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Original Article

Dentistry Section

Correlation of Salivary and Serum Calprotectin Levels in Patients with Periodontitis - Stage II and III, before and after Non Surgical Periodontal Therapy: A Clinicobiochemical Case Control Study

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ABSTRACT

Introduction: Calprotectin (CPT), a major cytoplasmic protein in neutrophils, is released from myeloid cells during inflammation. It exerts pro-inflammatory effects on various cell types and plays a prominent role in tissue breakdown. CPT levels in Gingival Crevicular Fluid (GCF) have been proposed as a sensitive biomarker for monitoring the effects of periodontal therapy at the site level. The present study aimed to evaluate the diagnostic and prognostic utility of salivary CPT as a biomarker in periodontitis.

Aim: To compare CPT levels in the saliva and serum of patients with Stage II and III periodontitis, before and after Non Surgical Periodontal Therapy (NSPT).

Materials and Methods: This preliminary case-control study was conducted at the Outpatient Department (OPD) of Periodontology at Tamil Nadu Government Dental College and Hospital, Chennai, Tamil Nadu, India, from July 2022 to May 2023. A total of 20 subjects were assigned to two groups: Group I-10 subjects with a healthy periodontium, and Group II-10 subjects with periodontitis (Stages II and III) according to the 2017 World Workshop Classification of Periodontal and Peri-Implant Diseases and Conditions. Clinical parameters were recorded, and saliva and serum samples were collected at baseline (T0), one month (T1) and three months (T2) after NSPT for the estimation of CPT levels using an Enzyme Linked Immunosorbent Assay (ELISA). Correlations between salivary and serum CPT levels and clinical parameters were assessed before and after NSPT. Repeated-measures Analysis of Variance (ANOVA) was employed for intra-group comparison. The correlation between salivary and serum CPT levels was evaluated using the Pearson's correlation coefficient. For all statistical analyses, $p<0.05$ was considered statistically significant.

Results: Intergroup comparisons of clinical and biochemical parameters at T0 demonstrated statistically significant differences in all parameters ($p<0.001$) except serum CPT. Salivary CPT levels were higher in periodontitis than in healthy individuals (p-value=0.018); and the levels decreased following NSPT. There was a negligible positive correlation between salivary and serum CPT levels at T0 ($r=0.017$); and a weak negative correlation at T1 ($r=-0.242$) and T2 ($r=-0.203$), indicating no significant linear relationship at any time point assessed.

Conclusion: Salivary CPT levels showed a clear reduction in patients with periodontitis at one and three months after non surgical therapy. Within the confines of this study, it could be presumed that salivary CPT may be considered a potential diagnostic and prognostic biomarker for periodontitis.

Keywords: Biomarkers, Disease progression, Periodontal diseases, Scaling and root planing

INTRODUCTION

Periodontitis is a chronic, multifactorial inflammatory disease associated with dysbiotic plaque biofilms and characterised by progressive destruction of the tooth-supporting apparatus [1]. The primary features of periodontitis include reduced periodontal support, evidenced by clinical attachment loss and radiographic alveolar bone loss, gingival bleeding on probing, and periodontal pocket formation. Owing to its high prevalence and its negative impact on quality of life (such as decreased masticatory efficiency, impaired aesthetics, tooth loss and disability, and effects on general health and systemic diseases), periodontitis is considered a major global health issue. Early diagnosis and prompt treatment are imperative to limit the adverse consequences of periodontitis.

Conventional periodontal diagnosis is typically based on clinical parameters such as Probing Pocket Depth (PPD) and Clinical Attachment Level (CAL), together with standard dental radiography. However, these diagnostic approaches are inherently limited to assessing disease history rather than current disease activity. Innovative diagnostic techniques which are capable of detecting ongoing disease activity, forecasting future disease progression and assessing the effectiveness of periodontal therapy, thereby improving the clinical management of periodontitis and reducing its global burden, are desirable. [2].

Biomarkers have the potential to enhance diagnostic precision by providing information on current disease activity and associated risk factors, thereby enabling earlier diagnosis and more effective treatment planning [3]. The 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions – Staging and Grading of Periodontitis anticipated the future inclusion of specific biomarkers and their corresponding thresholds in the grading criteria, as supporting evidence continues to emerge [4].

A multitude of studies have focused on defining biomarkers for periodontal diagnosis and monitoring [5-9]. Various enzymes, cytokines, receptors and proteins have been explored for their potential role in the diagnosis of periodontitis and monitoring the treatment response [3].

Calprotectin - a member of S100 family, also known as myeloid related protein (MRP) is a major cytoplasmic protein in neutrophils. It is secreted from myeloid cells during inflammation and exerts proinflammatory effects on various cell types, triggering signal transduction and inducing leukocyte recruitment and cytokine secretion at sites of inflammation. CPT plays an important role in tissue breakdown by inhibiting the activity of matrix metalloproteinases. Thus, it is an interesting biomarker for periodontitis [10].

The practicality of CPT as a diagnostic marker of periodontal inflammation has been supported by its strong correlation with clinical indices and collagenase activity [11]. CPT levels in Gingival Crevicular Fluid (GCF) have been proposed as a sensitive biomarker for monitoring the effects of periodontal therapy at the site level [12]. The diagnostic utility of CPT in serum and saliva in periodontitis has also been demonstrated [10].

The present study aimed to assess the diagnostic and prognostic potential of CPT by comparatively evaluating its levels in the serum and saliva of patients with Stage II and Stage III periodontitis, before and after nonsurgical periodontal therapy (NSPT). The objectives were: (i) to assess and compare salivary and serum CPT levels in subjects with a healthy periodontium and in patients with Stage II and Stage III periodontitis; (ii) to evaluate changes in CPT levels in subjects with periodontitis at baseline (T0), one month (T1), and three months (T2) following NSPT; and (iii) to determine the correlation between salivary and serum CPT levels.

Statement of hypotheses:

1. Comparison of CPT levels between groups.

Null hypothesis ($H0_1$):

There is no significant difference in salivary and serum CPT levels among subjects with a healthy periodontium and patients with periodontitis.

Alternative hypothesis (Ha_1):

There is a significant difference in salivary and serum CPT levels among subjects with a healthy periodontium and patients with periodontitis.

2. Changes in CPT levels following NSPT.

Null hypothesis ($H0_2$):

There is no significant change in salivary and serum CPT levels in patients with periodontitis from baseline (T0) to one month (T1) and three months (T2) following NSPT.

Alternative hypothesis (Ha_2):

There is a significant change in salivary and serum CPT levels in patients with periodontitis from baseline (T0) to one month (T1) and three months (T2) following NSPT.

3. Correlation between salivary and serum CPT levels.

Null Hypothesis ($H0_3$): There is no significant correlation between salivary and serum CPT levels.

Alternative Hypothesis (Ha_3): There is a significant correlation between salivary and serum CPT levels.

MATERIALS AND METHODS

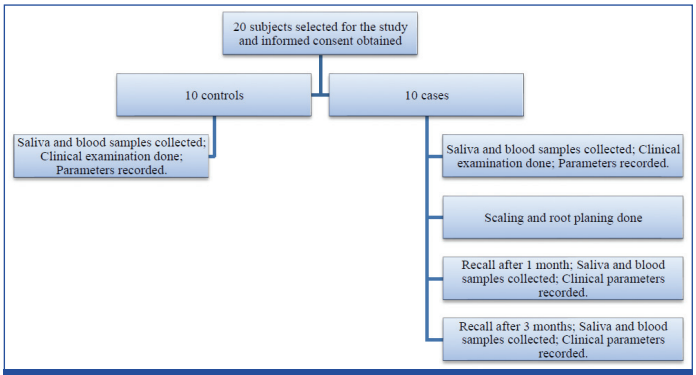
This preliminary case-control study was conducted at the Outpatient Department (OPD) of Periodontology at Tamil Nadu Government Dental College and Hospital, Chennai, Tamil Nadu, India, from July 2022 to May 2023. Following approval from the Institutional Ethical Review Board (IERB Reference No: 1/IERB/2022), the study was conducted as depicted in [Table/Fig-1]

Inclusion criteria: Participants who gave their willingness for the study were recruited considering criteria for inclusion such as systemically healthy individuals, free from systemic ailments that might impact the periodontal conditions, and have not received any treatment or medications in the previous six months. A total of 20 subjects were assigned to two groups according to the 2017 World Workshop Classification of Periodontal and Perilmplant Diseases and Conditions [4].

- Group I (control) - Periodontally healthy individuals, with no sites of clinical attachment loss, no site with probing depth > 3mm, no radiographic bone loss.
- Group II (case) - Subjects with periodontitis stage II and III based on the 2017 World Workshop of Periodontology classification [4].
    - Patients with bone loss of 15-33% restricted to middle third (measured in radiographs)
    - Clinical attachment level (CAL) > 3 mm, with or without tooth loss < 10 occluding pairs
    - Bleeding on probing score of more than 15%.

Exclusion criteria: Patients with a history of systemic diseases (such as diabetes mellitus, ischaemic heart diseases, atherosclerosis, stroke, hypertension, thyroid diseases, Crohn's disease, ulcerative colitis), metabolic bone disorders, acute bacterial, viral or fungal infection, former/current smokers, pregnant/lactating women were excluded. Additionally, patients who underwent periodontal intervention within the preceding six months and those under any medications in the last six months were also excluded.

Patients who attended OPD were randomly selected and enrolled in the study in accordance with the inclusion and exclusion criteria. The purpose of the study was explained to all participants, and informed consent was obtained. As this was a time-bound preliminary study, a formal sample size calculation was not performed; instead, a sample size feasible for biochemical analysis was chosen. All subjects who met the inclusion and exclusion criteria during the study period were included. The study was conducted as depicted in [Table/Fig-1].



[Table/Fig-1]: Study design flowchart.

Study Procedure

Saliva collection: Participants were instructed to refrain from eating, chewing gum, or brushing their teeth for one hour before saliva collection. They were seated with their heads tilted forward. A 5 mL sample of unstimulated whole saliva (initially swallowed and then allowed to pool on the floor of the mouth) was collected using a syringe and transferred to a sterile, capped tube. The saliva samples were centrifuged at 6000 rpm for 10 minutes to remove cellular debris, and the clear supernatant was transferred to Eppendorf tubes and stored at -20°C until analysis.

Serum collection: Blood samples were obtained from the antecubital vein using a 20-gauge needle and a 5 mL syringe. Before each sampling, the site was disinfected with isopropyl alcohol. The blood was allowed to clot at room temperature for 30 minutes and then centrifuged at 3000 rpm for 15 minutes. The separated serum was transferred to Eppendorf tubes and stored at -20°C until analysis.

Protocol for Non Surgical Periodontal Therapy (NSPT): Group II participants received NSPT (Phase I therapy), which included oral hygiene instructions, removal of plaque and calculus, and correction of overhanging restorations [13,14]. Following a test dose of 0.5 mL of 2% lignocaine solution, local anaesthetic was administered at the diseased sites. One-stage, full-mouth Scaling and Root Planing (SRP) was performed using ultrasonic instrumentation. A single

calibrated examiner carried out the procedure and assessed all clinical parameters to ensure consistency and minimise examiner-related variability.

Baseline (T0) saliva and serum samples were collected from participants in both group I and group II. Group II participants reported for review at one month (T1) and three months (T2). During these visits, saliva and blood samples were collected and clinical parameters such as Plaque Index (PI), Gingival Bleeding Index (GBI), Periodontal Probing Depth (PPD), Clinical Attachment Level (CAL), were recorded [15,16]. Quantification of CPT in saliva and serum was performed using ELISA, and the values were reported in ng/mL (nanograms per millilitre).

STATISTICAL ANALYSIS

Descriptive and inferential statistics were performed using IBM SPSS Statistics, version 25. Quantitative data (PI, GBI, PPD, CAL, salivary CPT, and serum CPT) were presented as mean and standard deviation. Student's t-test/independent samples t-test was used for inter-group comparisons (differences in parameters between the two study groups). Repeated-measures ANOVA with a post-hoc Tukey's honestly significant difference test was employed for intra-group comparisons (comparing salivary and serum CPT levels before and after treatment). The correlation between salivary and serum CPT levels was assessed using Pearson's correlation coefficient. For all statistical analyses, a p-value <0.05 was considered statistically significant.

RESULTS

The present clinicobiochemical study was conducted to estimate, compare, and correlate salivary and serum CPT levels in patients with Stage II and Stage III periodontitis before and after Phase I therapy. Intergroup comparisons of clinical and biochemical parameters at T0 [Table/Fig-2] showed statistically significant differences in all parameters (p≤0.001), except for serum CPT. Intragroup assessment of clinical and biochemical parameters for Group II is presented in [Table/Fig-3]. Changes in CPT levels at different time points are shown in [Table/Fig-4]. The correlation analysis between salivary and serum CPT is provided in [Table/Fig-5] and graphically illustrated in [Table/Fig-6-8].

Biochemical parameters	Groups	Mean± SD	Std. Error Mean	t-statistic	p-value
Periodontal Probing Depth (PPD)	Test	3.8742±0.1907	0.06031	14.45	<0.001
	Control	2.1795±0.31819	0.10062		
Clinical Attachment Level (CAL)	Test	3.1468±0.64822	0.20498	9.11	<0.001
	Control	0.6795±0.55939	0.17689		
Plaque Index (PI)	Test	1.379±0.26756	0.08461	4.2	0.001
	Control	0.6115±0.51277	0.16215		
Gingival Bleeding Index (GBI)	Test	85.4564±13.48188	4.26335	17.71	<0.001
	Control	6.416±4.17462	1.32013		
Salivary Calprotectin (ng/mL)	Test	22.887±17.58803	5.56182	2.84	0.018
	Control	6.7169±3.84495	1.21588		
Serum Calprotectin (ng/mL)	Test	9.0811±7.3814	2.3342	0.99	0.339 (NS)
	Control	6.3889±4.44457	1.4055		

[Table/Fig-2]: Intergroup comparison of clinical and biochemical parameters at baseline.
Independent t-test; p<0.05 shows statistical significance; NS: Not significant

The baseline (T0) salivary CPT values differed significantly between group I and group II (p=0.018). In group II, the mean reduction in salivary CPT from T0 to T1 and from T1 to T2 was 8.96 and 4.88, respectively; however, these reductions were not statistically significant (p=0.051).

The difference in baseline (T0) serum CPT values between group I and group II (2.7) was not statistically significant (p=0.336). The mean serum CPT levels at T1 and T2 were 9.12±8.37 and 7.92±4.91, respectively. There was a slight increase in serum CPT from T0 to T1 (0.04) and a decrease from T1 to T2 (1.2); however, these changes were not statistically significant (p>0.05).

Biochemical parameters	Time point	Mean±SD	F value	p-value
Periodontal Probing Depth	T0	3.87±0.19	42.371	0.001
	T1	3.39±0.27		
	T2	3.02±0.14		
Clinical Attachment Level	T0	3.15±0.65	3.96	0.03
	T1	2.77±0.57		
	T2	2.42±0.51		
Plaque Index	T0	1.38±0.27	28.19	0.001
	T1	0.4±0.31		
	T2	0.46±0.39		
Gingival Bleeding Index	T0	85.46±13.48	131.278	0.001
	T1	26.68±6.73		
	T2	19.38±8.54		
Salivary Calprotectin (ng/mL)	T0	22.89±17.59	3.309	0.051 (NS)
	T1	14.33±10.63		
	T2	9.05±4.61		
Serum Calprotectin (ng/mL)	T0	9.08±7.38	0.286	0.753 (NS)
	T1	9.13±8.38		
	T2	7.03±4.75		

[Table/Fig-3]: Intragroup assessment (Group II) of clinical and biochemical parameters.
Test: Repeated measures ANOVA; p-value <0.05 shows statistical significance; F: Fisher in ANOVA test; NS: not significant

Time point	Parameters	Mean±SD	Std. Error Mean	t statistics	p-value
T0	Salivary	22.89±17.59	5.56	2.289	0.034
	Serum	9.08±7.38	2.33		
T1	Salivary	14.33±10.63	3.06	1.185	0.251
	Serum	9.13±8.38	2.65		
T2	Salivary	9.05±4.61	1.46	0.526	0.605
	Serum	7.03±4.75	1.55		

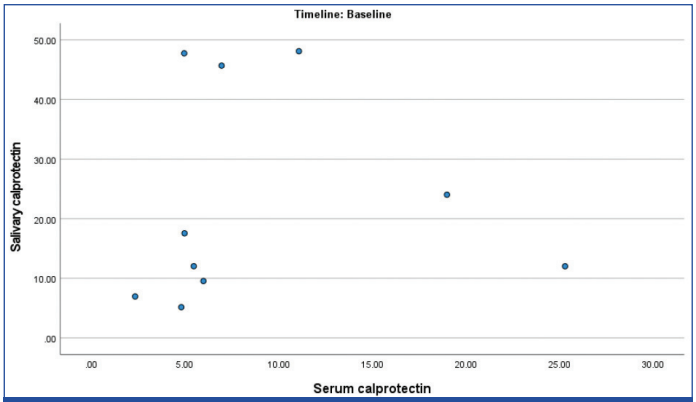
[Table/Fig-4]: Intragroup (Group II) comparison of salivary and serum calprotectin at different time points.
Test applied - Independent t-test; p<0.05 shows statistical significance

Variables correlated	Timeline	r -value	p-value
Salivary and Serum calprotectin	T0	0.017	0.962 (NS)
	T1	-0.242	0.5 (NS)
	T2	-0.203	0.573 (NS)

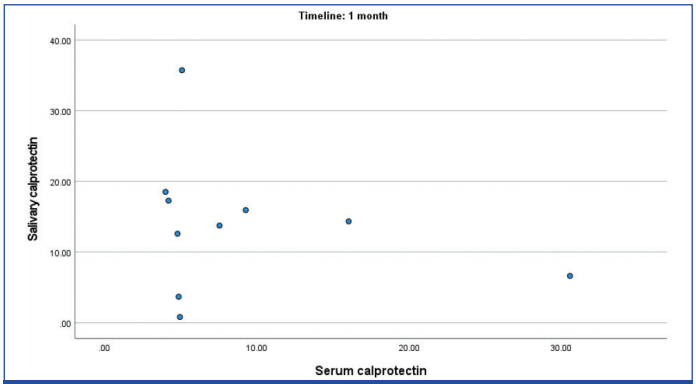
[Table/Fig-5]: Correlation analysis between salivary and serum calprotectin.
*The data shows that at T1 and T2 month salivary and serum CPT were inversely correlated (weak negative) but the correlation is not statistically significant; r – Pearson's correlation coefficient

Comparison of salivary and serum CPT levels: The mean T0 levels of salivary and serum CPT showed a statistically significant difference of 0.034. However, there was no significant difference in CPT levels between saliva and serum at T1 and T2 (p=0.251 and p=0.60, respectively). Despite the calculated mean±SD of salivary CPT at T0 being 39, a few patients exhibited higher individual values, which explains the extension of the scatter plot up to 50 [Table/Fig-6].

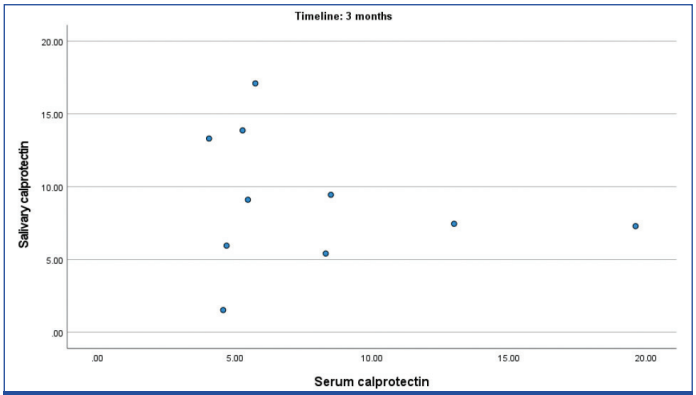
Correlation analysis revealed a negligible positive correlation between salivary and serum CPT levels at T0 (r=0.017), indicating



[Table/Fig-6]: Scatterplot showing correlation between salivary and serum calprotectin at Baseline (T0).



[Table/Fig-7]: Scatterplot showing correlation between salivary and serum calprotectin at one month (T1).



[Table/Fig-8]: Scatterplot showing correlation between salivary and serum calprotectin at 3 months (T2).

no meaningful association. At T1 (r=-0.242) and T2 (r=-0.203), a weak inverse correlation was observed. However, none of these correlations was statistically significant (p>0.05), suggesting that salivary and serum CPT levels did not show a significant linear relationship at any of the time points assessed.

The following inferences were drawn from the results of this study:

- There was a definitive improvement in clinical parameters such as PI, GBI, PPD and CAL in periodontitis patients after NSPT.
- Salivary CPT levels had statistically significant difference between the two groups.
- There was a definitive reduction in salivary CPT levels in Group II at T1 and T2 after non surgical therapy, although this reduction was not statistically significant.
- Serum CPT levels did not differ significantly between the two groups, and the changes in serum CPT levels in Group II after non surgical therapy were also not significant.

DISCUSSION

Periodontitis, a chronic multifactorial inflammatory disease destroying the tooth supporting apparatus, adds to the global burden of health issues. Technological progress, together with advances in diagnostic research, is promising for the development of methods to identify individual periodontal risk and quantify the risk by objective biomarkers [17].

Calprotectin, a heterodimer of calcium-binding proteins, is constitutively expressed by neutrophils, activated monocytes, epithelial cells and keratinocytes. Lipopolysaccharides of periodontal pathogens can provoke the release of CPT from neutrophils, macrophages and gingival epithelial cells in periodontal tissue sites. CPT has been investigated as a candidate marker in various inflammatory conditions. The faecal CPT level has been the gold standard diagnostic test in Inflammatory Bowel Disease (IBD), as it reflects disease activity and response to therapy in IBD. Recently, Khosravi P et al., had demonstrated CPT as a new potential clinical marker for multiple myeloma [18].

The association between CPT levels in GCF and periodontitis has been investigated in several studies (Kido J et al., Lundy FT et al., Kojima T et al., Andersen E et al., Nishikawa Y et al.,) [19-23]. CPT levels in saliva have been studied by Haririan H et al., Holmström SB et al., Kim HD et al., Kamatham SA and Chava VK, who have demonstrated CPT as a diagnostic marker [10,24-26]. The present study also explored the potential utility of salivary CPT in monitoring periodontal treatment response. A comparison of salivary CPT levels in healthy individuals with those of periodontitis patients exhibited a statistically significant difference with a p-value of 0.018. This is in accordance with the results of Haririan H et al., Kim HD et al., Karna S et al., [10,25,27]. Similar results were demonstrated in GCF by Andersen E et al., and Gao H et al., among others [22,28]. With respect to serum CPT, its levels were higher in periodontitis patients compared to healthy individuals; however, this difference was not statistically significant. The null hypothesis ($H0_1$)is rejected, as there is a difference in salivary and serum CPT levels between healthy subjects and periodontitis patients. NSPT has been extensively recognised for its ability to resolve inflammation and halt the progression of periodontal disease, resulting in a relative gain in clinical attachment and a reduction in probing depth [29]. The intragroup comparison of salivary CPT levels before and after NSPT supports this, demonstrating a decrease in salivary CPT levels from T0 to T1 and T2.

The intragroup comparison of serum CPT in periodontitis patients after NSPT revealed a slight increase from T0 to T1, followed by a decrease from T1 to T2. This may be explained in light of findings from Afacan B et al., who observed elevated GCF CPT levels despite clinical improvement after treatment [30]. They highlighted the antimicrobial activities of CPT, which may indirectly contribute to healing after periodontal therapy by suppressing destructive enzymes. These findings regarding serum CPT should be interpreted with caution because of the small sample size and biological variability. The null hypothesis ($H0_2$) is accepted, as neither salivary nor serum CPT levels in periodontitis patients showed statistically significant differences following NSPT.

The analysis of serum and GCF requires considerable time, financial investment and resource allocation, particularly when contemplating the clinical implementation of a biomarker-based screening model [31].

Saliva contains proteins derived from GCF and blood from the systemic circulation. Consequently, qualitative and quantitative alterations in the composition of saliva have diagnostic and therapeutic significance for periodontitis [32]. Because saliva can be easily collected by a non invasive method, without the need for trained dental professionals, it is feasible to develop a self-test kit for periodontitis using saliva as the specimen [33,34].

Regarding clinical parameters, there is a significant difference in PI, GBI, PPD and CAL in periodontitis patients after SRP from T0 to T1 and T2. The observed changes can be attributed to a reduction

in inflammation and the formation of reattachment following SRP. These findings are consistent with studies conducted by Cugini MA et al., and Haffajee AD et al., which have investigated follow-up periods ranging from one to 12 months [35,36].

In a study by Junior LR et al., the salivary CPT levels correlated with PI, but did not correlate with serum CPT or other clinical parameters. However, serum CPT correlated with BOP, PPD and CAL [37]. These discrepancies may be attributed to differences in study design, methods of sample collection, storage and analysis, as well as inter-examiner variability.

Kido J et al., reported that CPT levels correlated positively with PPD and were significantly higher at BOP-positive sites than at BOP-negative sites [19]. Thus, CPT may be useful for evaluating the extent of periodontal inflammation. Andersen E et al., demonstrated positive correlations between CPT concentrations in GCF and GI [22]. Zheng Y et al., reported positive correlations between GCF CPT levels and clinical indicators such as BOP, PPD and CAL [38]. Holmström SB et al., also found a positive correlation between CPT and BOP [24].

Correlation analysis between salivary and serum CPT levels revealed an inverse relationship; however, this association did not reach statistical significance. This suggests a complex interplay between local and systemic inflammatory pathways in periodontitis, which may not exhibit a direct linear relationship and may be influenced by multiple biological and host-related factors. The null hypothesis ($H0_3$) is accepted as there is no significant correlation between salivary and serum CPT levels.

Limitation(s)

A major limitation that might have affected protein detection is the long-term storage of samples. Because samples were stored for an extended period before analysis, this represents an inherent constraint of the study. The lack of standardisation of analytical techniques and the absence of a clear-cut reference range for CPT values constitute another important limitation. As a wide range of values has been reported in both health and disease, it is uncertain whether any single value can be used for diagnostic purposes. Moreover, CPT has been reported to be associated with various inflammatory and autoimmune conditions, adding multiple confounding factors such as subclinical systemic inflammation and individual variations in immune response, which could not be eliminated and may have distorted the true relationships.

As a preliminary study with a small sample size, the results cannot be generalised. In the future, multicentric studies with larger sample sizes, longer follow-up periods and multivariate data analysis should be conducted to further clarify the association between salivary CPT levels and the periodontal status of the patient, its role in the progression of periodontitis, the effectiveness of Phase I therapy in reducing CPT levels, and to establish standardised reference values for clinical application.

CONCLUSION(S)

The present study was conducted to evaluate CPT levels in the saliva and serum of patients with periodontitis, before and after NSPT. It was demonstrated that salivary CPT levels were elevated in patients with periodontitis compared with healthy individuals and that these levels decreased following therapy. Despite the absence of a statistically significant difference, it may be suggested that salivary CPT levels reflect periodontal inflammatory status and may serve as a potential biomarker for the diagnosis of periodontitis and for monitoring treatment outcomes. Point-of-care (POC) tests may facilitate the practical application of salivary CPT, supported by advances in science and technology.

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