Are diagnostic inflammatory biomarkers suitable to predict periodontal disease activity after non-surgical periodontal therapy?

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Abstract

Objective: Many biomarkers of periodontal diseases are inflammatory in nature and understandably decrease after non surgical periodontal therapy (NSPT). This may not reflect the true nature of the disease. The aim of the study was to compare the salivary and serum levels of biomarkers more likely associated with bacterial infection and inflammation before and after NSPT.

Methodology: 64 were enrolled and grouped based on their periodontal status as group I- periodontally healthy individuals (n=32) and group II- periodontitis stage II and III grade A (n=32). Plaque index, gingival index, bleeding index, probing depth (PD) and clinical attachment level (CAL) were recorded. The Periodontitis patients received NSPT. Salivary and serum samples were obtained at baseline and two weeks after treatment for analysis of Procalcitonin (PCT), C - reactive protein (CRP) and Interleukin (IL-1β) by enzyme linked immunosorbent assay (ELISA).

Results: Periodontitis individuals had significantly higher levels of PCT, CRP and IL-1β than non periodontitis group ($p < .05$). Significant positive correlation was found between the three biomarkers and the clinical parameters. A significant decrease in salivary and serum PCT, CRP and IL-1β was detected after periodontal treatment compared to baseline values in the periodontitis group ($p < .05$). Correlation analysis revealed serum PCT to be strongly associated with PD and CAL at $p < .05$ even after NSPT.

Conclusion: The present study found that NSPT resulted in reductions of both salivary and serum CRP, and IL-1β in periodontitis individuals within the normal range while the serum PCT levels still showed higher than normal values suggesting it to be a suitable marker for measuring periodontal disease activity.

Keywords: Procalcitonin; periodontitis; serum; saliva; inflammation.

Introduction

Periodontitis is a chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms and characterized by progressive destruction of the tooth-supporting apparatus (Papapanou *et al*., 2018). Epidemiologically, the interplay between bacterial species and host immune system in periodontal disease has been linked to cardio vascular (Dietrich *et al*., 2013, Beukers *et al*., 2017), chronic respiratory (Usher and Stockley., 2013) and diabetes mellitus (Nazir., 2017, Preshaw *et al*., 2020). Generally it causes systemic inflammatory overload through inflammatory biomarkers like the C-reactive protein (CRP), interleukin-1β (IL-1β), and interleukin-6 (IL-6). In majority of these studies, the transfer of these potentially pathogenic bacteria into the vascular pathway and their interaction with the vascular cells adds to the increased concentrations of these inflammatory mediators in the serum. Overgrowth of specific gram negative microorganisms in the biofilms and their interactions (Hajishengallis., 2015) have gained importance in understanding host bacterial interactions. Therefore mechanical debridement of the affected tooth surface with objective of

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reducing total bacterial load and changing the environmental conditions of these microbial niches forms the standard treatment for periodontitis. This microbial reduction followed by good maintenance has demonstrated clinical reduction in inflammatory signs and symptoms, but does not eliminate the bacteria persisting in inaccessible areas (Tonetti *et al*., 2015). A vast majority of clinical interventional studies has demonstrated a statistically verifiable added benefit in the use of adjunctive systemic antibiotic therapy even though the clinical relevance is controversial (Pretzl *et al*., 2019, Smiley *et al*., 2015, Eick *et al*., 2018). There is also lack of evidence for long term benefits (above 2 years) and indication stating differences on the effect of systemic antimicrobials in aggressive and chronic forms of periodontitis (Teughels *et al*., 2020), (D'Ercole *et al*., 2008). It is still unclear if microbial testing process can be applied to all forms of periodontitis since no evidence based conclusion or guidelines has been drawn from the extensive information published on decision making for usage of antibiotics with mechanical debridement. Currently, there is scarcity of data differentiating bacterial biomarkers and inflammatory biomarkers before and after non surgical periodontal therapy (NSPT) that reflects the residual bacterial load and periodontal disease activity. Procalcitonin (PCT) is a proven bacterial biomarker and is approved by the FDA for guiding antibiotic therapy (Meier *et al*., 2019). The hypothesis of the present study is that serum and salivary PCT, CRP and IL-1β may be increased in stage II and III periodontitis and these inflammatory biomarkers should decrease following NSPT. The bacterial biomarker should also be able to reflect on the residual bacterial infection present. Thus the aim of the study was to first compare the serum and salivary PCT, CRP and IL-1β levels between periodontitis patients and periodontally healthy individuals; Second, to evaluate if the changes in the saliva and serum levels of PCT, CRP, and IL-1β of the periodontitis patients after NSPT are able to reflect the disease activity.

Material and Methods

Study population

A total of 64 systemically healthy and non-smoker participants with age range between 25- 55 years were recruited for the present study from the Department of Periodontology, Bangalore Institute of Dental Sciences and Research Centre between February 2019 and June 2019. This study design was approved by the institutional ethical committee (BD/2017/1611). Before enrolment, the detail of the study was explained and the written informed consent was obtained from all the participants in accordance with declaration of Helsinki of 1975, as revised in 2013. Medical and dental histories of all the participants were taken, and patients were excluded if they had any systemic or debilitating diseases affecting periodontal health like diabetes mellitus, thyroid disease; pregnant or lactating women; recent history of or presence of acute bacterial infection or fever; former or current smokers; HIV infection. None of the participants gave history of use of antibiotics or immunosuppressant medication within last 3 months or treatment for periodontal disease within six months before the study.

Clinical examination and grouping

All the individuals had minimum of twenty teeth or more excluding third molars underwent detailed full mouth periodontal clinical and radiographic examination. Clinical periodontal evaluations consisted of measurements of Probing depth (PD), Clinical attachment level (CAL), Plaque index (Turesky *et al*., 1970), gingival index (Loe., 1967) and gingival bleeding index (Newbrun.,1996) scores. All the measurements were recorded at six sites around each tooth using UNC 15 manual periodontal probe by a two calibrated examiners (RM and SR).Considering the periodontal status, the participants were classified as periodontally healthy controls (n=32) and generalised stage II and III periodontitis (n=32). Diagnosis was based on the criteria set by the 2017 World Workshop on the Classification of Periodontal and Peri-implant diseases and Conditions (Caton *et al*., 2018, Papapanou *et al*., 2018). Healthy controls included volunteers with an intact periodontium who had BOP <10% and PD ≤ 3mm without clinical attachment loss or radiographic sign of alveolar bone loss. They also had no previous history of periodontitis. For stage II and III periodontitis grade A the inter dental CAL 3-4mm or ≥5mm, maximum Probing depth ≤ 5 mm or ≥ 6mm and radiographic bone from coronal third (15%-33%)or extending to middle or apical third of the root. They showed no more than 4 teeth loss.

Examiner calibration

Two examiners (RM and SR) were calibrated prior to the study. Five subjects not included in the study were requested to volunteer and calibrations exercise for clinical parameters recording before the actual study was done. The probing depth estimation was judged to be reproducible if the intra examiner agreement was within ± 1 mm between the repeated measurements was at least 80%. The kappa value for intra examiner variability was recorded to be 0.89. The inter examiner calibration was done using Cronbach's alpha and the scores was between 0.8-0.9.

Saliva and Serum sampling

All the samples were collected a day after the clinical measurements to avoid contamination of blood. The samples were collected in the morning after overnight fast. To obtain salivary samples the protocol set by Navazesh (Navazesh M and Satish KM., 2008) was followed. Individuals were instructed to refrain from eating, chewing gums and brushing their teeth for 1 hour prior to the saliva sample collection. 5ml of un-stimulated whole saliva was collected by passive drooling method using polyethylene centrifuge tubes. The procured salivary samples were centrifuged to remove the cell debris using Remi-R 8C for 10min at + 400 C to obtain a clear supernatant. The samples was transferred to epondorff tubes and stored at -800 C until the assay was performed.

Following saliva sampling, 5ml of venous blood were taken from the antecubital vein by standard venipuncture method (Melissa K Tuck *et al*., 2009). Prior to each sampling the site was wiped with isopropyl alcohol to minimize the number of potential skin contaminants. Serum was isolated by centrifugation at 3000rpm for 15 minutes and separated using sterile pipette. It was then transferred to 1.5ml aliquots and stored at -80°C until the assay was performed.

Protocol for nonsurgical periodontal therapy

All periodontitis patients received nonsurgical periodontal therapy (NSPT) by the same researcher (RM) after the baseline serum and salivary sampling. NSPT included motivation, oral hygiene instructions and scaling and root planing (SRP) procedures. Full mouth SRP was performed using combination of ultrasonic and manual instruments. Root surfaces were instrumented using area specific curettes under local anaesthesia if necessary on the same day (under 24 hrs). No antibiotics or any other medicine was prescribed during the treatment. Motivation and reinforcement of oral hygiene education was done at all the recall intervals. At two weeks after completion of the treatment, clinical periodontal measurements were repeated and serum and salivary samples were taken in patients with periodontitis.

Analysis of PCT, IL-1β and CRP levels

Standard dilutions and reagents were prepared as mentioned in the respective kits. The frozen samples were thawed to room temperature before the assays. The levels of PCT, IL-1β and CRP in serum and saliva were determined using Enzyme linked immunosorbent assay (ELISA) using commercial available kits (KINESISDx, California, USA).The assays were carried out on 40µL samples of serum and 40µL of saliva for each molecule. Standards solution and sample solution were added using pipettes into the respective wells. Assay diluents were also added to all plates of the molecules and incubated at room temperature for 60 minutes. Then, the plates were washed and chromogen solution was added. The enzyme substrate reaction was terminated using a stop solution and after 15minutes, the absorbance was read at 450nm by ELISA plate reader. The detection ranges for PCT, CRP and IL 1β were 1 to 5.125125pg/ml, 0 to 2.00 pg/ml and 2 to 3.91pg/ml respectively.

Statistical analysis

Statistical power calculation to estimate minimal sample was performed using G power v3.1.9.2. According to this analysis, 32 individuals were needed per group to achieve a 85% of power at 0.72 effective size and margin of error at 5%. Statistical significance level (p value) was set at less than .05. The distribution of all the variables was examined by Shapiro – Wilk normality test. Since the data was not normally distributed, comparisons of biochemical and clinical parameters between the groups were determined using the Mann-Whitney U test. The Wilcoxon signed –rank test (paired observations) was performed to compare the baseline values with those after treatment. The Spearman's rank correlation was performed in all groups to detect the possible correlation of salivary and serum PCT, CRP and IL 1β with clinical parameters and also with each other. All the recorded clinical and biochemical parameters were statistically analyzed using SPSS software version 20 (IBM® SPSS® Statistics, IBM corporation, NY, USA).

Results

The demographic variables are presented as median values in Table 1. There are no significant differences in age, gender and number of teeth distribution between the study groups at p >.05. Full mouth clinical periodontal scores are outlined in Table 2. All periodontal parameters (PI, GI, GBI, PD and CAL) were significantly higher in periodontitis group than the healthy group (P <.05) (Table 2). Significant decrease was noted in PI, GI, GBI at two weeks post nonsurgical periodontal treatment in the periodontitis group (p <.05).The PD and CAL remained higher in periodontitis group even at the end of the study (Table 2). The biomarkers results for PCT, CRP and IL-1β in both serum and saliva are outlined in Table 3 for both the groups. The periodontitis group showed significant increase in all the three biomarkers than the healthy group (p < .05). Two weeks post nonsurgical periodontal therapy the levels of all the three biomarkers in saliva showed significant reduction for the periodontitis group (p < .05) .While in serum the reduction was significant at p < .05 for the

CRP and IL-1β and the same was not observed for the PCT. (Table 4). Correlation statistics revealed PCT, CRP and IL-1β in saliva to be positively correlated to the clinical parameters (p<.05, Table 5). Among these, the salivary PCT levels showed higher strong positive correlation levels with Bleeding Index than CRP and IL-1β. Significant positive correlations were also seen between the serum PCT, CRP and IL-1β to the clinical parameters (p< .05, Table 5). The PCT showed higher strong positive correlation with PI, GI, GBI, PD and CAL than CRP and IL-1β (p< .05, Table 5).

Table 1. Demographic Characteristics of the Study Groups.

Demographic Variables	Healthy (n=32)	Periodontitis (n=32)	
		Stage II (n=12)	Stage III (n=20)
Age (years)	35(22-48)	41.15(32-56)	42.44(33-59)
Sex (F/M)	17 F / 15 M	5/7	9/11
Number of teeth	27(25-28+	28 (25-28)	25 (22-25)

F- Female; M-Male; Data are expressed as median (minimum to maximum).

Table 2. Clinical Periodontal Measurements of the Study Groups.

PARAMETERS (Full mouth)	Healthy (n=32)	Stage II Periodontitis		Stage III Periodontitis	
		Baseline	Post NSPT	Baseline	Post NSPT
PI	.40 (.17-1.4)	2.37 (1.8-2.71) *	0.72 (0.33-1.02) #*	2.44 (1.8-2.91) *	0.74 (0.42-1.12) #*
GI	.45 (.09-1.52)	2.15 (1.1-2.80) *	0.56 (0.23-0.85) #*	2.43 (1.2-2.95) *	0.57 (0.21-0.91) #*
GBI(%)	8.17 (5.30-4.25)	90.5 (84-96) *	9.75 (7.12-11.43) #*	92.75 (85-98) *	9.9 (7.22-12.01) #*
PD(mm)	1.33 (.21-1.84)	4.74 (4.41-4.97) *	4.61 (4.41-5.87)	5.45 (4.93-5.83) *	5.01 (5.01-5.6)
CAL(mm)	0.00(0.00-0.00)	4.86 (4.45-5.1) *	4.79 (4.4-5.73)	5.72 (5.1-5.97) *	5.45 (4.3-5.61)

PI: Plaque Index; GI: Gingival Index; GBI: Gingival Bleeding Index; PD: Probing Depth; CAL: Clinical Attachment Loss. Data are expressed as median (minimum to maximum). Mann–Whitney U test (unpaired observations). * Significantly different from healthy controls. #* Significantly different from baseline.

Table 3. Clinical Parameters over time.

		(n=32)	Minimum	Maximum	Median	p value
SALIVA	PCT (ng/mL)	Healthy	0	0.09	0.03	0.01*
		Periodontitis	0.12	0.56	0.23	
	CRP (mg/L)	Healthy	0.03	0.4	0.08	0.01*
		Periodontitis	0.12	0.32	0.26	
	IL-1β (pg/dL)	Healthy	42.6	91.45	68.25	0.01*
		Periodontitis	210.45	341.2	284.94	
SERUM	PCT (ng/mL)	Healthy	0.02	0.09	0.05	0.01*
		Periodontitis	0.57	3.21	1.87	
	CRP (mg/L)	Healthy	1.1	2.78	1.71	0.01*
		Periodontitis	2.96	3.98	3.48	
	IL-1β (pg/dL)	Healthy	0.01	1.12	0.06	0.01*
		Periodontitis	0.8	2.68	1.59	

PCT: Procalcitonin; CRP: C-reactive protein;IL-1β: Interleukin 1β. Data are expressed as Median.
Mann–Whitney U test (unpaired observations)
* Significantly different from healthy controls

Table 4. Analysis of biomarkers two weeks after periodontal therapy.

			Median	Z value	p value
SALIVA	PCT (ng/mL)	Pre	0.23	-4.95	0.02*
		Post	0.10		
	CRP (mg/L)	Pre	0.26	-4.9	0.03*
		Post	0.22		
	IL-1β (pg/dL)	Pre	284.94	-4.8	0.02*
		Post	195.81		
SERUM	PCT (ng/mL)	Pre	1.87	-1.91	0.05*
		Post	0.89		
	CRP (mg/L)	Pre	3.48	-4.9	0.01*
		Post	2.93		
	IL-1β (pg/dL)	Pre	1.59	-4.54	0.01*
		Post	1.45		

PCT:Procalcitonin; CRP: C-reactive protein;IL-1β: Interleukin 1β.
.-Wilcoxon signed-rank test (paired observations).
*significantly different between pre and post NSPT

Table 5. Spearman's Correlation between clinical parameters and PCT, CRP and IL-1β at baseline (pre test).

Parameters		Saliva		Serum	
		r value	p value	r value	p value
PI	PCT(ng/mL)	0.74	0.74	0.74	0.74
	CRP(mg/L)	0.67	0.67	0.67	0.67
	IL-1β(pg/dL)	0.72	0.72	0.72	0.72
GI	PCT(ng/mL)	0.74	0.74	0.74	0.74
	CRP(mg/L)	0.60	0.60	0.60	0.60
	IL-1β(pg/dL)	0.71	0.71	0.71	0.71
GBI (%)	PCT(ng/mL)	0.81	0.81	0.81	0.81
	CRP(mg/L)	0.66	0.66	0.66	0.66
	IL-1β(pg/dL)	0.77	0.77	0.77	0.77
PD (mm)	PCT(ng/mL)	0.76	0.76	0.76	0.76
	CRP(mg/L)	0.72	0.72	0.72	0.72
	IL-1β(pg/dL)	0.77	0.77	0.77	0.77
CAL (mm)	PCT(ng/mL)	0.75	0.75	0.75	0.75
	CRP(mg/L)	0.72	0.72	0.72	0.72
	IL-1β(pg/dL)	0.76	0.76	0.76	0.76

PI: Plaque Index; GI: Gingival Index; GBI: Gingival Bleeding Index; PD: Probing Depth; CAL: Clinical Attachment Loss; PCT: Procalcitonin; CRP: C-reactive protein; IL-1β - Interleukin1β. *Statistically significant (P< 0.05).

Discussion

The results of our study demonstrate that the serum and salivary PCT, CRP and IL-1β levels is significantly higher in periodontitis patients compared to periodontally healthy individuals. The raised inflammatory markers reduce significantly after NSPT. Strong positive correlation is seen between these inflammatory markers and the clinical periodontal parameters. Interestingly two weeks post NSPT, the CRP and IL-1β showed complete resolution comparable to periodontally healthy individuals, while the serum PCT levels are still higher than normal which reflects a possible residual bacterial infection.

Meta analysis report shows PCT as a promising biomarker specific for bacterial infection (Simon *et al*., 2004).To the best of our knowledge, this is the first study presenting data on serum and salivary levels of bacterial (PCT) and inflammatory biomarkers (CRP and IL-1β) in periodontal disease.

Early identification of infection is still a challenge for clinicians; the general consensus is to avoid antibiotics for every suspected infection because of emerging issues with bacterial resistance. Therefore identification of a reliable biomarker for early periodontal loss and personalizing the use of antibiotics based on the PCT levels could be of great benefit in clinical practice. For our research, both serum and salivary samples were obtained from participants. Serum and saliva reflect the disease activity better than crevicular fluid which is secreted in minuscule amounts making its collection time consuming and tedious. Serum reflects systemic bacteremia and inflammatory status of the individuals whereas whole saliva represents pooled sample of all periodontitis sites reflecting the cumulative effect of the disease. In our study the PCT values (Saliva- 0.23ng/ml and Serum- 1.87ng/ml) in periodontitis Stage II and III is higher than the PCT values of periodontally healthy individuals (Saliva-03ng/ml and Serum- 0.05ng/ml). This is in accordance with studies done by Selvadurai *et al*., 2019, Hendek *et al*., 2015. Contradictory results have been shown in salivary PCT levels by Yousefimanesh *et al*., 2015. The possible reason for low salivary PCT levels may be due to difference in the section of the periodontitis subjects included (mean CAL of 3.5mm versus 5.4mm) and the sample storage affecting potency (-200 versus -800). Based on the guidelines and review on serum PCT values (Bouadma *et al*., 2010, Andriolo *et al*., 2017) it can be postulated from our study that periodontitis patients with serum PCT levels less than 0.5ng/dL need not use antibiotics. Two weeks after NSPT, the PCT values decreases to 0.89± 0.51ng/ml, which happens to still higher than the levels of periodontal healthy individuals. This can be explained as there is resolution of microbial inflammatory, load (Matwiyoff *et al*., 2012).

C-reactive protein (CRP) is considered as a key biomarker of inflammation. CRP takes 12-24 hours to rise in inflammation or tissue damage and remains elevated for up to 3-7 days (Redman *et al*., 2016). It returns to normal once the inflammation subsides. The results of our study show that in periodontal health the serum and salivary levels are 1.71mg/L and 0.08mg/L respectively, which is well within the normal CRP range in the body. Whereas, in periodontitis stage II and III individuals it is 3.48mg/L and 0.26mg/L in serum and saliva respectively. This can be due to low levels of bacteremia, lipopolysaccharides, and other bacterial components providing a stimulus for systemic inflammatory responses leading to an increase in production of CRP by activation of inflammatory cascade. Many studies are available in literature which echo similar views (Gomes-Filho *et al*., 2011, Shojaee *et al*., 2013, Out *et al*, 2012, Noack *et al*., 2001). However CRP is not a very accurate biomarker for periodontal disease because the levels in serum may vary between individuals and fluctuate due to other factors such as high blood pressure, alcohol use, smoking, chronic fatigue, diabetes, sleep disturbances and depression. CRP levels range from 4.1mg/L in hypertensive periodontitis patients (Alade *et al*., 2018), to 32.55 ± 13.773 mg/L in diabetics with severe periodontitis (Dholey *et al*., 2017). The results of CRP after two weeks of nonsurgical periodontal therapy in this study demonstrate that serum and salivary values reduces to 2.93mg/L and 0.22mg/L respectively which coveys that there is complete resolution of inflammation. It is of importance to note that the PCT values after NSPT declines but not to the normal range indicating the persistence of bacteria thus making it more reliable than CRP.

However, the use of both markers together increases the predictive value in periodontitis individuals.

Members of the interleukin-1 family are thought to be key mediators of host response to microbial invasion, inflammation and immunological reactions. IL-1β is an important mediator of inflammatory response in periodontitis as it is involved in cellular proliferation, differentiation, and apoptosis. (Faizuddin *et al*., 2003). In our study the serum and salivary levels of IL-1β in periodontal health is 0.06 pg/mL and 68.25pg/mL where as in periodontitis stage II and III individuals it is 1.59pg/mL and 284.94pg/mL respectively. This is in accordance with several studies. (Talvan *et al*., 2017, Scannapieco *et al*., 2007, Gursoy *et al*., 2009). Post NSPT the results of our study show reducing IL-1β levels to 1.45pg/mL and 195.81pg/mL in serum and saliva. There is wide disparity in literature reporting salivary IL-1β values in periodontitis ranging from 280pg/mL to 2500pg/mL (Teles *et al*., 2009, Tobo´n-Arroyave *et al*., 2008). This variation can be attributed to factors such as individual variation in immunological response and quality of local bacterial challenge (Gursoy *et al*., 2009, Tobo´n- Arroyave *et al*., 2008). Moreover it is noted that levels may vary from site to site in the same periodontium as local inflammatory response can vary. Hence site specific GCF analysis may not be as reliable as pooled salivary sample with respect to IL-1β.

Both inter group and intra group comparison of all the three biomarkers between periodontal health and periodontitis stage II and III in our study suggest that all the selected biomarkers are highly significant and they correlate well with the disease severity (Simon e*et al*., 2008, Selvadurai *et al*., 2019, Hendek *et al*., 2015, Yousefimanesh *et al*., 2015).

Our data in this study elucidate the substantial effectiveness of monitoring levels of PCT pre and post non surgical therapy. Furthermore, the clinical utility of PCT as a biomarker for infection and to guide antibiotic therapy will minimize unwanted use of antibiotics. Therefore we consider serum PCT levels to be beneficial in both diagnosis of periodontitis and a guide for effective periodontal therapy. We chose two weeks post treatment as

the ideal follow up period to observe the variations in these acute phase protein markers specifically PCT as it could guide us to the usage of antibiotics which would have been lost in longer follow ups.

Limitation of this study is not having a bacterial culture results and limited follow up with no antibiotic intervention for those individuals showing higher serum PCT values. Nevertheless future studies with PCT levels in larger population with different stages of periodontal disease in systemically compromised individuals and comparing the results to blood culture or other methods of identifying disseminated bacteria, needs to be focused on.

Conclusions

The present investigation demonstrates the markedly elevated levels of serum and salivary PCT, CRP and IL-1β and their significant reduction post periodontal therapy in periodontitis stage II and III Grade A patients compared to periodontal healthy controls suggest a close association between all three biomarkers to the periodontal status. However as the inflammation subsided two weeks after therapy, the CRP and IL-1β levels were comparable to periodontally healthy individuals failing to reflect the continuous disease activity and questioning the predictability of conventional diagnostic biomarkers. The serum PCT level was sensitive enough to relate to the residual bacterial load after NSPT suggesting it to be a more useful biomarker for predicting periodontal disease activity. Further longitudinal studies with larger sample size and relating PCT to bacterial culture could validate serum PCT as a biomarker for bacterial infection in periodontal disease.

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