made to the classification system of periodontal and peri-implant diseases and conditions since its introduction in 2017, but current clinical diagnosis methods (such as inspection, palpation, periodontal probing, and radiography) are still unable to meet the needs of periodontitis detection and diagnosis. This has increased the need for new diagnostic procedures, one of which is the utilization of biomarkers found in oral fluids such as gingival crevicular fluid and saliva to make up for the issues inherited in the currently used methods (4).

Around 90% of the organic matrix in bones is composed of collagen type I, which is more common in osseous tissue. Trypsin and bacterial collagenase break down type I collagen of bone to release a 12–20 kD fragment known as ICTP. As periodontal disease progresses, increased ICTP levels are directly correlated with increased collagen degradation (5,6).

Deoxypyridinoline (DPD) is a particular biomarker of bone resorption. It is generated by the reaction of the side chains of a lysine molecule and two hydroxylysine molecules. DPD is mainly found in bones, but not so much in dentine (7), and it is specific for bone breakdown (8). To the best of our knowledge, no study until now investigates these biomarkers as diagnostic biomarkers for periodontitis, therefore, the present
study aimed to investigate the diagnostic potential of salivary concentration of ICTP and DPD in differentiating periodontitis from periodontal health.

**Materials and Methods**

**Study population**

A case-control, observational study was carried out in the Baghdad teaching hospital, dental department. The study was started from January to June 2022. An informed consent form was signed by each patient after all information had been provided describing the aim as well as the nature of the study. Meanwhile, this study was also conducted following ethical guidelines, including the World Medical Association Declaration of Helsinki. Granted by the relevant Ethics Committee of the College of Dentistry, University of Baghdad (ref. number: 449, Date: 19/1/2022).

Before enrolment with the current study, the dental and medical history of each patient was recorded using a questionnaire including age, sex, the medication used, and the full medical history of the subject as well as the history of the previous periodontal treatment. Next, salivary samples were taken from each patient, and a clinical examination was conducted after that. The periodontitis case was determined if there was: 1- "Interdental clinical attachment loss (CAL) is detectable at ≥ 2 non-adjacent teeth, or 2 teeth with buccal or oral CAL ≥ 3 mm with probing pocket depth (PPD) ≥ 3 mm was detectable at ≥ 2 teeth"\(^{(1)}\). While individuals with healthy periodontium should be with intact periodontium with a probing pocket depth of less than 3mm and "bleeding on probing" of less than 10 % \(^{(9)}\). Regarding study design and results reporting, this clinical study adheres to "STROBE, or Strengthening the Reporting of Observational Studies in Epidemiology".

**Inclusion criteria**

Participants selected for the study should be: systemically healthy, and have a minimum of 20 teeth. Not under medications in the last 3 months. Furthermore, periodontitis cases were generalized periodontitis with more than 30% of teeth included in loss of attachment exhibiting unstable status (PPD ≥5mm or PPD 4mm with BOP).

**Exclusion criteria**

Including smoking and those with bowel inflammatory diseases, such as "Crohn's disease or diabetes mellitus", liver or renal malfunction, a history of organ transplantation or cancer treatment, or a history of cardiovascular disease and hyperparathyroidism. Furthermore; any substantial periodontal therapy that has been administered in the past or ongoing periodontal therapy. Patients who have used immunosuppressant or antimicrobial medicine within the last three months.

**Sample size and grouping of subjects**

A pilot study was conducted using the first samples collected from each group following an allocation ratio of 1:1 (periodontal health: periodontitis). The samples were analysed in the laboratory by Enzyme-Linked Immunosorbent Assay (ELISA) methods. Then the mean and standard deviation values obtained from this pilot study were used to determine the sample size required. thus, it was determined according to the equation mentioned by Sharma et al., 2020\(^{(10)}\). The required sample size was 80 participants who were divided into two groups: cases (40 patients with periodontitis). While the control group included 40 subjects with healthy periodontium.
Clinical evaluation

A complete mouth examination was done for all subjects included in the study by a calibrated examiner using a periodontal probe (UNC-15) used for this purpose. Including dichotomous plaque index (+/-) by using disclosing agents (PLI)\(^{(11)}\), bleeding on probing percentage (BOP\%)\(^{(12)}\), as well as CAL \(^{(13)}\), and PPD \(^{(14)}\) recorded to the nearest millimetre was measured on six facets of the tooth except for plaque index which was done on four surfaces. Additionally, Body mass index (BMI) was calculated to exclude overweight and obese participants.

Saliva collection

Unstimulated saliva was gathered in a sterile container by passive drooling method for the collection of 3 ml of saliva, following all the necessary instructions to prevent contamination of samples such as using sterile cups as well as waering gloves for samples handiling. Samples were collected in the morning hours; the patients were told not to take any food or drink at least for 1 hour before the time collection of saliva as well as before the performance of periodontal examinations. Patients were instructed to sit in an upright position and asked to spit into plastic sterile containers after rinsing their mouths with water. after that, the sample was put in a centrifuge at 3000 rpm for about 15 minutes to isolate the cellular debris from salivary supernatants. Furthermore, The salivary fluid after being centrifuged and separated from the cellular debris, was aspirated slowly to avoid formation of bubbles. and stored in a clean sterile Eppendorf tube, then labelled by giving a sepecial number for each tube, and kept frozen at about -80°C till analysed by the ELISA.

Principle of analysis

After the completion of the sampling procedure, the samples were thawed and left for a few minutes to reach room temperature, then centrifuged again to ensure sample clearance from any debris. A commercially available ELISA kit, purchased from (Shanghai YL Biont, China) was used for determining protein levels in salivary samples. The analysis was done following the manufacturer’s instructions for each kit. These kits used ELISA which depends on the biotin double antibody sandwich method to assess the Human ICTP and DPD. The (ICTP, DPD) was applied to the wells, which were coated previously with (ICTP, DPD) monoclonal antibody, after that they were incubated. Then, anti-ICTP antibodies and anti-DPD antibodies were added with labelled biotin to combine with streptavidin-HRP, which makes an immune complex. The free enzymes after incubation were eliminated by washing. Finally, A and B substrates were added which turn the solution yellow from blue with the acid effect. There was a positive association between the colour of the solution and the ICTP and DPD concentration.

Calibration:

Ten patients with periodontitis who have not been involved in the study underwent calibration sessions for periodontal metrics, for inter examiner’s calibration, the periodontal parameters were measured by the researcher and specialist periododontist at the same time. Calibration sessions were done for the measurement of PLI, PPD and CAL while for BOP, the measurement between the researchers was conducted after two hours to avoid false positive results if it’s conducted immediately. While for intra examiner calibration, first the examiner was trained for the exact probing force on a sensitive scale, between 0.2-0.25 N. The examiner’s repeatability was evaluated by the collection of clinical periodontal data. In a single visit, each individual was evaluated twice during 1 hour. To hide the results of the first evaluation, a second set of measurements was taken. The percentage of sites evaluated where the scores were identical or within 1 mm was calculated for each site to determine the data collection’s reproducibility. Finally, intra-class correlation coefficients were used for continuous data of >0.90, while kappa coefficients were used for dichotomous data of >85%.
Statistical analysis

Graph pad prism 9.5.1, was used to analyse the findings. First, descriptive statistics including means and standard deviations were illustrated then the Shapiro-Wilk test was done to assess the normality of the data distribution. Then to compare the measured variables among groups, an unpaired t-test was done to test any statistically significant difference between each group. Furthermore, ROC curve was done to differentiate periodontal health from periodontitis. The significance level was established at 5%.

Results

One hundred patients were examined seeking their eligibility in the study, 20 of them were excluded either due to systemic causes as mentioned in the exclusion criteria or because they refuse to participate in the study. only 80 patients with an age range of 40-60 years were included in the present study and they were distributed equally among the two groups with an allocation ratio of 1:1. Concerning the demographic data, the mean age and BMI among groups were non-significant while regarding periodontal parameters (PLI, BOP%, PPD, CAL) was higher in periodontitis group and statistically significant in comparison with the healthy group. Regarding salivary biomarkers ICTP and DPD, it was found that their salivary concentrations were higher in the periodontitis group and statistically significant in comparison with the healthy group. As shown in Table (1).

<table>
<thead>
<tr>
<th>Variables</th>
<th>healthy periodontium</th>
<th>Periodontitis</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>43.4±4.3</td>
<td>50.9±5.1</td>
<td>0.08</td>
</tr>
<tr>
<td>BMI</td>
<td>29.3±1.7</td>
<td>28.5±2.2</td>
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<tr>
<td>PLI</td>
<td>14.0±6.1</td>
<td>85.8±28.4</td>
<td>&lt;0.001</td>
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<tr>
<td>BOP %</td>
<td>5.5±0.1</td>
<td>46.9±19.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PPD</td>
<td>1.7±0.6</td>
<td>5.8±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CAL</td>
<td>-</td>
<td>5.2±1.9</td>
<td>-</td>
</tr>
<tr>
<td>ICTP (ng/ml)</td>
<td>4.6±1.4</td>
<td>8.7±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DPD(nmol/L)</td>
<td>392.1±142.8</td>
<td>188.4±49.1</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values were recorded in means ± standard deviation. Statistical analysis was done using a t-test for all variables; BMI: body mass index, PLI: plaque index, BOP: bleeding on probing, PPD: probing pocket depth, CAL: clinical attachment level, ICTP: Pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen, DPD: deoxypyridinoline.

* Significant at p-value ≤ 0.01 by using unpaired t-test

Based on the result from the ROC curve, which was used to differentiate periodontal health from periodontitis, the result of AUC for ICTP was 0.99. The proposed cut-off point was 6.6 ng/ml. (Figure 1, Table 2). Meanwhile, the result from the ROC curve, for DPD, was 0.95. The proposed cut-off point was 211.5 nmol/L (Figure 2, Table 2).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>AUC</th>
<th>Cut off point</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICTP</td>
<td>0.97</td>
<td>0.97</td>
<td>0.99</td>
<td>6.6 ng/ml</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DPD</td>
<td>0.95</td>
<td>0.74</td>
<td>0.95</td>
<td>211.5 nmol/L</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

ICTP: Pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen, DPD: Deoxypyridinoline, AUC: area under the curve.
Discussion

Diagnosing periodontitis using biomarkers is gaining prominence in periodontal research, and our study contributes to this area by examining the diagnostic potential of ICTP and DPD. These cytokines, relatively new in diagnostic studies, particularly in the context of the 2017 classification system, were investigated to determine their ability to differentiate between periodontitis and periodontal health. Our results revealed significant difference in PLI, and BOP between periodontitis and healthy groups, indicative the role dental biofilm or plaque-related bacteria in periodontal disease pathogenesis. The aetiology and pathophysiology of periodontal disease have been strongly connected to a number of the main subgingival microbiome pathogens that have been known as having pathogenicity potential (15), which coincide with a study done by Lertpimonchai et al (16), these parameters increase in periodontitis than healthy periodontium the same with studies done by Fadhil et al (17), Ibraheem et al (18) and Dahash (19).
Concerning PPD and CAL, the present study shows that there was a significant difference between periodontitis and healthy periodontium groups. This could be due to an increase in plaque and bacterial invasion with its toxin, which induces further damage to the alveolar bone tissue, sulcular and junctional epithelium, and other supporting tissue, ultimately increasing the supply of nutrients required for the multiplication of bacteria (20). Related to higher levels of IL-1, IL-6, IL-8, IL-10, TNF-alpha, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor, which produce bone resorption in alveolar bone, activate mature osteoclasts, control bone cell proliferation, and induce osteoclasts (21,22), destroying collagen, in particular type I collagen, the main form of collagen in bones (23). This has coincided with studies done by Juluri et al (24) for PPD, Gondim et al (25) for CAL and Mahmood (26) for PPD and CAL.

As far as the diagnosis of periodontitis is the main concern of the present study, the selected biomarker showed high sensitivity and specificity in the diagnosis of periodontitis and distinguish it from healthy periodontium at the cut-off point (6.657 ng/ml of ICTP). It could be regarded as a particular periodontal tissue breakdown biomarker (27), and clinically it can be used to distinguish healthy from advanced periodontitis (28). Patients with periodontitis showed higher salivary levels of ICTP as compared with healthy periodontium. The increased level of ICTP suggested the presence of a type I collagen breakdown product in the alveolar bone, which was more prevalent in patients with periodontitis, while periodontally healthy patients had the lowest concentration, in physiologic bone metabolism, ICTP gives slow and slight responses, but in contrast in pathological bone resorption, the response is significantly higher (29). So, ICTP is a sensitive bone resorption biomarker and can represent the amount of bone change. In periodontitis, there is rapid collagen matrix degradation and osteoplastic resorption, which leads to the formation of amino- and carboxy-terminal cross-linked telopeptides of type I collagen, deoxypyridinoline, and pyridoxine, which end-up in the bloodstream (30). These substances are formed as a result of post-translational alteration of collagen molecules. Furthermore, these molecules are released into the circulation and cannot be used once more in the formation of collagen as a result of the collagen matrix breakdown in response to periodontal disorders. This result coincides with the study done by Mishra et al (31).

Concerning, DPD the results showed high sensitivity and specificity in the diagnosis of periodontitis and distinguish it from healthy periodontium at the cut-off point (211.561 nmol/L of DPD). Mean awhile, the salivary level of this biomarker was higher in the periodontitis group than healthy periodontium group, pyridinium crosslinks, including deoxypyridinoline, hold type I collagen fibrils together. These collagen fibril crosslinks will deteriorate concurrently with the deterioration of collagen. The breakdown by-products are discharged into the serum in the free form (unbound to other proteins), and they are also present in oral fluids including saliva and gingival fluid (32, 33). Furthermore, it has been discovered that the level of deoxypyridinoline in the oral fluid increases in proportion to the periodontal index. This was consistent with the study done by Syed et al (34).

However, our study has several limitations that warrant acknowledgement. Firstly, the use of a case-control observational design limits the ability to establish causal relationships between exposures and diseases. Prospective longitudinal studies incorporating known confounders would provide more robust evidence of causality, albeit at potentially higher costs. Additionally, the generalizability of our findings may be constrained by the specific study population and setting, necessitating further research in diverse populations to validate our results.

Conclusion

ICTP and DPD were higher in periodontitis patients in comparison with healthy control and had an excellent ability to diagnose periodontitis and distinguish it from healthy periodontium.
**Data availability statement:**

On request, the data used in this investigation are available.

**Conflict of interest:**

The authors state no potential conflict of interest.

**Author contributions**

AM and RF; study conception and design, Methodology, statistical analysis and interpretation of results AM; data collection, writing - review, original draft manuscript preparation. MM and HA; editing, supervision, and Both authors reviewed the results and approved the final version of the manuscript to be published.

**Acknowledgement and funding**

No grant or financial support was received from any governmental or private sector for this study.

**References**


البيبتيد الكاربوكسي الطرفي المترابط عبر البيريدينول (ICTP) (P. 3-26).


المستخلص:
التهاب دواعم السن هو مرض التهاب دواعم السن الذي تسببه مجموعة متنوعة من الكائنات الحية الدقيقة المسببة للأمراض التي تؤدي إلى فقدان التعلق وارتشاف العظم السنخي.

الغرض من البحث: القدرة التشخيصية للتركيز اللعابي لـ ICTP و DPD في التفريق بين التهاب دواعم السن وبين صحة اللثة المرضية. تم إجراء دراسة مراجعة الحالات بالملحق في قسم طب الأسنان في مستشفى بغداد التعليمي. شملت الشتلة على 80 مريضاً مقسمة إلى مجموعتين (المجموعة الأولى كانت مرضى بالتهاب دواعم السن والمجموعة الثانية كانت مرضى يعانون من أمراض دواعم السن الصحية). تم تسجيل التاريخ السني والطبي لكل مريض باستخدام استبيان، ثم تم أخذ عينات اللعاب من كل مريض، ثم تجميده عند حوالي -80 درجة مئوية. تم استنشاق السائل اللعابي من كل مريض بعد طرده وفصله عن الحطام الخلوي، مرة أخرى وتخزينه في أنبوب إيبندورف نظيف ثم تحليله بواسطة اختبار الممرض المناعي المرتبط بالإنزيم (ELISA). أظهرت النتائج أن المؤشرات الحيوية هي مؤشرات حيوية يمكن استخدامها لتشخيص التهاب دواعم السن. كان التركيز اللعابي لـ ICTP، DPD أعلى في مرضى التهاب دواعم السن مقارنة بالأفراد الأصحاء. كذلك، كان لديهم قدرة ممتازة في تشخيص التهاب دواعم السن والقدرة على استخدامها لتشخيص التهاب دواعم السن. المستخلص: النتائج تظهر أن التركيز اللعابي لـ ICTP، DPD أعلى في سلال من مرضى التهاب دواعم السن مقارنة بالأفراد الأصحاء. فضلاً عن ذلك، كانت لديهم قدرة ممتازة في تشخيص التهاب دواعم السن والقدرة على استخدامها لتشخيص التهاب دواعم السن.