DOI: 10.7860/JCDR/2021/47123.15181

Original Article

Dentistry Section

Evaluation of Salivary Procalcitonin and Macrophage Activating Factor in Generalised Chronic Periodontitis Patients with and without Type 2 Diabetes Mellitus

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ABSTRACT

Introduction: Periodontitis is a polymicrobial and multifactorial oral disease and is the sixth complication of diabetes mellitus. Early diagnosis is important, and the use of non invasive biomarkers are highly useful for this purpose. The level of Macrophage Activating Factor (MAF) and Procalcitonin (ProCT) corresponds to the intensity of the inflammatory response and the severity of infection; thereby indicating that an increase in concentration or persistence of high values is considered as a prognostic indicator for severity of infection with an adverse outcome.

Aim: To assess the periodontal parameters and quantify the levels of MAF and ProCT in saliva samples of generalised chronic periodontitis subjects with and without type 2 diabetes mellitus and to correlate these levels with the periodontal parameters.

Materials and Methods: The study was a single centre cross-sectional study carried out at the Department of Periodontology, Meenakshi Ammal Dental College and Hospital, Chennai, Tamil Nadu, India, from November 2018 to November 2019. A total of 80 subjects with generalised severe chronic periodontitis were selected and divided into two groups. Group I comprised of 40 subjects who were diagnosed with generalised chronic periodontitis without type 2 diabetes mellitus, whereas group II comprised of 40 subjects with generalised chronic periodontitis who had already been diagnosed with type 2 diabetes mellitus. Periodontal parameters such as Plaque Index (PI), Bleeding on Probing (BOP), Probing Pocket Depth (PPD) and Clinical Attachment Level (CAL) were recorded. The collected samples were subjected to molecular analysis for evaluating ProCT and MAF using Enzyme-Linked Immunosorbent assay (ELISA). Statistical analysis was done using Statistical Package for Social Sciences (SPSS) version 25.1 (Chicago, USA Inc). Student's Independent t-test was used to compare the mean values for the variables in the control and test group. The Pearson's correlation test was used to evaluate correlation between all the variables. The p-value <0.05 was set as the level of significance.

Results: On comparing the periodontal parameters between group I and group II, there was no significant difference between the groups p-value >0.05. The mean salivary ProCT level in group I and group II was 268.76±152.78 ng/mL and 785.75±244.37 ng/mL, respectively. The mean salivary MAF level in group I and group II was 7.15±2.02 ng/mL and 26.56±9.12 ng/mL, respectively. On comparing MAF and ProCT value between group I and group II, there was a statistically significant increase in group II (p-value <0.001) and a weak correlation value with the periodontal parameters was seen.

Conclusion: There was a significant difference in levels of MAF and ProCT in saliva samples of generalised chronic periodontitis subjects with and without type 2 diabetes mellitus, however the periodontal variables in each group did not correlate with MAF and ProCT.

Keywords: Biomarker, Calculus, Diabetes, Gingivitis, Inflammation, Periodontium, Plaque

INTRODUCTION

Gingivitis and periodontitis are most common forms of periodontal disease and periodontitis has been referred as the "sixth classic complication" of diabetes [1]. Periodontitis is initiated by a group of periodontopathic bacteria which generate Lipopolysaccharide (LPS). LPS activates macrophages through toll-like receptors and activated macrophages secrete inflammatory cytokines. These inflammatory responses induce an imbalance between osteoblasts and osteoclasts and result in alveolar bone resorption [2].

Macrophage are the sentinels of the innate immune system monitoring for early signs of infection or tissue damage [3]. Macrophages are hence essential not only for immunity, but also for development and tissue homeostasis [4]. These cells are usually at rest, but can be stimulated through the immune response by variousstimuli [5,6]. MAF, Macrophage Chemotactic Factor (MCF) and Macrophage Migration-Inhibitory Factor (MMIF) are three important mediators involved in macrophage accumulation, activation and function [7,8].

ProCT is a 116 amino acid peptide belonging to the calcitonin superfamily of peptides that has an average Molecular Weight (MW) of 14.5 kDa [9]. It is seen that the serum concentrations in healthy individuals are extremely low, <0.05 ng/mL, or sometimes even immeasurable. In systemic inflammation, particularly in bacterial infections, under the influence of inflammatory cytokines and bacterial endotoxin, it is produced in a number of tissues including lung, liver, kidney, adipose tissue and goes into circulation, when its level can increase upto 1000 times [10]. The cause for this nearly ubiquitous constitutive secretion may be due to changes in the promotor for the ProCT gene, responding to intestinal translocation of LPS or other bacterial constituents, or by a secondary proinflammatory cytokine stimulus such as tumour necrosis factor-α. The very first measurable parameters are displayed within 2-4 hours of stimulation and the peak within 6-24 hours after stimulation [10].

ProCT is a recently used biomarker of severe systemic inflammation, infection and sepsis [11]. ProCT is useful both for early detection of sepsis and for tracking the regimen of antimicrobial care [9]. Salivary ProCT levels also have been shown to be higher in patients with moderate to severe periodontitis than in patients with an essentially healthy periodontium [9,10]. The literature suggests that salivary and/

or serum ProCT might be a predictor of periodontitis with systemic inflammatory conditions such as cardiovascular disease, diabetes, arthritis, etc., [7-9].

The aim of the study was to analyse saliva MAF and ProCT in generalised chronic periodontitis patients with and without diabetes to investigate the relationship of MAF and ProCT with periodontal parameters.

MATERIALS AND METHODS

This was a single centered cross-sectional study carried out at the Outpatient facility of the Department of Periodontology, Meenakshi Ammal Dental College, Chennai, Tamil Nadu, India, from November 2018 to November 2019. The study was approved by the Institutional Review Board MAHER Deemed to be University, Chennai (Protocol No: MADC/IRB- XVI/2018/306). The subjects were explained about the study and written informed consent was obtained from those who agreed to voluntarily participate in this study.

Inclusion criteria: Inclusion criteria were patients willing to participate in the study within the age group of 20-65 years (both male and female), having ≥10 natural teeth, generalised chronic periodontitis patients (involving 30% or more sites according to APP 1999 classification) [12], For group II, patients who had already been diagnosed and under medications for type 2 diabetes mellitus (those having HbA1c ≤7 were included in the study) [13].

Exclusion criteria: Exclusion criteria were patients with systemic conditions such as respiratory diseases, renal disease, liver disease, rheumatoid arthritis, allergy, advanced malignancies and Human Immunodeficiency Virus (HIV) infection, patients on drugs such as corticosteroids, antibiotics, aspirin, in the past three months, active smokers, people who started smoking less than six months ago, patients who underwent periodontal treatment during the preceding six months, pregnant women.

Sample size calculation: Sample size was calculated using the G power software with the mean and standard deviation of the previous studies as reference. The power analysis was done using the formula:

$$n = \left(\frac{Z_{1-\alpha/2} + Z_{1-\beta}}{ES} \right)^2$$

Where, α is the meaning level chosen and $Z_{1\text{-alpha}/2}$ is the value below it from the regular normal distribution holding 1-alpha/2. It showed 95% power with minimum sample size of 40 subjects in each group.

Group I: 40 generalised chronic periodontitis subjects without type 2 diabetes mellitus.

Group II: 40 generalised chronic periodontitis subjects diagnosed with type 2 diabetes mellitus.

Collection of Saliva Samples

Participants were instructed to refrain from eating, drinking, chewing gum and brushing their teeth in the morning of the saliva sample collection. Unstimulated whole saliva samples were obtained by expectorating into polypropylene tubes; clinical periodontal measurements and any necessary periodontal interventions were then carried out. For removing cell debris, saliva samples were clarified by centrifugation (3000 rpm) for three minutes at +4°C, the clear supernatant was transferred to the eppendorf tubes and stored at -80°C until an assay was performed [14].

Periodontal Screening and Examination

Periodontal examination was conducted in the Outpatient Department of Periodontology, Faculty of Dentistry, Meenakshi Ammal Dental College and Hospital, Chennai. Williams periodontal probe was used for the examination and recorded to the nearest millimetre. Periodontal parameters assessed were PI (Silness and Loe, 1964) [15], Bleeding on Probing (BOP), (Ainamo and Bay, 1975) [16], Probing Pocket Depth (PPD) and Clinical Attachment Level (CAL).

Bleeding on Probing (BOP)

The presence or absence of BOP was determined by gentle probing of the gingival crevice with a periodontal probe. A positive score was demonstrated by the presence of bleeding within 10 seconds, calculated as a percentage of the total number of gingival margins examined.

Protein Analysis of Procalcitonin (ProCT) and Macrophage Activating Factor (MAF) by ELISA

Protein concentration of ProCT and MAF in the saliva of test groups was analysed by ELISA [7]. Kits were used according to the manufacturer's instructions and the samples were analysed for protein quantification.

STATISTICAL ANALYSIS

Statistical analysis was done using Statistical Package for Social Sciences (SPSS) version 25.1 (Chicago, USA Inc). Mean and standard deviation for periodontal parameters, MAF and ProCT were estimated for both the groups. The Pearson's correlation test was used to evaluate correlation between all the variables. Student's Independent t-test was used to compare the mean values for the variables in the control and test group. In the present study, p-value <0.05 was considered as the level of significance.

RESULTS

Mean age in group I was 43.65±8.92 years and in group II was 49.12±6.26 years. Gender distribution in group I was 55% male (n=25) and 45% (n=15) female and in group II was 57% male (n=28) and 43% female (n=12) patients. The mean Plaque Index (PI) in group I and group II was 2.07±0.38 and 2.08±0.33, respectively. On comparing the PI between group I and group II the mean difference was -0.0045 and it was statistically not significant (p-value=0.955). The mean PPD in group I and group II was 4.53±0.53 and 4.74±0.98, respectively. On comparing the PPD between group I and group II the mean difference was -0.20 and it was statistically not significant (p-value=0.248) [Table/Fig-1].

Parameter	Group I (n=40) Mean±SD	Group II (n=40) Mean±SD	Mean difference	p-value
PI	2.07±0.38	2.08±0.33	-0.0045	0.955 (NS)
PPD (mm)	4.53±0.53	4.74±0.98	-0.20	0.248 (NS)
CAL (mm)	4.75±0.64	4.86±0.99	-0.11	0.555 (NS)
BOP (%)	80.30±17.39	83.0±17.09	-2.70	0.486 (NS)
MAF (ng/mL)	7.15±2.02	26.56±9.12	-19.40	<0.001**
ProCT (ng/mL)	268.76±152.78	785.75±244.37	-516.98	<0.001**

[Table/Fig-1]: Intergroup comparison of mean, standard deviation and test of significance of Plaque Index (PI), Bleeding on Probing (BOP), Probing Pocket Depth (PPD), Clinical Attachment Level, Macrophage Activating Factor (MAF), Procalcitonin (ProCT) Between Group I and Group II.
**Level of significance p-value ≤0.05 (student's independent t-test)

The mean CAL in group I and group II was 4.75±0.64 and 4.86±0.9934 respectively. On comparing the CAL between group I and group II the mean difference was -0.11 and it was statistically not significant (p-value=0.555). The mean BOP in group I and group II was 80.30±17.39 and 83±17.09 respectively. On comparing the BOP between group I and group II the mean difference was -2.7 and it was statistically not significant (p-value=0.486) [Table/Fig-1]. The mean salivary MAF level in group I and group II was 7.15±2.02 ng/mL and 26.56±9.12 ng/mL, respectively. On comparing the salivary MAF level between group I and group II the mean difference was -19.40 ng/mL and it was statistically significant (p-value <0.001 [Table/Fig-1].

The mean salivary ProCT level in group I and group II was 268.76±152.78 ng/mL and 785.75±244.37 ng/mL, respectively. On

comparing the salivary ProCT level between group I and group II the mean difference was -516.98 ng/mL and it was statistically significant (p-value <0.001) [Table/Fig-1].

The correlation coefficient value of PI with salivary MAF in group I was -0.096 and in group II it was -0.079 which suggested weakly negative association and it was non-significant. Correlation value of PI in salivary ProCT in group I was -0.063 which suggested weakly negative association and group II it was 0.206 which was weakly positive but the values were not significant [Table/Fig-2].

Variables	MAF Group I	MAF Group II	ProCT Group I	ProCT Group II
r-value	-0.096	-0.079	-0.063	0.206
p-value	0.554 (NS)	0.628 (NS)	0.699 (NS)	0.203 (NS)

[Table/Fig-2]: Correlation between Plaque Index (PI) and Macrophage Activating Factor (MAF) and Procalcitonin (ProCT) in Group I and Group II.
S: Statistically significant; NS: Not significant; Level of significance p-value ≤0.05

The correlation value of PPD with salivary MAF in group I was -0.027 and in group II it was -0.139, respectively which suggested weakly negative correlation that was non-significant. The correlation value of PPD with salivary ProCT in group I was 0.286 and in group II was 0.132, respectively which suggested weakly positive non-significant correlation [Table/Fig-3].

Variables	MAF Group I	MAF Group II	ProCT Group I	ProCT Group II
r-value	-0.027	-0.139	0.286	0.132
p-value	0.867 (NS)	0.393 (NS)	0.074 (NS)	0.417 (NS)

[Table/Fig-3]: Correlation between Probing Pocket Depth (PPD) and Macrophage Activating Factor (MAF) and Procalcitonin (ProCT) in Group I and Group II.
S: Statistically significant; NS: Not significant; Level of significance p-value ≤0.05

The correlation value of CAL with salivary MAF in group I was -0.044 and in group II it was -0.059 respectively which suggested no significant correlation. CAL in salivary ProCT in group I was 0.267 and in group II it was 0.137, respectively which suggested no significant correlation [Table/Fig-4].

Variables	MAF Group I	MAF Group II	ProCT Group I	ProCT Group II
r-value	-0.044	-0.059	0.267	0.137
p-value	0.785 (NS)	0.717 (NS)	0.096 (NS)	0.400 (NS)

[Table/Fig-4]: Correlation between Clinical Attachment Level (CAL) and Macrophage Activating Factor (MAF) and Procalcitonin (ProCT) in Group I and Group II.
S: Statistically significant; NS: Not significant; Level of significance p-value ≤0.05

The correlation value of BOP with salivary MAF in group I was 0.169 and in group II it was -0.083 respectively which suggested no significant correlation. BOP in salivary ProCT in group I was -0.077 and in group II it was -0.071, respectively which suggested no significant correlation [Table/Fig-5].

Variables	MAF Group I	MAF Group II	ProCT Group I	ProCT Group II
r-value	-0.169	-0.083	-0.077	-0.071
p-value	0.297 (NS)	0.611 (NS)	0.636 (NS)	0.664 (NS)

[Table/Fig-5]: Correlation between Bleeding on Probing (BOP) and Macrophage Activating Factor (MAF) and Procalcitonin (ProCT) in Group I and Group II.
S: Statistically significant; NS: Not significant; Level of significance p-value≤0.05

DISCUSSION

The study analysed salivary MAF and ProCT in generalised chronic periodontitis patients with and without diabetes to correlate the relationship of these biomarkers with periodontal status.

The periodontal parameters such as PI, bleeding index, PPD and CAL were assessed in both the groups which showed no significant difference. This was in accordance with studies done by Sbordone L and Ramaglia L, Oliver RC and Tervonen T, Sznajder N et al., Kawamura M et al., Salvi GE et al., Siudikiene J, Khader YS and Albashaireh ZSM and Janket SJ et al. who showed no significant difference in the average PI between diabetics and non-diabetics [17-24]. Also, current study results were similar to study conducted by Kamil MA, which showed no significant difference in mean PI score for the diabetic and non-diabetic groups, even though the mean PI score was observed to be slightly higher in the diabetic group, the difference was statistically non-diabetic [25].

BOP findings were in contrast to findings of Tchobroutsky G who showed the degree of gingival bleeding was more in diabetics than non-diabetics [26]. In our study, group II showed higher percentage of BOP than group I but it was not statistically significant. Similar observations were made by Sayeeganesh N et al., and Katagiri S et al., who found that successful glycaemic control strengthened BOP lesions by ameliorating inflammation of periodontal tissues in type 2 diabetic patients with periodontitis [27,28]. This might be the reason for the reduced BOP percentage in group II in the present study.

Previous study done by Wernicke K et al., showed that HbA1c levels (average HbA1c reduction 1.8%) were positively associated with deeper PPD in patients with non-insulin-dependent diabetes mellitus [29]. Thus, it seems that controlled diabetes does not impair periodontal health as regards to pocket formation. This is in accordance with Tervonen T and Knuuttila M. and Campus G et al., who showed well-controlled diabetic patients had better periodontal health than the controls [30,31].

In the study conducted by Awartani FA, the researchers demonstrated a significant association of the loss of attachment level (3-4 mm) with periodontal disease in poorly controlled diabetic patients, as compared to better controlled patients (p-value <0.05). Thus, it is not unusual to find diabetic patients under control with normal or almost normal gingiva and supporting structures. These cases coincide rather consistently with reduced levels of plaque and calculus [31,32].

Several studies showed that macrophages played a role in the aggravation of inflammation in diabetics and are identified as critical markers of regulation at cellular signal transduction [33-35]. To become resident macrophages, macrophages are recruited to peripheral tissues and contribute to local inflammation, insulin resistance production or even pancreatic dysfunction. In addition, the accumulation of evidence has played an important role in macrophage polarisation in the development of metabolic diseases [33]. Results of our study were in accordance with the study conducted by Kraakman MJ et al., who showed that in diabetes there is an imbalance in the ratio of M1/M2 macrophages, with M1 “proinflammatory” macrophages being enhanced compared with M2 “antiinflammatory” The downregulation of macrophages contributes to chronic inflammation and the spread of metabolic dysfunction [33]. Lew JH et al., stated that condition such as hyperglycemia induced IL-1β and sIL-6R production from macrophages in inflammatory periodontal tissues which exacerbates the periodontitis synergistically via MMP-1 production from Human Gingival Fibroblasts (HGFs) [34]. Another study conducted by Zhang P et al., showed that salivary MAF levels were positively correlated with the progression of periodontitis (p-value<0.05, r=0.779) [35]. This might be due to activated macrophages resulting in release of inflammatory cytokines, including MAF into the periodontal tissue, with a consequential induction of more osteoclasts. Thus, the inflammatory response and the increased MAF levels create a feedback loop that regulate the progression of periodontal disease [35]. Pussinen PJ et al., suggested that the infected/inflamed area in periodontitis is associated with macrophage activation via increased concentration of LPS [2]. Almubarak A et al., showed that increase in the percentage of inflammatory monocytes and macrophages has been reported in the circulation of patients with T2DM [36]. Thus, in the present study MAF was higher in both the groups but it was comparatively higher in diabetes group and it was statistically significant [2,35-37].

The mean value of ProCT in group I and group II was statistically significant and the difference between the groups was statistically significant (p-value <0.05). Periodontitis in diabetes may act as a stimulus for ProCT production, since endotoxin is a potent stimulator

for the production of ProCT and can promote the systemic release of calcitonin precursors from nearly all tissues of the body [38-40]. Periodontitis is initiated and promoted by pathogenic Gram-negative, endotoxin-producing bacterial infections, and may promote a local upregulation of ProCT in saliva [39]. The correlation between salivary ProCT and HbA1c values may be another supportive indication of the underlying connection between the local and systemic inflammatory states of periodontitis and type II diabetes [40,41]. Increased proximal expression of ProCT was postulated with deteriorating diabetic status or periodontitis. ProCT levels in extremity wound effluent have recently been measured in this regard and have been associated with dehiscence in wartime extremity injuries, indicating local ProCT development at the extremity wound [40]. Study conducted by Uzzan B et al., showed that although ProCT level changes during infections, it may change under conditions without infection [42]. Our study correlated with study by Bassim CW et al., in which ProCT level was studied in patients with periodontitis and type II diabetes [43]. Their findings showed a significant increase in ProCT level in patients with periodontitis and diabetes as compared with control group (241±71 vs. 77±516 pg/mL, p-value=0.02) [43]. Also, the results of our study are in accordance with the study done by Hendek MK et al., who evaluated ProCT level in periodontal disease showed that there was a weak correlation between the mean salivary ProCT level and periodontal disease (r-value-0.09, p-value <0.05) [14]. Both groups showed higher levels of ProCT value; but in diabetes group it was significantly higher. Periodontal therapy was shown to decrease the salivary ProCT level in patients diagnosed with periodontal disease [44].

To the best of our knowledge this was the first study to evaluate saliva ProCT and MAF in generalised chronic periodontitis patients with and without type 2 diabetes in a single study. Analysis of these biomarkers showed that it could provide useful information on the initial stage of periodontal destruction in patients with and without diabetes.

Limitation(s)

However, our study would have been better with larger sample size and longer follow-up period. Also, cases of different grades of periodontal inflammation should have been selected such as severe and moderate periodontitis cases to correctly assess whether MAF and ProCT levels vary due to diabetic condition or periodontal inflammation.

CONCLUSION(S)

There was a significant difference in MAF and ProCT in diabetics and non-diabetics in general chronic periodontitis cases. Therefore, salivary ProCT and MAF may have a potential role as biomarkers for systemic inflammation such as diabetes however the periodontal variables in each group did not correlate with MAF and ProCT. Therefore, the relationship of these factors with periodontal status must be further explored.

Acknowledgement

We like to thank our patients and our Department for complete support during this study process.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
• Plagiarism X-checker: Oct 09, 2020
• Manual Googling: Nov 19, 2020
• iThenticate Software: Jun 30, 2021 (25%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
• Financial or Other Competing Interests: None
• Was Ethics Committee Approval obtained for this study? Yes
• Was informed consent obtained from the subjects involved in the study? Yes
• For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: Oct 08, 2020
Date of Peer Review: Nov 19, 2020
Date of Acceptance: Feb 12, 2021
Date of Publishing: Jul 01, 2021