Evaluation of association between Porphyromonas gingivalis and visfatin levels in chronic periodontitis patients

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INTRODUCTION

Periodontal disease is one of the noncommunicable prevailing diseases, where the soft- and hard-tissue destruction occurs due to the direct effect of microorganisms and indirectly by the stimulation of host cells by these microorganisms. The inflammatory lesion is confined to gingiva in gingivitis, wherein periodontitis the inflammatory process produces additional results in alveolar bone and periodontal ligament.

Visfatin is one of the adipokines; it is encoded by NMPT gene. Visfatin derived its name since they are derived from the visceral adipose tissue. It is secreted not only by adipose tissue but also by the inflammatory cells infiltrated in the adipose tissue. Visfatin itself is a proinflammatory cytokine, and it also secretes the release of more proinflammatory cytokines. Proinflammatory cytokines released during the periodontal inflammation also induces the secretion of visfatin. Visfatin also contributes to periodontal inflammation by the production of collagenase-1 and monocyte chemoattractant protein-1. Moreover, visfatin also inhibits the neutrophils apoptosis, thereby prolonging the duration of neutrophils, leading to tissue destruction.

Hence, assessing this novel biomarker may help to diagnose periodontal destruction. Porphyromonas gingivalis is a primary periopathogen in the periodontal disease progression. It is also considered as a key pathogen, since it has an ability to alter the normal microflora to the dysbiotic community. This organism has high proteolytic activity, and this property provide nutrients for their growth. It is one of the red complex microorganism primarily involved in patients with periodontal disease capable of releasing virulence factors and proteases resulting in periodontal tissue destruction.

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Porphyromonas gingivalis also induces visfatin secretion, and it is suggested that the immunologic and microbial signals utilize the detrimental effect of visfatin for periodontal tissue destruction. Gingival crevicular fluid (GCF) eventually comes out from the connective tissue and reaches the gingival sulcus. GCF is in intimate contact with periodontal tissue; hence, GCF levels depict this tissue status in a better way compared to saliva. Hence, assessing GCF visfatin serves as a potential inflammatory marker for periodontal disease. The diverse inflammatory action of visfatin made this adipokine a potential periodontal biomarker of choice along with the periopathogen Porphyromonas gingivalis. Limited studies are available in the literature to know the relationship between visfatin and Porphyromonas gingivalis levels in the South Indian population. Hence, this study was carried out to evaluate the visfatin levels in GCF and Porphyromonas gingivalis levels in subgingival plaque in periodontal health and disease.

MATERIALS AND METHODS

The study population comprised sixty participants with the between the age of 30–55 years involving males and females. Ethical board of our university approved the study. All the patients were chosen from the periodontics outpatient clinic, in a dental college and hospital, Chennai. Recruited participants were divided into two groups of thirty participants each as Group I – patients who have healthy periodontium and Group II – patients with generalized chronic periodontitis. The clinical parameters assessed for the participants were plaque index (PI), papillary bleeding index (PBI), probing pocket depth (PPD), and clinical attachment loss (CAL).

The eligibility criteria for Group I participants were good oral hygiene, without any visual signs of inflammation and CAL. Group II participants had CAL ≥3 mm and PPD of ≥5 mm in ≥30% of the sites. The exclusion criteria consisted of patients with a minimum of ≤15 teeth, aggressive periodontitis, smoking and tobacco chewing habits, overweight/obese participants, diabetic patients, pregnant or lactating women, and patients who received antibiotics or underwent periodontal treatment in the previous 3 months. Patients recruited were conveyed about the protocol and got the written consent, and they were scheduled for a sampling of GCF, subgingival plaque, and clinical parameters assessment at baseline.

Gingival crevicular fluid collection
GCF was collected in sites with the greatest CAL and before collection supragingival plaque was removed. Calibrated micropipettes were used, and extracrevicular method was used to collect GCF. Eppendorf tubes were used to transfer GCF and stored till the time of assay at −70°C.

Subgingival plaque collection
The subgingival plaque was collected after the removal of supragingival plaque by scaler. Graceycurette was inserted into the pocket without giving pressure on the tooth surface, to avoid the dislocation of subgingival plaque into the depth of the pocket. One millilitre of Rapid Perferinges medium was added into the container as the transportation media. The samples were transported to the laboratory for microbial assessment.

Estimation of gingival crevicular fluid visfatin
GCF visfatin was assessed by the enzyme-linked immunosorbent assay [Figure 1]. The human visfatin antibody coated in the microtiter plate wells was treated with sample containing visfatin. Biotinylated human visfatin antibody was appended, and it binds to sample containing visfatin. Then, Streptavidin-Horse Radish Peroxidase addition binds the biotinylated visfatin antibody. The addition of substrate results in blue color, and the severity of the color is related to the visfatin quantity [Figure 2]. Acidic stop solution terminates the reaction and measured at an absorbance of 450 nm [Figure 3]. A standard curve for each standard was constructed by marking the mean concentration on the X-axis and optical density on the Y-axis [Figure 4].

Detection of Porphyromonas gingivalis by the conventional polymerase chain reaction
The red complex organism Porphyromonas gingivalis was identified and assessed using the polymerase chain reaction (PCR) in both Group I and Group II. Genomic DNA was extracted using a QIAamp DNA mini kit (QIAGEN Inc., USA). All the steps were carried out at the room temperature. To the samples in a centrifuge tube, 180 µl buffer ATL and proteinase K were vortexed and later incubated at 56°C (1–3 h). Buffer AL with the quantity of 200 µL was incubated at 70°C. Then, ethanol with 200 µL quantity was vortexed for 15 s. It was poured onto the QIAamp Mini spin column and later centrifugated at 8000 rpm for 1 min. QIAamp Mini spin column was kept in 2 ml tube and 500 µL AW1 was centrifugated at 8000 rpm. 500 µL AW2 buffer in QIAamp Mini column was centrifugated at 14,000 rpm. It was again centrifugated for 1 min to remove AW2 buffer carry over. Finally, the addition of 200 µL AE buffer DNA was eluted.

Conventional polymerase chain reaction procedure
Conventional PCR was done using Taq DNA Polymerase 2x Master Mix RED which is a 2x reaction mix with the Ampliqon Taq DNA polymerase, NH4+ buffer system and magnesium chloride present. Twenty five µl of the 2x Master Mix RED was used for each reaction. The primer sequence used was Forward: 5’-AGG CAG CTT GCC ATA CTG CG-3’ Reverse: 5’-ACT GTT AGC AAC TAC CGA TGT-3’. The agarose mix was prepared in 1x–TAE buffer in a microwave oven to get a clear homogeneous solution. The solution was cooled, and ethidium bromide was added. Then, the solution was poured into the gel mold with the combs placed. After allowing it to settle for 30 min, the comb was removed carefully, and tapes were peeled off from the gel casting tray. The gel was kept completely submerged in the buffer. Each of the PCR products with a quantity of 10 µl was taken and mixed with gel loading dye and then loaded to ethidium bromide incorporated in standard 2% agarose gel (0.5 µg/ml). A DNA marker (100 base pair ladder) was at the same time loaded in a separate lane. The gel was run at 80V for 30 min or until dye reaches two-third of the gel. PCR products in the gel were viewed on an ultraviolet transilluminator (BIORAD) [Figures 5 and 6].

RESULTS
The data were analyzed statistically to find the mean, standard deviation, and test of significance for the various parameters between the groups, using the SPSS software (21 version) IBM corporation, Armonk, New York, USA. To assess the mean
difference in the PI scores, PBI scores, PPD, CAL, and visfatin levels in GCF between healthy and chronic periodontitis groups, the Mann Whitney U-test was utilized. The frequency of *P. gingivalis* presence between study and control groups, Chi-square test was applied. The correlation between clinical parameters and GCF visfatin was tested using the Spearman’s rank correlation test. The clinical parameters and GCF visfatin levels between the groups showed a notable significant difference [Table 1]. *P. gingivalis* levels between the two groups demonstrated a notable difference [Table 2]. The comparison of clinical parameters and GCF visfatin with *P. gingivalis* levels in Group I and II showed a significant difference [Table 3]. A significant positive correlation was inferred on the correlation of GCF visfatin levels with clinical parameters [Table 4].

**DISCUSSION**

Various cytokines in GCF have been used as a potential biomarker for periodontal destruction. Lately, it has been observed that adipokines are released following host inflammatory response in periodontal disease. Visfatin also termed as pre-B-cell colony-enhancing factor, is implicated in B-lymphocytes formation, and it also has a role in the cell cycle regulation. Visfatin is secreted by monocytes, lymphocytes, dendritic cells, and macrophages, and also suggested that it has a role in regulating immune function.\(^1\)
The excessive release of adipokine visfatin is said to be correlated with age and body mass index, accordingly in this study, the probable outcomes of the factors considered are avoided by including participants with similar BMI and age. The mean GCF visfatin level in Group II was higher than Group I with a statistically notable difference. The present study results are comparable to the previous studies, who had observed increased GCF visfatin levels in chronic periodontitis patients compared to periodontally healthy participants. Raghavendra et al. also showed that elevated visfatin levels in periodontitis patients attained levels seen in healthy individuals after appropriate periodontal treatment. Another study also reported that salivary visfatin level was observed to be an inflammatory marker in periodontal disease and salivary visfatin levels were reduced after periodontal treatment. In addition, the proportionate rise in the levels of GCF from healthy gingiva to periodontitis groups proved that visfatin was highly expressed predominantly by the cells of the periodontal disease. This increase might be caused due to the synthesis of visfatin from cells of the periodontium as a response to inflammation.

On comparison of \( P. \) gingivalis levels between Group I and Group II showed that 27 participants (90%) in Group I and two participants (6.6%) in Group II tested negative for \( P. \) gingivalis. Twenty-eight participants (93.4%) in Group II and three (10%) participants in Group I tested positive for \( P. \) gingivalis, and the difference between the two groups was found. This inference is comparable to the observations in previous study by Özcan et al., who described that the \( P. \) gingivalis levels in the subgingival plaque were higher in chronic periodontitis participants compared to periodontally healthy participants. The outcomes of our study are in accordance to Krishnan et al., who described the increased odds of detecting \( P. \) gingivalis in participants with periodontitis as compared to the healthy participants.

\( P. \) gingivalis produces modulatory effect on host immunoinflammatory response, by acting on immune as well as nonimmune cells present in the periodontal tissues. \( P. \) gingivalis may also influence the proinflammatory cytokines secreted by the immune cells such as tumor necrosis factor-\( \alpha \) and interleukin-1 \( \beta \). The comparison of clinical parameters and \( P. \) gingivalis levels in both group participants showed that the periodontal parameters and GCF visfatin levels were more for study participants with positive \( P. \) gingivalis when compared to those who had negative \( P. \) gingivalis, and the differences were statistically significant. This inference agree to the study done by Özcan et al. who described that the proinflammatory cytokines mainly interleukin-1 \( \beta \) released following \( P. \) gingivalis colonization, increase visfatin synthesis. Hence, the proinflammatory cytokines emanated by the immune cells against the periodontopathogens may have

### Table 1: Comparison of clinical parameters and gingival crevicular fluid visfatin levels between Group I and II

| Parameters | Group | Mean±SD | Mean rank | \( P \) |
|------------|-------|---------|-----------|--------|
| PI         | I     | 0.51±0.24 | 15.50     | 0.001* |
|            | II    | 2.57±0.39 | 45.50     |        |
| PBI        | I     | 0.56±0.28 | 15.50     | 0.001* |
|            | II    | 3.07±0.39 | 45.50     |        |
| PPD        | I     | 2.04±0.57 | 15.50     | 0.001* |
|            | II    | 7.18±0.72 | 45.50     |        |
| CAL        | I     | 2.04±0.57 | 15.50     | 0.001* |
|            | II    | 6.18±0.72 | 45.50     |        |
| GCF Visfatin| I    | 0.43±0.11 | 15.50     | 0.001* |
|            | II    | 0.95±0.23 | 45.50     |        |

*Level of significance: \( P<0.05 \) significant. PI = Plaque index; PBI = Papillary bleeding index; PPD = Probing pocket depth; CAL = Clinical attachment loss; SD = Standard deviation; \( \text{P} \) value = Probability value; GCF = Gingival crevicular fluid

### Table 2: Comparison of \( P. \) gingivalis visfatin levels between Group I and II

| Porphyromonas gingivalis | Groups | Total | \( \chi^2 \) | \( P \) |
|--------------------------|-------|-------|-------------|--------|
|                          | I     | II    |             |        |
| Negative                 | 27    | 2     | 29          | 41.713 | 0.001* |
| Positive                 | 3     | 28    | 31          |        |
| Total                    | 30    | 30    | 60          |        |

*Level of significance: \( P<0.05 \) significant. \( \text{P} \) value = Probability value

### Table 3: Comparison of clinical parameters and gingival crevicular fluid visfatin with \( P. \) gingivalis levels in Group I and II

| Variable | Porphyromonas gingivalis | Mean±SD | Mean rank | \( P \) |
|----------|--------------------------|---------|-----------|--------|
| PI       | Negative                 | 0.66±0.55 | 18.03    | 0.001* |
|          | Positive                 | 2.36±0.77 | 42.16    |        |
| PBI      | Negative                 | 0.74±0.80 | 17.78    | 0.001* |
|          | Positive                 | 2.81±0.79 | 42.40    |        |
| PPD      | Negative                 | 2.29±1.30 | 16.31    | 0.001* |
|          | Positive                 | 6.78±1.55 | 43.77    |        |
| CAL      | Negative                 | 2.22±1.07 | 16.31    | 0.001* |
|          | Positive                 | 5.87±1.29 | 43.77    |        |
| Visfatin | Negative                 | 0.46±0.12 | 17.55    | 0.001* |
|          | Positive                 | 0.90±0.30 | 42.61    |        |

*Level of significance: \( P<0.05 \) significant. SD = Standard deviation; \( \text{P} \) value = Probability value; PI = Plaque index; PBI = Papillary bleeding index; PPD = Probing pocket depth; CAL = Clinical attachment loss

### Table 4: Correlation of clinical parameters with gingival crevicular fluid visfatin levels between Group I and II

| Variables | PI | PBI | PPD | CAL |
|-----------|----|-----|-----|-----|
| Visfatin  |    |     |     |     |
| Correlation coefficient | 0.732 | 0.737 | 0.794 | 0.794 |
| Significant (two tailed) | 0.001* | 0.001* | 0.001* | 0.001* |
| \( n \)   | 60 | 60  | 60  | 60  |

*Level of significance: \( P<0.05 \) significant. PI = Plaque index; PBI = Papillary Bleeding Index; PPD = Probing pocket depth; CAL = Clinical attachment loss; \( \text{P} \) value = Probability value

On comparing the periodontal parameters such as PBI and PI, the mean scores were lower in Group I than in Group II, where a statistically significant difference was seen. This observation was also seen in the study done by Özcan et al., who showed that the mean PI, PBI scores were higher in chronic periodontitis patients than in healthy patients. The plaque being the disease causing factor which, in turn, cause a transition from healthy gingiva to inflamed and then periodontitis. Gingipain, an important virulence factor of \( P. \) gingivalis, affects vascular permeability of periodontal sites, by activating the plasma kallikrein and bradykinin. It also degrades the fibrinogen and increases the bleeding at the site, thereby increasing the store of hemin needed for \( P. \) gingivalis. In this study, the mean scores of PPD and CAL were more profound in Group II than in Group I with notable difference.
elevated the visfatin release indirectly, and these periodontal pathogens may also likely to upregulate the visfatin secretion by its direct effect. Deschner et al. also have suggested that the visfatin levels can be influenced by the host-associated factors and also the bacteria.[20] Previous studies have also showed an increased visfatin secretion in the periodontal ligament cells and gingival fibroblasts, due to *P. gingivalis*.[21,22] Therefore, it is accepted that *P. gingivalis* occupied in the deep subgingival pocket could elevate the GCF visfatin secretion in direct as well as indirect ways.

On comparison of GCF visfatin levels with *P. gingivalis* in Group I and Group II, the mean visfatin levels were greater for study participants with positive *P. gingivalis* when compared to those who had negative *P. gingivalis*, and these changes were statistically significant. *P. gingivalis*, a genuine pathogen and prominent inflammation cytokine interleukin-1 beta activate the visfatin present in periodontal ligament, which in turn stimulates collagenase-1 and chemoattractant protein, will bring about increased destructive activity in periodontal ligament.[21]

On the correlation of GCF visfatin levels with PI, PPD, PBI, and CAL, the results showed a strong direct correlation, which was statistically significant. Visfatin causes the modulation of nuclear factor kappa B signaling pathways, thereby impeding apoptosis of neutrophils and also enhances the release of adhesion and inflammatory proteins.[23] The present study results were related to early studies done by Tabari et al. and Özcan et al., who reported that visfatin levels were correlated directly with the severity of periodontal destruction.[14,19] Our present work shows that the visfatin concentration in GCF and *P. gingivalis* levels were increased in periodontitis.

**CONCLUSION**

It has been proven from our study results that *Porphyromonas gingivalis* plays a significant role in modulating the visfatin levels in periodontal disease participants and GCF visfatin can also be evaluated as a biomarker to understand periodontal destruction. Periodontal destruction may be a risk indicator for many prevailing noncommunicable systemic conditions and hence controlling periodontal destruction may help to reduce the load of inflammatory in these vulnerable participants.

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**Conflicts of interest**

There are no conflicts of interest.

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