

Estimation of GCF and Salivary Levels of NT-proBNP in Systemically Healthy Subjects with Severe Chronic Periodontitis Before and After Periodontal Flap Surgery

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Purpose: The aim of the study was to estimate and compare the Saliva and GCF levels of NT-proBNP in systemically healthy subjects with severe chronic periodontitis before and after periodontal flap surgery.

Materials and Methods: Twenty subjects were selected and divided into two groups based on inclusion and exclusion criteria. Healthy Controls: 10 periodontally and systemically healthy subjects. Presurgery Group: 10 systemically healthy subjects with severe chronic generalized periodontitis. Postsurgery Group included Presurgery Group subjects who will undergo periodontal flap surgery. After the periodontal parameters were measured, GCF and saliva samples were collected. Postsurgery Group subjects underwent periodontal flap surgery and both periodontal parameters and GCF and saliva levels were reassessed after 6 months.

Results: Presurgery Group showed a higher mean value of plaque index, modified gingival index, probing pocket depth and clinical attachment level when compared to Healthy Controls and it was found to decrease after periodontal flap surgery (Postsurgery Group). Intergroup comparison (Presurgery Group vs Postsurgery Group) of the mean difference of salivary NT-proBNP was found to be statistically significant. GCF levels of NT-proBNP also decreased after periodontal flap surgery but the difference was not statistically significant.

Conclusion: NT pro-BNP levels were found to be higher in periodontitis group as compared to the controls. The levels decreased following surgical periodontal therapy, elucidating the role of periodontal treatment on the expression of NT-proBNP as a salivary and GCF marker. NT-proBNP could serve as a potential biomarker for periodontitis in saliva and GCF in future.

Keywords: biomarker, GCF, NT-proBNP, natriuretic peptides, periodontal disease, periodontal flap surgery, saliva

Introduction

Periodontitis is a chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms and characterized by progressive destruction of the tooth-supporting apparatus.¹

As the bacteria multiply, the plaque becomes more mature which increases the concentration of gram-negative bacteria like *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. These bacteria release various chemicals like hydrogen sulphide, proteases and ammonia which not only damages the periodontium but also triggers the immune system to produce proinflammatory cytokines like IL-1 β , IL-6, IL-12 IFN- γ and TNF- α .

To isolate these mediators, biological fluids like saliva, serum, GCF can be used. Saliva and GCF are commonly used fluids because they can be easily collected and are rich in locally and systemically derived markers of periodontal disease. Hence, they have a great potential for the assessment of patient-specific biomarker in the diagnosis of periodontitis and other systemic diseases.²

Many pathways have been proposed to explain the association between periodontal and cardiovascular disease. Since periodontitis causes both a local and systemic inflammatory and immune response, there is an increase in cardiovascular risk. There is an increase in white blood count, C-reactive protein, fibrinogen, cell adhesion molecules and pro-inflammatory cytokines in response to these inflammatory changes. Also, the bacteria associated with periodontal disease like *Porphyromonas gingivalis* has been isolated from atherosclerotic plaques. In addition, serum IgA antibodies to *Porphyromonas gingivalis* are increased in myocardial infection patients.^{3,4} Biomarkers of endothelial dysfunction and dyslipidemia such as CRP, t-PA, and LDL-C, which are known risk factors for cardiovascular disease has shown statistically significant association with periodontal disease, which provides a link between periodontal and cardiovascular disease.⁵

De Bold in 1979 discovered and isolated a polypeptide hormone atrial natriuretic peptide (ANP) which is secreted by heart muscle cells. Later, B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) was isolated.⁵ BNP was first isolated from porcine brain, and it was thought to be a neurotransmitter. So, it was named as brain natriuretic peptide. But later, it was shown to be 10-fold more abundant in the heart than in the brain, and hence renamed as B-type natriuretic peptide. ProBNP is processed within the human heart to form BNP with 32 amino acids (amino acids 77–108 of its 108 amino acid prohormone), and an N-terminal proBNP peptide (amino acids 1–76; NT-proBNP), both of which circulate in humans. BNP is produced by direct synthesis in response to the degree of ventricular stretch and also upregulated in failing ventricular myocardium. The messenger RNA for proBNP is unstable, so there is active regulation of BNP levels according to ventricular wall tension. Hence, it acts as a reliable biomarker of ventricular dilatation.⁶ In patients with stable coronary artery disease, both BNP and NT-proBNP are potent prognostic indicators. Their association with end points of death and heart failure has been extensively proved. Increased levels of salivary NT-proBNP have been detected in heart failure patients compared to healthy control subjects.⁷

Leira and Blanco in 2018 showed that the serum NT-proBNP increases with increasing severity of periodontal destruction.⁸ This was in accordance with our previous study where we compared the salivary and serum levels of NT-proBNP in otherwise systemically healthy patients with mild, moderate and severe chronic periodontitis.⁹ The mean value of NT-proBNP increased with the increasing severity of the periodontal disease. Karl Pearson correlation analysis between salivary and serum NT-proBNP and other periodontal parameters such as plaque index, modified gingival index, probing pocket depth, and clinical attachment level showed a very strong positive correlation ($P < 0.001$). In this study, we evaluate the levels of NT-proBNP in GCF and comparing the levels of NT-proBNP before and after periodontal flap surgery.

Hence, this study was done to assess and compare the NT-proBNP levels in saliva and GCF in systemically healthy subjects with severe chronic periodontitis before and after periodontal flap surgery.

Materials and Methods

Study Design

From the out-patient Department of Periodontology, Meenakshi Ammal Dental College and Hospital, the study subjects were enrolled. A total of 20 systemically healthy subjects were selected, out of which 10 were periodontally healthy. The remaining 10 had severe chronic generalized periodontitis. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Meenakshi Ammal Dental College and Hospital (protocol code MADC/IRB-XXVII/ 2019/424 approved on 20/3/19) for studies involving humans. The power of the study was calculated to be 95% when 20 subjects were included. After explaining the subjects about the study, informed consent was signed by those who were willing to take part in this study.

The criteria for inclusion included for the study were those who were systemically healthy and willing to participate in the study, subjects in the age range of 35–55 years and having at least 10 teeth. Subjects were classified based on the

AAP International Workshop for classification of Periodontal Diseases 1999. Healthy control group included subjects with clinically healthy periodontium, PD \leq 3 mm, with no attachment loss and bone loss on radiographs (Group I). Severe chronic generalized periodontitis pretreatment group included subjects with probing pocket depth \geq 5mm in more than 30% of the sites and clinical attachment loss (AL) \geq 5 mm, and bone loss evident on radiograph (Presurgery Group). Postsurgery Group included Presurgery Group subjects who underwent periodontal flap surgery. Subjects were excluded if they had smoking habit, were pregnant or those who had taken any medication which can affect the periodontal status during the past 1 month and those who were treated for periodontal disease during the past 6 months.

To measure the parameters, University of North Carolina –15 (UNC-15) probe was used and the values were rounded off to the nearest millimetre. The following periodontal parameters were estimated: Plaque Index (Silness and Loe, 1964),¹⁰ Modified Gingival Index,¹¹ Probing Pocket Depth measured around six sites per tooth (PPD) and Clinical Attachment Level (CAL).

Sample Collection

After assessing the periodontal parameters, saliva and GCF samples were obtained from all the subjects. GCF was collected on a day other than the day of clinical examination to avoid contaminating the GCF with blood caused by contact with areas of inflammation. Two sites with deepest pocket were chosen for collection of GCF. The site was carefully dried and isolated with cotton roll before the collection of the fluid using microcapillary pipette. The pipette was placed gently at the entrance of the gingival sulcus. Each sample was collected for a maximum of 5–20 minutes. Into a plastic vial, the collected GCF was transferred and stored at -80°C till the time of assay.

Five milliliters of unstimulated whole saliva was collected in the morning, 2 hours after the last meal to standardize the collection of saliva according to the circadian rhythm. Before sample collection, distilled water was used to rinse the mouth. They were instructed to let the saliva pool on the floor of the mouth and then expectorate into the collecting vessel till the desired quantity of saliva was collected. The sample was shifted to Eppendorf tubes for centrifugation at 1000 rpm for 1 minute and kept at -80°C until the time of assay.

Pre-Surgical Procedure

After collection of saliva and GCF and recording of clinical parameters, Phase I therapy (full mouth scaling, root planing and oral hygiene instructions) was performed. To assess the response to Phase I therapy and also to evaluate their oral hygiene practice, Presurgery Group subjects were recalled. Periodontal flap surgery was planned after 2 weeks and from here they were placed in Postsurgery Group.

Surgical Procedure

Two percent lignocaine hydrochloride with adrenaline (1:200,000) was used to anesthetize the surgical site. On the facial and lingual/palatal surface of each tooth segment or area involved, crevicular incisions were made using Bard Parker No.15 blade. Periosteal elevator was used to raise a full thickness flap taking care to preserve the maximum amount of gingival connective tissue in the flap. Granulation tissue was removed, and roots were thoroughly planed. To minimize bleeding, the granulation tissue was also removed from the under surface of the flap. The site was thoroughly irrigated with saline. Then, suturing of the flap was done and haemostasis was achieved. The same procedure was repeated in all the other quadrants.

Post-Operative Instructions

During the first post-operative day, the patients were instructed to avoid chewing on the surgical area. Antibiotics and mouth rinses were not prescribed, but they were instructed to follow their routine mechanical plaque control measures.

After 7 days, suture removal was done. During the first month, recall appointments were planned once in 10 days and oral hygiene was monitored. At the sixth month, clinical parameters were reassessed and Saliva and GCF collection was collected. The pre -and post-operative values were then statistically analysed.

NT-proBNP Analysis

Saliva and GCF samples were analysed for NT-proBNP using the Human NT-proBNP enzyme linked immunosorbent assay (ELISA) kit (Abbkine). Manufacturer's instructions were followed during the analyses. After preparing the standard solutions, the samples were rested to bring it to room temperature before starting the assay procedure. Into the testing sample well, 40 μL of the sample diluent was added followed by addition of 10 μL of sample. After covering the well with a plate cover, it was incubated for 45 minutes at 37°C. Using 250 μL of wash buffer solution, each well was washed four times for a duration of 1–3 minutes per time. Following this, 50 μL of HRP-Conjugated detection antibody was added into the well. It was again covered with a plate cover and incubated for 30 minutes at 37°C. The wash process was repeated. After this, to each well 50 μL of chromogen solution A and chromogen solution B was added, gently mixed and incubated for 15 minutes at 37°C. The color in the wells changed from blue to yellow, after which 50 μL of stop solution was added. ELISA plate reader (LABSERV) at 450 nm was used to measure the optical density.

Statistical Analysis

SPSS (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY: IBM Corp. Released 2019) was used to analyse data. The results of Normality tests and Kolmogorov–Smirnov and Shapiro–Wilks tests showed that all variables followed normal distribution. Parametric methods were used to analyse the data. Independent *t*-test was used to compare the intragroup mean values. Paired *t*-test was done to compare the mean values pre and post treatment. Significance level was fixed as 5% ($\alpha = 0.05$).

Results

The mean value of plaque index, modified gingival index, probing depth and clinical attachment level in Healthy Control Group were 0.308 \pm 0.10, 0.101 \pm 0.06, 2.047 \pm 0.11 and 2.047 \pm 0.11 mm, respectively. The mean value of plaque index, modified gingival index, probing depth and clinical attachment level in Presurgery Group were 2.860 \pm 0.26, 3.573 \pm 0.20, 7.01 \pm 0.58 and 8.092 \pm 0.71 mm, respectively. Comparison of the mean values of the periodontal parameters was found to be statistically significant (Table 1).

NT-proBNP level in saliva and GCF was also evaluated. Salivary NT-proBNP level was 4.59 \pm 0.81 pg/mL in Healthy Group and 18.45 \pm 6.19 pg/mL in Presurgery Group. Comparison of the mean values of the NT-proBNP in saliva

Table 1 Mean and Standard Deviation of Periodontal Parameters and Salivary and GCF Levels of NT-ProBNP in Healthy Control Group and Presurgery Group

Parameter	Group	N	Mean	Std Dev	p-value
Plaque Index	Healthy Control	10	0.308	0.10983	<0.001
	Presurgery	10	2.86	0.26432	
Modified Gingival Index	Healthy Control	10	0.101	0.06967	<0.001
	Presurgery	10	3.573	0.20413	
Probing Depth	Healthy Control	10	2.047	0.11216	<0.001
	Presurgery	10	7.01	0.58395	
Clinical Attachment Level	Healthy Control	10	2.047	0.11216	<0.001
	Presurgery	10	8.092	0.71069	
Salivary NT-proBNP levels (pg/mL)	Healthy Control	10	4.59	0.81028	<0.001
	Presurgery	10	18.45	6.19682	
GCF NT-proBNP levels (pg/mL)	Healthy Control	10	16.75	6.71338	0.066
	Presurgery	10	22.25	5.81784	

Table 2 Mean and Standard Deviation of Periodontal Parameters and Salivary and GCF Levels of NT-ProBNP in Presurgery Group and Postsurgery Group

		N	Mean	Std Dev	p-value
Plaque Index	Presurgery	10	2.86	0.26432	<0.001
	Postsurgery	10	0.85	0.19579	
Modified Gingival Index	Presurgery	10	3.573	0.20413	<0.001
	Postsurgery	10	1.51	0.29609	
Probing Depth	Presurgery	10	7.01	0.58395	<0.001
	Postsurgery	10	4.981	0.44428	
Clinical Attachment Level	Presurgery	10	8.092	0.71069	<0.001
	Postsurgery	10	5.43	0.29458	
Salivary NT-proBNP levels	Presurgery	10	18.4500	6.19682	<0.001
	Postsurgery	10	9.7700	3.91494	
GCF NT-proBNP levels (pg/mL)	Presurgery	10	22.2500	5.81784	0.008
	Postsurgery	10	18.8500	4.05552	

was found to be statistically significant. GCF levels of NT-proBNP was 16.75 ± 6.71 pg/mL in Healthy Group and 22.25 ± 5.81 pg/mL in Presurgery Group. Comparison of the mean values of NT-proBNP in GCF was not statistically significant (Table 1).

Postsurgery Group included subjects from Presurgery Group who underwent periodontal flap surgery. All the periodontal parameters and salivary and GCF levels of NT-proBNP were assessed 6 months after periodontal flap surgery.

The mean value of plaque index, modified gingival index, probing depth and clinical attachment level in Presurgery Group were 2.86 ± 0.26 , 3.5730 ± 0.20 , 7.01 ± 0.58 and 8.092 ± 0.71 mm respectively. The mean value of plaque index, modified gingival index, probing depth and clinical attachment level in Postsurgery Group were 0.85 ± 0.19 , 1.51 ± 0.2 , 4.981 ± 0.4 and 5.43 ± 0.29 mm respectively. The reduction in the values of periodontal parameters at the sixth month was found to be statistically significant (Table 2).

NT-proBNP levels in saliva and GCF was also evaluated at the sixth month. Salivary NT-proBNP levels in Postsurgery Group was 9.77 ± 3.91 pg/mL. Comparison of the mean values of the NT-proBNP in saliva between Presurgery Group and Postsurgery Group was found to be statistically significant. GCF levels of NT-proBNP in Postsurgery Group was 18.85 ± 4.05 pg/mL. Comparison of the mean values of NT-proBNP in GCF between Presurgery Group and Postsurgery Group was not statistically significant (Table 2).

All the periodontal parameters significantly reduced following periodontal flap surgery. Salivary and GCF NT-proBNP levels also reduced following periodontal flap surgery. This shows that NT-proBNP levels can be potentially considered as a biomarker to identify periodontitis patients who are at a risk of developing cardiovascular diseases.

Discussion

The inflammatory condition of the periodontium is limited not only to the destruction of periodontal tissues but it can lead to intravascular coagulation via mediators and pro-inflammatory cytokines, and can increase the risk of atherosclerosis resulting in its consequences such as acute myocardial infarction (MI) or stroke. Periodontal pathogens may directly act by damaging the vascular endothelium or indirectly by activating hypercytokinemia (inflammatory-cytokine storm). The bacterial lipopolysaccharides play an important role in this process which mainly originates from periodontal bacteria. LPS is known to strongly activate host immune response. Monocytes produce significantly more inflammatory

mediators such as tumor necrosis factor- α (TNF- α), prostaglandin E2 (PGE-2), interleukin-1 β (IL-1 β) and proteolytic enzymes – matrix metalloproteinases (MMP). In due course of chronic periodontitis, an increased level of TNF- α and IL-6 are found in the gingival crevicular fluid. These cytokines besides having destructive effects on the periodontium, also have a vital role in pathogenesis of arteriosclerosis and cardiovascular diseases. TNF- α causes an increase in fibrinogen level in plasma and blood vessel permeability which stimulates the production of IL-1, IL-6, IL-8 by endothelial cells leading to increase in osteoclasts' activity. Furthermore, TNF- α and IL-1 enhance the aggregation of platelets and their adhesion to the vessel walls and also facilitate the formation of lipid and cholesterol deposits.¹²

In the course of chronic periodontitis, an increased concentration of fibrinogen and von Willebrand factor (vWD) in serum occurs in response to inflammatory factors (including IL-6 and IL-1). In systemic blood circulation, this phenomenon is regarded as a factor which can lead to coronary disease. The increased concentration of fibrinogen is associated with an increased aggregation of erythrocytes, and with an intensified viscosity and resistance in micro-circulation. It has been proven that in coronary diseases, fibrinogen is a stronger risk factor than cholesterol. Fibrinogen and CRP are biomarkers of systemic inflammatory response and hence they can be used to assess risk of acute atherosclerotic complications, for instance acute coronary syndrome.

The regulation of cardiovascular homeostasis and extracellular fluid volume is an important function of the natriuretic peptides. ANP, BNP, and CNP are the three structurally homologous structures.¹³ The main function of these peptides is to support cardiovascular homeostasis by influencing the central and peripheral hemodynamics. The intravascular volume is influenced by vasodilatory, diuretic and natriuretic effects of the three natriuretic peptides. The levels of NT-proBNP (76 AA) or BNP (32 AA) in serum can be used to diagnose heart failure (HF).¹⁴ The relationship between inflammation and high NT-proBNP levels in patients with sepsis, endocarditis or rheumatoid arthritis can be attributed to the hydrodynamic stress experienced by these patients. Therefore, inflammation through its effect on hemodynamic load results in a correlation between the levels of CRP and BNP or NT-proBNP.¹⁵

A correlation between periodontitis and concentration of brain natriuretic peptide type B (BNP) has also been suggested. BNP is thought to inhibit cardiomyocytes and fibroblasts augmentation and hinders collagen synthesis not only in connection with cardiac muscle but also with periodontal tissues. Moreover, it inhibits synthesis and activity of MMP's, limits neutrophil granulocytes activity and decreases the activity of platelets. BNP level can be increased by factors such as smoking, stress, diabetes and age, which are also risk factors in periodontal diseases.¹⁶

To invade the immune cells, the periodontal pathogens first invade the vascular endothelial cells. *P. gingivalis* invades the endothelial cells using hemagglutinin A, hemagglutinin B and fimbriae A. Expression of adhesion molecules and inflammatory cytokines like IL-6, IL-8 and cyclo-oxygenase-2 are upregulated by Fimbriae A. Together with LPS, it also upregulates the proinflammatory stimulation of the arterial endothelium by *P. gingivalis*. There is an upregulation in the formation of these adhesion molecules by *Aggregatibacter actinomycetemcomitans*, but to a lesser amount. This also suggests that there is a bidirectional relationship between CVD and periodontal disease.⁸

In a previous cross-sectional study, we have evaluated the levels of NT-proBNP in serum and saliva in otherwise systemically healthy subjects with varying degree of periodontal disease severity. The levels of NT-proBNP increased in both serum and saliva with increasing severity of disease progression.⁹

In this study, we have also estimated the levels of NT-proBNP in GCF. Collection of GCF is a non-invasive and widely used method to explore potential biomarkers of periodontal disease. It can also be used to detect diagnostic markers that indicate the presence of a disease process before extensive clinical damage has occurred. A recent systematic review examined the diagnostic relevance of periodontitis-specific biomarkers in the GCF and concluded that their quantification in the GCF can provide an outlook of the changes associated with periodontitis and have diagnostic value.¹⁷

GCF levels of NT-proBNP at baseline in Presurgery Group subjects was higher compared to Healthy Control Group, but this difference was not statistically significant. Fazal et al also reported increased GCF levels of NT-proBNP in generalized chronic periodontitis subjects.¹⁴ Lipopolysaccharides from periodontal pathogens like, *Porphyromonas gingivalis* spreads into the bloodstream through the periodontal epithelium by binding to the endothelial cells, monocytes and macrophages. Due to this, there is an increase in the production of pro-inflammatory cytokines. So, it could be speculated that periodontal inflammation may contribute to increased levels of NT-proBNP in serum and GCF as a result of systemic inflammation.

A systematic review analysed the potential value of saliva biomarkers in the diagnosis, management and prognosis of heart failure (HF).¹⁸ They concluded that salivary BNP levels can be used for heart failure monitoring. This highlights the use of saliva to estimate the levels of biomarkers and hence salivary levels of NTpro-BNP was also evaluated. Salivary levels of NT-proBNP at baseline was higher in Presurgery Group compared to Healthy Control Group, and difference was also statistically significant. This is in accordance with the study done by Liera Y et al and Vijayaraj et al who reported increased salivary NT-proBNP levels in severe chronic periodontitis patients compared to healthy controls.^{8,9} The diagnostic potential of saliva lies in the fact that it contains 20% similar proteins as blood. NT-proBNP has a slow clearance rate from blood, about 60–90 minutes. This slow clearance rate allows it to flow into the saliva via GCF.

After collecting the baseline samples, subjects in Presurgery Group underwent periodontal flap surgery. All the periodontal parameters and GCF and salivary levels of NT-pro BNP were evaluated after 6 months. All the periodontal parameters were reduced in Postsurgery Group compared to Presurgery Group.

This study is the first of its kind to assess GCF and salivary levels of NT-pro BNP Pre and post periodontal flap surgery. The intergroup comparison of mean difference of salivary and GCF levels of NT-ProBNP between Presurgery Group and Postsurgery Group were found to decrease after periodontal flap surgery. Surgical debridement leads to complete removal of local factors and healing of the epithelial attachment. The reduction in inflammation leads to decreased NT-proBNP levels in GCF and saliva. This is in accordance with the study done by Fazal et al where there was statistically significant decrease in the NT-pro BNP levels before and after SRP. They concluded that SRP leads to resolution of inflammation leading to cessation of periodontal destruction thereby creating a direct impact on this biomarker, reducing its levels in serum.¹⁴

This study is the first of its kind to assess the salivary and GCF levels of NT-proBNP in periodontitis patients before and after periodontal flap surgery. Further studies with larger sample size are required to confirm these results. Periodontal disease may significantly enhance the risk for coronary heart disease and CHD –related diseases like angina, infarction, etc. So, detection of this CVD biomarker in saliva and GCF can help educate the patients on their risk for future CVD diseases.

Conclusion

The present study was conducted with the main objective of estimation of GCF and salivary levels of NT-proBNP in otherwise systemically healthy subjects with severe chronic periodontitis before and after periodontal flap surgery. The GCF and salivary levels of NT-proBNP increased with the severity of periodontal disease and decreased after flap surgery. Our findings suggest that NT pro-BNP levels in saliva and GCF could serve as potential biomarkers for periodontitis. This study also provides an understanding and awareness about the potential role of NT-proBNP in periodontal disease progression.

Informed Consent Statement

Written informed consent has been obtained from the patients to publish this paper.

Funding

This research received no external funding.

Disclosure

The authors report no conflicts of interest in this work.

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