Interleukin-34 Levels in Gingival Crevicular Fluid and Plasma in Healthy and Diseased Periodontal Tissue in Presence or Absence of Obesity: A Clinico-biochemical Study

C N Guruprasad and A R Pradeep

Department of Periodontology, Government Dental College and Research Institute, Bengaluru, Karnataka, India

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Abstract

Interleukin (IL)-34 has recently been identified as an alternative ligand for colony-stimulating factor-1 receptor and plays an important role in osteoclastogenesis. The aim of this study was to assess and compare IL-34 levels in gingival crevicular fluid (GCF) and plasma in obese individuals in the presence or absence of periodontal disease and to determine whether they showed a correlation with disease severity. Forty patients aged between 25 and 40 yr were enrolled and categorized into 4 groups: 10 non-obese patients with healthy periodontium (Group I); 10 obese patients with healthy periodontium (Group II); 10 non-obese patients with chronic periodontitis (Group III); and 10 obese patients with chronic periodontitis (Group IV). Demographic variables such as age and body mass index score were recorded and assessed, together with clinical periodontal parameters such as the gingival index, probing pocket depth, and clinical attachment level scores in all groups. The GCF and plasma levels of IL-34 were quantified using enzyme-linked immunosorbent assay. The results showed that the mean IL-34 concentrations in GCF or plasma were highest in Group IV, followed by Group III, Group II, and Group I, with the difference among them being statistically significant (p<0.05). These results suggest that obese individuals with periodontitis have higher GCF and plasma IL-34 levels than non-obese individuals with healthy periodontium. This suggests IL-34 as a potential inflammatory marker of periodontal disease and obesity.

Key words: Obesity — Chronic periodontitis — Gingival crevicular fluid — Plasma — Body mass index

Introduction

Obesity is a systemic disease that has been identified as a risk factor for the development of type 2 diabetes, dyslipidaemia, hypertension, cardiovascular disease, respiratory problems, certain forms of cancer, and, most recently, periodontitis. Adipose tissue acts as an endocrine organ. As such, it secretes a number of bioactive molecules, including...
adipocytokines and adipokines, such as visfatin, resistin, adiponectin, leptin, chemerin, and apelin, together with cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6, which are responsible for dysregulation of immune responses\(^{15,23}\). Obesity plays a role in modulating the initiation and progression of periodontal disease. Recent meta-analyses have validated a positive association between being overweight or obese and periodontitis\(^{6,14,27}\).

Periodontitis is an inflammatory disease that results in the destruction of the supporting connective and bony tissues of the teeth. Human periodontal ligament cells play a role in osteoclastogenesis through expression of receptor activator of nuclear factor kappa B ligand (RANKL) on the cell surface in response to exposure to periopathogenic factors and inflammatory cytokines\(^{3}\). Studies using bone cell models have demonstrated that formation of osteoclasts requires interaction between nuclear factor kappa B (NF-κB), which is expressed on the surface of osteoclasts, and RANKL in the presence of macrophage colony-stimulating factor (M-CSF)\(^{26}\). The latter is required for osteoclastogenesis, stimulating both adhesion and proliferation of osteoclast precursors\(^{9}\).

Functional screening of a library of secreted proteins has resulted in the identification of a novel cytokine, IL-34, which stimulates the viability of monocytes and macrophage colony formation from bone marrow cells. Interleukin-34 mRNA is expressed in various tissues, including heart, brain, lung, liver, kidney, spleen, thymus, testes, ovary, small intestine, prostate, and colon, and is most abundant in the spleen. The receptor of IL-34 was discovered by screening of extracellular domains of transmembrane proteins, and was found to be already known as the M-CSF receptor\(^{16}\).

Interleukin-34 plays an important role in RANKL-induced osteoclastogenesis, as it can substitute for M-CSF and support osteoclast differentiation in the same way\(^{9}\). It is likely that IL-34 plays a role in inflammation, as it increases IL-6 and chemokine levels in human whole blood\(^{11}\). Interleukin-34 is also expressed in rheumatoid arthritis (RA) synovium, where it has been shown to be associated with severity of synovitis, and is elevated in serum and synovial fluid from RA patients\(^{8}\). Furthermore, IL-34 is produced by synovial and gingival fibroblasts in response to TNF-α and IL-1β through the NF-κB and c-Jun N-terminal kinase (JNK) pathways\(^{5,9}\). Expression of IL-34 is also up-regulated in intestine in patients with inflammatory bowel disease (IBD)\(^{31}\) and in inflamed salivary glands in patients with Sjögren’s syndrome\(^{18}\). Moreover, IL-34 is expressed in human adipose tissue. The circulating concentration is significantly elevated in obese patients and is associated with insulin resistance\(^{7}\).

To our knowledge, however, no studies to date have compared IL-34 levels in gingival crevicular fluid (GCF) or plasma between healthy and diseased periodontium; nor has its association with obesity been investigated in individuals with periodontal disease. The purpose of the present study, therefore, was to investigate the role of IL-34 in the pathogenesis of periodontal disease and to study the effect of obesity on IL-34 levels in periodontally diseased individuals.

**Materials and Methods**

1. **Study population**

The study population comprised 40 patients of both sexes aged between 25 and 40 yr. All were selected from the outpatient pool of the Department of Periodontology of the Government Dental College and Research Institute, Bengaluru, India. The research protocol was submitted to the Institutional Ethical Committee and Review Board of the same institution and ethical clearance obtained (Approval no: GDCRI/ACM/PG/PhD/10/2013-2014). The study was conducted between March 2015 and August 2015. The study protocol was explained and written informed consent obtained from all participants prior to commencement.
2. Selection criteria

Only patients with a minimum number of 20 natural teeth and the clinical signs required for their respective groups were included in the study. The exclusion criteria were as follows: chronic inflammatory disease, such as RA or IBD; a state of immunodeficiency due to infection with HIV; pregnancy; giant-cell tumor of the bone; respiratory disease, such as chronic obstructive pulmonary disease, asthma, and bronchitis; coronary heart disease; hypertension; aggressive periodontitis; diabetes mellitus; smoker; betel/areca nut chewing; alcoholism; use of steroids, contraceptives, anti-inflammatory drugs, or antibiotics; and periodontal treatment in the preceding 6 months.

The patients were selected randomly and categorized into the following 4 groups: Group I, comprising 20 samples (10 GCF and 10 plasma) obtained from 10 non-obese patients with healthy periodontium; Group II, comprising 20 samples (10 GCF and 10 plasma) obtained from 10 obese patients with healthy periodontium; Group III, comprising 20 samples (10 GCF and 10 plasma) obtained from 10 non-obese patients with chronic periodontitis; and Group IV, comprising 20 samples (10 GCF and 10 plasma) obtained from 10 obese patients with chronic periodontitis. The patients were categorized as having healthy periodontium or chronic generalized periodontitis based on bleeding on probing, gingival index (GI)\(^\text{18}\), pocket depth (PD), and clinical attachment level (CAL)\(^\text{13}\) scores together with radiograph evidence of bone loss. A GI score of 0, PD \(\leq 3\) mm, CAL = 0, and no evidence of bone loss on a radiograph was considered to indicate a healthy periodontium. A GI score of \(\geq 1\), PD \(\geq 5\) mm, CAL \(\geq 3\) mm, and radiographic evidence of bone loss at more than 30% of sites was taken to indicate chronic generalized periodontitis\(^\text{11}\). The CAL was measured as the distance in millimetres from the cemento-enamel junction to the bottom of the periodontal pocket. Overweight and obesity were defined as a body mass index (BMI) in the range of >25 kg/m\(^2\) and waist circumference of >90 cm in men and >80 cm in women\(^\text{20}\). The BMI was calculated by dividing weight in kilograms by height in meters squared.

3. Selection of site and GCF fluid collection

Group allocations and sample site selections were performed by one examiner (ARP). Clinical parameters GI, PD, and CAL were evaluated by a calibrated examiner (CNG) using a periodontal probe (UNC PCP-15, Hu-friedy, Chicago, IL, USA). The same examiner also evaluated the radiographs and collected the GCF samples. Intra-examiner calibration was achieved by examining each of a total of 30 sites twice at an interval of 24 hr before the start of the study. If measurements at baseline and 24 hr were within a range of 1 mm, calibration was accepted at the 95% level. The GCF was collected from the site with the maximum CAL in Groups III and IV, whereas in the periodontally healthy groups, it was collected from multiple sites to ensure an adequate amount. To avoid the effect of circadian variation on GCF volume, it was collected in the morning at the same time of the day. Each selected site was first cleaned, isolated, and air dried using sterile cotton rolls. Supragingival plaque was then removed gently using a Universal curette (Columbia #4R/4L, Hu-friedy, Chicago, IL, USA) to avoid contamination of the paper strips. The paper strips were placed gently at the entrance of the gingival sulcus/crevice until light resistance was detected\(^\text{17}\) and then left in place for 60 sec. Care was taken to avoid mechanical injury and the absorbed GCF volume of each strip was determined by electronic impedance (Periotron 8000, ProFlow Inc., Amityville, NY, USA), with a digital readout being converted to microliters using software (MLCONVERT.EXE software version 2.52, Oraflow Inc., Amityville, NY, USA). Samples contaminated with blood or saliva were excluded from the study. After collection of the gingival fluid, the 4 periopaper strips used to absorb GCF from each patient were pooled and immediately transferred to microcentrifuge tubes containing 200μl
phosphate buffer saline and stored at $-70^\circ$C for subsequent analysis.

4. Blood collection and plasma extraction

Using a 2-ml syringe with a 20-gauge needle, 2 millilitres of blood was collected from the antecubital fossa by venepuncture and immediately transferred to EDTA containing vials. Plasma was separated from blood by centrifuging at 3,000 rpm for 5 min. The plasma was immediately transferred to a plastic vial and stored at $-70^\circ$C until the time of assay.

5. IL-34 analysis

The GCF and plasma samples were assayed for IL-34 levels using a highly sensitive enzyme linked immunosorbent assay (ELISA) kit (human IL-34 Catalog Number: DY5265, R & D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. The samples were run in duplicate and the mean was taken into consideration.

6. Statistical analysis

The statistical analysis was performed using SPSS statistical software (SPSS version 18.0, SPSS Inc., Chicago, IL, USA). Based on a pilot study comprising 5 patients in each group, the sample size was estimated to be 10 patients in each group to achieve 90% power to detect a difference of $0.5 \pm 0.687$, with a significance level (alpha) of 0.05 using a two-sided, two-sample t-test. The mean values of the demographic and clinical parameters were compared by using a one-way ANOVA. The GI and CAL were compared between Group III and Group IV by using an independent sample t-test. A pair-wise comparison of IL-34 concentration in GCF and plasma was performed using the Mann-Whitney U test between groups. A p-value of $<0.05$ was considered to indicate statistical significance. An intra-group correlation of GCF or plasma concentration of IL-34 with the clinical parameters was performed using Spearman’s rho correlation test. The mean intra-examiner standard deviation of differences in repeated PD and CAL measurements was obtained using single passes of measurements (correlation coefficients between duplicate measurements; $r = 0.95$).

Results

Table 1 shows the descriptive statistics (mean ± SD) of the study population. A total of 40 patients (20 men and 20 women) were included in the study. The mean age in Group I ($33.90 \pm 4.48$) was higher than in the other groups. Similarly, the mean BMI scores in Groups II and IV were higher than in Group I or III, and the difference was statistically significant ($p<0.001$). The comparison of mean GI and CAL scores between Group III and Group IV showed no statistically significant difference (Table 2). The mean IL-34 concentrations in GCF and plasma were highest in Group IV, and the difference was statistically significant, with a p-value of $<0.001$ (Table 1). Further multiple comparisons

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Group I (n=10)</th>
<th>Group II (n=10)</th>
<th>Group III (n=10)</th>
<th>Group IV (n=10)</th>
<th>f-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>33.90 ± 4.48</td>
<td>32.00 ± 4.40</td>
<td>31.80 ± 4.96</td>
<td>31.40 ± 6.04</td>
<td>0.492</td>
<td>0.690</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PD (mm)</td>
<td>1.80 ± 0.63</td>
<td>2.10 ± 0.74</td>
<td>6.40 ± 1.71</td>
<td>7.00 ± 1.56</td>
<td>26.106</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.04 ± 1.46</td>
<td>28.16 ± 1.56</td>
<td>20.25 ± 1.34</td>
<td>28.01 ± 1.45</td>
<td>99.488</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GCF IL-34 (pg/ml)</td>
<td>358.80 ± 29.68</td>
<td>446.18 ± 35.80</td>
<td>899.20 ± 67.83</td>
<td>969.44 ± 143.27</td>
<td>141.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma IL-34 (pg/ml)</td>
<td>77.66 ± 5.48</td>
<td>184.36 ± 62.60</td>
<td>641.76 ± 165.8</td>
<td>774.02 ± 215.47</td>
<td>59.459</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*p<0.001 is significant*
using the Mann-Whitney U test were carried out to find out which pair or pairs differ significantly. A statistically significant difference was observed in IL-34 concentration in GCF and plasma (p < 0.05), except between Group I and Group II and between Group III and Group IV, respectively (Tables 3 and 4). Spearman’s rho correlation demonstrated no statistically significant correlation between the clinical parameters and IL-34 concentration in GCF or plasma (Table 5).

### Table 2  Independent samples t-test to compare mean values between Group III and Group IV

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Group III (n = 10)</th>
<th>Group IV (n = 10)</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI score</td>
<td>2.360±0.38</td>
<td>2.520±0.50</td>
<td>0.666</td>
<td>0.425</td>
</tr>
<tr>
<td>CAL in mm</td>
<td>4.90±1.52</td>
<td>5.60±1.78</td>
<td>0.895</td>
<td>0.357</td>
</tr>
</tbody>
</table>

p<0.001 is significant

### Table 3  Pair-wise comparison using Mann-Whitney U test for IL-34 concentration in GCF

<table>
<thead>
<tr>
<th></th>
<th>Group II Mean Diff</th>
<th>p-value</th>
<th>Group III Mean Diff</th>
<th>p-value</th>
<th>Group IV Mean Diff</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>106.69</td>
<td>0.334</td>
<td>540.40</td>
<td>&lt;0.001*</td>
<td>610.64</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Group II</td>
<td>—</td>
<td>—</td>
<td>453.02</td>
<td>&lt;0.001*</td>
<td>523.26</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Group III</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>70.24</td>
<td>0.245</td>
</tr>
</tbody>
</table>

* p<0.001 is significant

### Table 4  Pair-wise comparison using Mann-Whitney U test for IL-34 concentration in Plasma

<table>
<thead>
<tr>
<th></th>
<th>Group II Mean Diff</th>
<th>p-value</th>
<th>Group III Mean Diff</th>
<th>p-value</th>
<th>Group IV Mean Diff</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>87.38</td>
<td>0.102</td>
<td>564.09</td>
<td>&lt;0.001*</td>
<td>696.36</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Group II</td>
<td>—</td>
<td>—</td>
<td>457.40</td>
<td>&lt;0.001*</td>
<td>589.66</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Group III</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>132.26</td>
<td>0.166</td>
</tr>
</tbody>
</table>

* p<0.001 is significant

Discussion

Being obese or overweight has been suggested to be associated with periodontitis, because obesity due to inflammatory mediators may affect systemic health through influencing host susceptibility to periodontitis. Periodontitis is a common chronic inflammatory oral disease of the adult population characterized by a gingival inflammatory response to pathogenic bacterial microflora, resulting in alveolar bone loss and eventually tooth loss. The link between periodontitis and obesity may have relevant public health implications as both diseases are important risk factors for cardiovascular disease.

The recently discovered cytokine IL-34 is an alternative ligand for the M-CSF receptor, and is now considered to constitute a novel
non-canonical pathway of osteoclast formation, as it can substitute for M-CSF in osteoclast differentiation and play an important role in RANKL-induced osteoclastogenesis. Patients with inflammatory periodontal disease often have elevated serum levels of proinflammatory cytokines. Bloemen et al. found that inflammation and osteoclastogenesis were triggered by proinflammatory cytokines and brought about alveolar bone resorption in periodontitis.

To our knowledge, however, the present cross-sectional study is the first to evaluate IL-34 levels in GCF and plasma in obese and non-obese individuals with or without periodontal disease and correlate disease severity with these values in an Indian population. The influence of age and sex on IL-34 levels was minimized by including an equal number of men and women and selecting patients within a specific age group (25 to 40 years).

A comparison of BMI as a demographic variable revealed a higher mean value in Group II and Group IV. One advantage of the protocol used here was that using absorbent filter paper strips allowed collection of GCF to be performed quickly and easily from individual sites, which also allows minimum trauma when done correctly. The results showed that the mean IL-34 levels in GCF (969.44 ± 143.27 pg/ml) and plasma (774.02 ± 215.47) were increased in patients who were obese and had periodontitis (Group IV) compared to in the other groups, and this difference was statistically significant (p<0.001). Chang et al. also found IL-34 expression in human adipose tissue, and the circulating concentration was significantly elevated in obese patients and was associated with insulin resistance. Zorena et al. concluded that IL-34 could serve as an additional potential inflammatory biomarker, with a cut-off value of 91.2 pg/ml for prediction of risk of vascular diabetic complications.

The pro-inflammatory cytokines TNF-α and IL-1β regulate IL-34 expression in synovial and gingival fibroblasts by a mechanism involving NF-κB and MAPK. Martinez et al.

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**Table 5** Spearman’s rho correlation of IL-34 levels in GCF and plasma to clinical parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma (in pg/ml)</th>
<th>GI (in mm)</th>
<th>PD (in mm)</th>
<th>CAL (in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation</td>
<td>p-value</td>
<td>Correlation</td>
<td>p-value</td>
</tr>
<tr>
<td>Group I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCF (in pg/ml)</td>
<td>–0.088</td>
<td>0.810</td>
<td>0.613</td>
<td>0.059</td>
</tr>
<tr>
<td>Plasma (in pg/ml)</td>
<td>0.332</td>
<td>0.349</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCF (in pg/ml)</td>
<td>0.546</td>
<td>0.103</td>
<td>0.379</td>
<td>0.279</td>
</tr>
<tr>
<td>Plasma (in pg/ml)</td>
<td>0.467</td>
<td>0.174</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCF (in pg/ml)</td>
<td>0.273</td>
<td>0.445</td>
<td>–0.005</td>
<td>0.050</td>
</tr>
<tr>
<td>Plasma (in pg/ml)</td>
<td>0.278</td>
<td>0.436</td>
<td>0.693</td>
<td>0.026</td>
</tr>
<tr>
<td>Group IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCF (in pg/ml)</td>
<td>0.571</td>
<td>0.085</td>
<td>0.684</td>
<td>0.791</td>
</tr>
<tr>
<td>Plasma (in pg/ml)</td>
<td>0.528</td>
<td>0.117</td>
<td>0.466</td>
<td>0.174</td>
</tr>
</tbody>
</table>

p<0.001 is significant
found that CSF-1 and IL-34 had complementary roles in periodontal disease, with IL-34 in steady-state and CSF-1 in inflammation. Ma et al. also found that IL-34 mRNA expression in periapical lesions was significantly higher than that in healthy periodontal ligament tissue, and concluded that IL-34 may be closely related to inflammation of chronic apical periodontitis. Thus, the increase in concentration of IL-34 in GCF or plasma in chronic periodontitis and obesity observed in the present study can be attributed to the proinflammatory properties of this protein.

These findings indicate that obesity and periodontitis can, independently or jointly, alter GCF and plasma IL-34 levels, mostly in favour of pro-inflammation. The concentration of IL-34 was higher in GCF than in plasma, which could be explained by local production of IL-34 in diseased periodontal tissue, suggesting that IL-34 levels might serve as a marker for local disease activity. An increase was observed in GCF IL-34 levels in obese patients with healthy periodontium compared to in non-obese patients with healthy periodontium, suggesting that obesity upregulates IL-34 secretion.

To our knowledge, this is the first study to evaluate IL-34 levels in GCF and plasma in obese and non-obese patients with or without periodontal disease and correlate disease severity with these values in an Indian population. Further longitudinal, prospective, multicenter studies, along with other markers of obesity, should be carried out to clarify the role of IL-34 as an inflammatory marker in obesity and chronic periodontitis.

**Conclusion**

Within the limitations of this study, the present results suggest IL-34 as a potential inflammatory marker of periodontal disease and obesity. The levels of IL-34 were highest in patients with chronic periodontitis and obesity, which may indicate an active inflammatory process, both systemically and locally in periodontal tissue. Determining IL-34 levels could, therefore, be valuable in detecting individuals at high risk of periodontitis and obesity. Further multicenter, longitudinal, prospective studies must be carried out to confirm these findings, along with other markers of obesity, to elucidate the possible role of IL-34 in the pathogenesis of periodontal disease and obesity.

**Conflict of Interest**

The authors declare that they have no conflict of interest. They declare no financial support or relationships that may pose a conflict of interest.

**References**


Correspondence:
Dr. C N Guruprasad
Department of Periodontology,
Government Dental College and
Research Institute,
Fort, Bengaluru, Karnataka, India
E-mail: periodonticsgdcri@gmail.com