

Short-term Effects of Non Surgical Periodontal Therapy on Oral Fluid Calprotectin Levels in Stages III or IV Periodontitis: A Longitudinal Observational Study

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ABSTRACT

Introduction: Periodontal diseases cause destruction of the tooth supporting structures and contribute to low-grade systemic inflammation. Calprotectin is a major cytosolic protein of immune cells; it is highly expressed in sites of inflammation and has been explored as a biomarker in many inflammatory conditions including periodontitis. Cross-sectional studies have reported elevated calprotectin levels in oral fluids of periodontitis patients, but evidence regarding the impact of periodontal therapy on Gingival Crevicular Fluid (GCF) and salivary levels of calprotectin is inconclusive. Present study was conducted to assess whether calprotectin levels in oral fluids would be altered by Non Surgical Periodontal Therapy (NSPT).

Aim: To observe the short-term impact of NSPT on the GCF and salivary levels of calprotectin.

Materials and Methods: The present longitudinal observational study was carried out in the Department of Periodontology Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences, Chennai, Tamil Nadu, India, over a period of four months from January to April 2023. A total of 11 patients clinically diagnosed with stages III or IV periodontitis and who met inclusion and exclusion criteria were enrolled in the study. Gingival Index (GI), mean Probing Pocket Depth (PPD), mean Clinical Attachment Loss (CAL), percentage of sites with PPD ≥ 5 mm and sites with CAL ≥ 5 mm were recorded. GCF and salivary samples were collected to measure calprotectin levels by Enzyme Linked Immunosorbent Assay

(ELISA). Professionally administered oral hygiene instructions, mechanical supragingival plaque removal (PMPR) and sub gingival instrumentation were performed for these patients. They were recalled for their first follow-up visit after a period of six weeks. Re-evaluation of periodontal parameters was done and GCF and salivary samples were collected to estimate calprotectin levels. Mean and standard deviation was used to summarise the variables. The comparison between baseline and follow-up periodontal parameters were done using non parametric Wilcoxon signed rank tests. Oral fluid calprotectin levels were compared using paired t-test. The p-values less than 0.05 were considered statistically significant.

Results: A total of 11 participants were recruited for the study. Only 10 participants returned for the first follow-up visit at six weeks. Statistically significant differences were seen in pre and post-treatment values for GI, mean PPD, mean CAL, percentage of sites with PPD ≥ 5 mm and sites with CAL ≥ 5 mm. Salivary calprotectin was 1160.17 ± 596.18 at baseline and was lowered to 1100.46 ± 604.75 pg/ μ L at follow-up. ($p=0.74$) GCF calprotectin was 1605.11 ± 79.29 before NSPT and decreased to 1556.24 ± 37.56 pg/ μ L post therapy (0.092).

Conclusion: This study observed a significant improvement in all clinical periodontal parameters at six weeks after NSPT. Calprotectin levels in both saliva and GCF decreased but the differences were not statistically significant. Calprotectin levels in oral fluids of stages III or IV periodontitis patients was not significantly altered at six weeks after NSPT.

Keywords: Biomarkers, Gingival crevicular fluid, Periodontal pocket, Saliva

INTRODUCTION

Periodontal diseases involve inflammation in the tooth supporting structures leading to progressive loss of connective tissue and alveolar bone ultimately leading to tooth loss. This immunoinflammatory process is initiated by pathogenic bacteria in the plaque biofilm. But the tissue destruction seen in the disease is largely due to dysregulated responses of the host, determined by individual host susceptibility. Periodontal diseases affect more than half of the world's adult population with 11.2% diagnosed with advanced form of the disease [1,2]. Periodontal diseases affect aesthetics, speech and mastication and impairs quality of life. Periodontal inflammation leads to the dissemination of microbes and/or their structural components and their enzymes into the systemic circulation. Also, the inflammatory mediators produced locally in response to the microbes are 'spilled' into circulation contributing to low-grade systemic inflammation. This low-grade systemic inflammation partially explains the association between periodontitis and systemic diseases such as diabetes mellitus,

cardiovascular diseases, premature rupture of membrane and pre-term labor in pregnant females, respiratory diseases, Alzheimer's disease, cancers, autoimmune diseases such as rheumatoid arthritis and Inflammatory Bowel Disease (IBD) and increased incidence of adverse outcomes and mortality in COVID-19 [3].

Precision periodontics entails tailoring therapeutic modalities specific to individual patient characteristics such as genetic profile, environmental and behavioural factors [4]. Incorporation of validated biomarkers into periodontal practice is a key pillar of precision periodontics. Personalised periodontics will be based on diagnostic test results obtained from genetic or proteomic studies of oral fluids [5]. The most recent World work shop classification on the diagnosis of periodontal and peri-implant diseases and conditions also welcomes the use of biomarkers in the diagnosis and classification of periodontal diseases [6,7]. Periodontal research has identified a number of potential biomarkers and biomarker panels [8]. Biomarkers may be used in periodontics for identifying individual disease susceptibility, screening for the presence of

disease, diagnosis and disease classification, monitoring treatment responses and determining prognosis.

Calprotectin is a 36-kDa, calcium binding heterodimeric complex (MRP8/14 or S100A8/9) which is chiefly expressed by the phagocytes during inflammation. The homodimers-also known as Calgranulins A and B [9] or S100A8 and S100A9 [10] or MRP8 and MRP14 [11] are made up of amino acid residues compactly folded into a helix-loop-helix structure [10]. These α -helices allow binding of transition metal ions. Calprotectin and its homodimers constitute more than 40% of cytosolic proteins of neutrophils. Evidence points out that calprotectin levels in body fluids and faeces is indicative of neutrophil numbers and activity [11,12]. Intracellularly calprotectin is important for modulation of the cytoskeleton and regulation of intracellular pathways. This protein complex is highly expressed by neutrophils, monocytes and macrophages during inflammation. It is a potent chemoattractant for neutrophils. Calprotectin binds chiefly to Toll-Like Receptor-4 (TLR-4) leading to the activation of nuclear factor- κ B and/or MyD88 pathways leading to the generation of pro inflammatory cytokines such as IL-6, IL-8 and TNF- α [10]. Calprotectin can sustain and augment inflammation.

By virtue of its affinity for metal ions such as Ca, Zn and Mn, it is regarded as an antimicrobial peptide. Evidence also points out that cytosolic calprotectin confers resistance to pathogenic microbes [13]. Fecal calprotectin is being used as a biomarker for IBD [10] and to distinguish between Irritable Bowel Syndrome (IBS) and IBD. This protein is being investigated for its biomarker potential in other inflammatory conditions such as rheumatoid arthritis, psoriasis, synovitis and peritonitis [13]. Serum calprotectin levels have been reported to be higher in patients with severe COVID-19 when compared to those with milder symptoms of the disease [14].

Oral fluids such as saliva and GCF are in close proximity to the periodontium and could house mediators of periodontal inflammation. Calprotectin levels in GCF and saliva has been investigated in previous studies [15-17], and evidence points out that calprotectin levels in oral fluids are elevated in periodontal diseases [16,17]. NSPT is the first step in periodontal treatment sequence and has to be delivered for all patients with periodontitis irrespective of type, stage or grade of disease. This phase of treatment accomplishes alteration or reduction of microbial flora and controls inflammation. Recent studies have pointed out that calprotectin may be used as a marker to monitor treatment response in patients with periodontitis [17,18]. However, reports on the impact of NSPT on oral fluid calprotectin levels have yielded mixed results [17-19]. The present study was carried out in subjects with advanced periodontal destruction, clinically diagnosed as Stages III or IV periodontitis [7]. The aim of this study was to investigate the impact of NSPT on oral fluid calprotectin levels. The objective was to estimate and compare GCF and salivary levels of calprotectin before and at six weeks after NSPT. Null hypothesis of the study was that NSPT would not significantly alter oral fluid calprotectin levels and the alternate hypothesis was that periodontal therapy would alter oral fluid calprotectin levels significantly.

MATERIALS AND METHODS

The present longitudinal observational study was conducted among patients who sought periodontal treatment at the Department of Periodontology Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences, Chennai, India, over a period of four months from January to April 2023. The study protocol received approval and clearance from the Institutional Ethics Committee (IEC) and Scientific Review Board (SRB) of the dental school at which it was carried out. (IHEC/PhD/PERIO-1621/21/230). The study was registered in the Clinical Trial Registry of India {CTRI/2022/11/047620 (Registered on: 24/11/2022)} The study strictly followed all ethical guidelines for medical research in humans laid down by the most recently amended version (2024)

of the World Medical Association, declaration of Helsinki. It was reported according to Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. All participants were informed about the study and written informed consent was taken.

Sample size calculation: The minimum sample size required was estimated using G Power open-source software [20]. The sample size was estimated to achieve 80% power and a type 1 error rate of 5%. An input effect size of 1.73 was used based on results from a previous study [21]. A minimum sample size of 10 patients per group was finalised. Eleven patients were recruited at base-line and one sample each of GCF and saliva were collected from each patient. At six weeks after NSPT, one patient did not report as advised and 10 GCF and 10 salivary samples were collected.

Study Procedure

The current study consecutively enrolled systemically healthy patients of ages between 25-60 years who were diagnosed clinically with stages III or IV periodontitis [7]. Smokers and obese individuals [22] {(weight (kg)/height (m)²= Body Mass Index (BMI), (Obese-those with BMI >30)} were excluded from the study. Subjects who gave a medical history of any systemic co-morbidities such as diabetes mellitus or any drug history such as anticoagulants were excluded. Those subjects who gave history of any acute or chronic illness in the preceding three months and received therapy for the same were excluded. Pregnant and lactating females were also excluded. Detailed periodontal examination and recording was then done. Participants were informed about their periodontal status [23] and oral hygiene instructions were given. Saliva and GCF samples were collected to estimate calprotectin levels.

Periodontal examination: Patients were seated comfortably on the dental chair and oral and periodontal examination was done under illumination using a mouth mirror and University of North Carolina (UNC 15) periodontal probe. GI [24] was recorded. PPD, gingival margin level (GML) as plus, minus or at the cemento-enamel junction and CAL were recorded at six sites per tooth (Mesiobuccal, mid-buccal, distobuccal, mesiolingual, mid-lingual and distolingual) on all permanent teeth present except the third molars. Mean PPD and mean CAL was calculated. Percentage of sites (% sites) with PPD ≥ 5 mm and % sites with CAL ≥ 5 mm were recorded in each patient. Stages III and IV periodontitis was defined as per the criteria of staging by the American Academy of Periodontology (AAP) and European Federation of Periodontology (EFP) World Workshop on classification of periodontal and peri-implant diseases and conditions in 2017 [7]. Stages III and IV periodontitis were defined clinically as presence of interproximal attachment loss ≥ 5 mm on two non-adjacent teeth.

Saliva sample collection, processing and storage: Following periodontal charting and diagnosis, patients were asked to swish and rinse their mouth with a glass of normal water in order to wash out any blood or debris. They were then seated comfortably in the waiting area. They had not performed any brushing or flossing in the two hours prior to sampling. They were advised not to have any coffee, tea or alcohol during this period prior to sample collection [25]. Patients were called from the waiting area and were seated comfortably on the dental chair. Unstimulated whole saliva was collected. To enable saliva sampling by passive drool, participants were asked to gently close their eyes, tip their head slightly forward and were asked to recollect the food they enjoyed. Saliva which passively drooled on to the floor of the mouth for five minutes was collected from the corners of the mouth into 15 mL prerrefrigerated tubes. The tubes were immediately placed in an ice box. The tubes were labelled with samples specifics such as name, age, gender of patient and time and date of sample collection. Ice boxes with the samples were transferred immediately to the Biochemistry Department where the sample were centrifuged at 1500 rotations per minute (rpm) at 40°C for 15 minutes [25]. The aliquots were transferred into a 2 mL Eppendorf tube which was specifically

labelled for each patient sample and stored at -80°C till assay by Enzyme-Linked Immunosorbent Assay (ELISA).

GCF sampling, processing and storage: Following saliva sample collection, GCF samples were collected from maxillary sites or mandibular sites with greatest probing depths. 2 µL microcapillary tubes were used for (Drummond Microcaps) GCF sample collection. A white marking was placed at the exact the center of the pipette length to get the 1 µL mark. Potential sampling sites were chosen and supra gingival debridement was done with a universal scaler. The sites were then dried and isolated with absorbent cotton rolls, the microcapillary pipette was then carefully slid into the periodontal pocket with minimal force ensuring that the pipettes did not injure the lateral aspects or base of the periodontal pocket. The pipette was hand held in the pocket by the operator until GCF ascended the transparent tubes up to the 1 µL mark or alternatively for a maximum of period of 10 minutes [26]. In cases where GCF could not be obtained at end of 10 minutes, alternate sites of sampling were chosen and sample collection was repeated. The tubes were then withdrawn gently and placed into a labelled 2 mL Eppendorf tube and the samples were transferred immediately to the Biochemistry Department. A 200 µL of Phosphate Buffered Saline (PBS) at 7pH was added into the Eppendorf tubes and the contents of the pipette were washed out into the PBS. The GCF samples were then refrigerated at -80 degrees centigrade (°C) until protein estimation by ELISA.

NSPT was carried out for all participants and comprised of oral hygiene instructions and supragingival and subgingival instrumentation. Professional oral hygiene instructions were first given to all patients by qualified dental graduates in the Department of Periodontology. Supra gingival Professional Mechanical Plaque Removal (PMPR) [23] (supragingival scaling) followed by subgingival instrumentation was carried out within a 24-hour period [27]. Oral hygiene instructions were reinforced and participants were advised to report for their first follow-up at six weeks post-NSPT [28]. A pack each of tooth paste and brush was given to all participants. The necessity and importance of follow-up and re-evaluation was explained to them. 10 patients reported for follow-up as advised at six weeks post-NSPT. Periodontal parameters such as GI, mean PPD and CAL were recorded. Percentage of sites in each patient with PPD ≥5 mm and CAL ≥5 mm was also re-assessed. Salivary and GCF samples were collected for estimation of calprotectin levels. All participants were then carefully evaluated for further periodontal treatment.

ELISA for estimation of calprotectin: was carried out using ELISA kit systems (R&D systems, Minneapolis, MN, USA). All components and reagents of the kits were brought to room temperature. The standards were reconstituted as provided in the ELISA kit according to the manufacturer's instructions to generate standard curves for calprotectin. GCF and saliva samples were also brought to room temperature. ELISA protocols specified in the catalogue for the quantitative estimation of calprotectin as provided by the manufacturer were meticulously followed. Concentration of calprotectin in GCF and saliva in picograms/milliliters (pg/mL) were calculated according to Optical Density (OD) values obtained and read at 450 nm.

STATISTICAL ANALYSIS

The data was entered into a Microsoft excel for windows version 2016 spreadsheet and analysed using IBM Statistical Package for Social Sciences (SPSS) statistics version 25. Means and standard deviations were used to summarise variables. For periodontal parameters, the comparison between baseline and follow-up were done using non parametric Wilcoxon signed rank tests. Pre and post-therapy GCF and salivary levels of calprotectin were compared using the paired t-test. The p-values less than 0.05 were considered statistically significant.

RESULTS

The present study recruited 11 patients with stages III or IV periodontitis. A total of 22 samples-11 GCF and 11 salivary samples were collected at baseline, prior to NSPT. After six weeks following NSPT, one patient was lost to follow-up, 20 samples-10 GCF and 10 salivary samples were collected.

Periodontal parameters: The mean GI was 2.46±0.36 at baseline and decrease to 1.72±0.24 at follow-up. Mean PD was 5.10±0.34 at base line and decreased to 4.39±0.19 at follow-up. Mean CAL was 4.06±0.70 at baseline and 3.70±0.60 at follow-up. Percentage of sites with PPD ≥5 mm and CAL ≥5 mm were 45.25±6.88 and 47.88±4.79 at baseline and was 31.3±5.83 and 44.7±3.94) at follow-up, respectively. All changes in periodontal parameters were statistically significant (p>0.05) [Table/Fig-1].

Outcomes	Baseline n=11 Mean (SD)	Follow-up n=10 Mean (SD)	p-value*	Z statistic
Gingival Index (GI)	2.46 (0.36)	1.72 (0.24)	0.005	-2.82
PPD	5.10 (0.34)	4.39 (0.19)	0.005	-2.82
CAL	4.06 (0.70)	3.70 (0.60)	0.007	-2.68
% sites with PPD ≥5 mm	45.25 (6.88)	31.3 (5.83)	0.005	-2.84
% sites with CAL ≥5 mm	47.88 (4.79)	44.7 (3.94)	0.005	-2.81

[Table/Fig-1]: Changes in periodontal outcomes following Non Surgical Periodontal Therapy (NSPT).

*Wilcoxon signed rank test, p<0.05 is considered statistically significant, n-number, SD-standard deviation, PPD: Probing pocket depth, CAL: Clinical attachment loss

Salivary calprotectin was 1160.17±596.18 at baseline and was lowered to 1100.46±604.75 pg/µL at follow-up visit. (p=0.74) GCF calprotectin was 1605.11±79.29 before NSPT and decreased to 1556.24±37.56 pg/µL post therapy (0.092) [Table/Fig-2].

Characteristic	Baseline n=11 Mean (SD)	Follow-up n=10 Mean (SD)	p-value*	t statistic
Salivary Calprotectin	1160.17 (596.18)	1100.46 (604.75)	0.74	0.347
GCF Calprotectin	1605.11 (79.29)	1556.24 (37.56)	0.092	1.887

[Table/Fig-2]: Changes in salivary and GCF values of calprotectin following Non Surgical Periodontal Therapy (NSPT).

*Paired t-test, p<0.05 is statistically significant, n-number, SD: Standard deviation; GCF: Gingival crevicular fluid

DISCUSSION

The present study investigated the changes in salivary and GCF calprotectin levels in periodontitis patients following the 1st and 2nd steps of periodontal therapy that together encompasses the non surgical phase of treatment. Periodontal therapy were performed according to the S3 level clinical practice guidelines for the treatment of Stage I-III periodontitis [23]. The study observed significant changes in periodontal parameters at the re-evaluation appointment six weeks post-NSPT. Study results point out that although salivary and GCF levels of calprotectin decreased following periodontal therapy, the observed differences were not statistically significant. As the study did not observe statistically significant differences in oral fluid calprotectin levels at the 6-week follow-up after NSPT, the null hypothesis could not be rejected.

The precise role of calprotectin in periodontal pathogenesis is yet to be precisely unraveled. Higher amounts of GCF calprotectin were reported in Generalised Aggressive (GAP) and chronic periodontitis than in healthy or gingivitis [29]. Evidence also points out that GCF calprotectin levels positively correlated with clinical parameters and biochemical markers of periodontal tissue destruction [30]. Calprotectin acts as an alarm in inflammation and is largely expressed by the granulocytes. Its measures in oral fluids may be reflective of the innate immune responses mounted against dysbiotic plaque microflora.

Kaner D et al., reported that GCF calprotectin concentration and amount/site positively correlated with depth of pockets at baseline

and at three months after NSPT among patients with GAP [12]. They also observed that GCF calprotectin could predict disease activity at both site and patient levels among patients treated for GAP [11]. Andersen E et al., reported that GCF calprotectin levels were higher in the periodontally diseased than healthy subjects and that the level of this protein in GCF decreased following periodontal therapy. They suggested that calprotectin may be used to evaluate response to periodontal therapy [21]. A recent study also reported higher GCF levels of calprotectin in patients with periodontitis and decreased levels at three months after NSPT [17]. Contrary to these reports, Afacan B et al., reported that GCF calprotectin levels increased following periodontal therapy and attributed this finding to the potential role of calprotectin in periodontal healing [19]. This study observed a decrease in GCF calprotectin levels at six weeks after initial periodontal therapy. Decrease of GCF calprotectin in parallel with the reduced clinical periodontal parameters suggests that the role of calprotectin in periodontitis may be predominantly proinflammatory. The lack of statistically significant difference maybe because of persisting inflammation in deeper pockets in the stages III or IV periodontitis patients who constituted the study group. Calprotectin is relatively stable in biofluids and may take longer than six weeks to be reduced significantly. Microcapillary pipettes were used in this study for GCF collection and it was observed that GCF collection was easier from maxillary sites with deeper pockets and visible signs of inflammation.

Salivary calprotectin was reported to be elevated in periodontitis patients when compared to healthy subjects and the level of this protein was seen to be positively correlated with microbiologic and clinical markers of periodontal tissue damage [31]. Lira-Junior R et al., observed that salivary calprotectin levels can be indicative of distinct periodontal case phenotypes and specific treatment responses [18]. This study did not observe significant decrease in salivary calprotectin after NSPT and the findings study are similar to those reported by Menon P et al., [17]. Considerable variation was seen in salivary calprotectin values. Although GCF collection methods are more demanding in terms of time and technique, GCF composition may be more reflective of inflammatory changes brought about by resident and recruited cells in the subjacent periodontium.

Que ML et al., in their longitudinal observation of GCF calprotectin levels in experimentally induced gingivitis observed that high or low calprotectin measures were associated with distinct response patterns among study participants. GCF calprotectin may identify individual patient response to plaque and may be reflective of patient susceptibility [32].

The present study assessed calprotectin levels in both saliva and GCF of stages III or IV periodontitis patients before and after NSPT. Periodontitis case definitions were done according to the most recent new international classification system. Standardisation of cases were done at baseline as the study has included only systemically healthy, stages III or IV periodontitis patients. The study excluded obese patients, smokers and those who had any systemic illness. Obesity may have an effect on systemic inflammation and GCF calprotectin may reflect serum levels of calprotectin. By excluding such participants, potential effects of these confounders on the oral fluid calprotectin levels have been reduced. Most of studies that investigated calprotectin levels in either GCF or saliva have had their follow-up assessment at a three month period while this study has assessed calprotectin in oral fluids at the first evaluation visit, at six weeks post NSPT [28]. According to the background literature review for this study, the simultaneous estimation of calprotectin levels in both GCF and saliva before and after NSPT in a cohort of patients with advanced periodontal destruction has not been done earlier. Meticulous attention to sample collection and rapid processing of samples was another merit of the present study.

Limitation(s)

The present study has evaluated the impact of NSPT on oral fluid calprotectin levels at one follow-up visit only. This is a longitudinal observational study and not a randomised-controlled clinical trial, the strength of evidence would have been better in a randomised controlled trial, but the present study could not have a control group, as all patients who were clinically diagnosed with stages III or IV periodontitis must receive NSPT as soon as possible. Exclusion of systemic factors has been made only on self-report by the participants. Potential confounders such as insulin resistance or varied dietary habits may have been present in the study. One patient was lost to follow-up as he moved to another state due to personal reasons.

CONCLUSION(S)

Clinical periodontics would be immensely benefitted from the identification of biomarkers which can mirror periodontal inflammation. Omics studies in oral fluids may the path to the identification of validated biomarker for periodontal inflammation. In this study, GCF and salivary calprotectin levels are lowered at six weeks post-NSPT. Statistically significant differences between pre and post-treatment values were not observed. Future clinical trials could investigate oral fluid calprotectin levels in larger patient groups, at multiple follow-up intervals and after complete clinical resolution of periodontal inflammation. Oral fluid levels of calprotectin merits more intensive research as the protein may be an important modulator of periodontal inflammation.

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