Original Article

# Association of Human Interleukin-35 Level in Gingival Crevicular Fluid and Serum in Periodontal Health, Disease, and after Nonsurgical Therapy: A Comparative Study

**Subash Chandra Raj, Subhashree Manaswini Panda, Muktikesh Dash[1], Kaushik Patnaik, Devapratim Mohanty, Neelima Katti, Annuroopa Mahapatra, Debasish Mishra[2], Kamdev Praharaj**

*Department of Periodontics, Sriram Chandra Bhanja Dental College and Hospital, Cuttack, [1]Department of Microbiology, Sriram Chandra Bhanja Medical College and Hospital, Cuttack, [2]Department of Periodontics, Kalinga Institute of Dental Science, Bhubaneswar, Odisha, India*

## Abstract

**Background:** Innovating newer methods to diagnose a multifactorial disease such as periodontitis is always challenging for a clinician. Gingival crevicular fluid (GCF) which is closely associated with the periodontal tissue environment has been used a viable alternative to saliva for the diagnosis of periodontitis. **Aim:** The aim of the present study was to estimate and compare the interleukin-35 (IL-35) levels in GCF and serum among healthy, gingivitis, and chronic periodontitis (CP) individuals as well as to evaluate the effect of nonsurgical periodontal treatment (NSPT) on IL-35 level among patients with CP. Settings and Design: The study was conducted at the Department of Periodontics, Srirama Chandra Bhanja Dental College and Hospital, Cuttack, Odisha, India. It is a comparative study. **Materials and Methods:** A total of 60 participants were divided into healthy (Group I; $n$ = 20), gingivitis (Group II; $n$ = 20), and CP (Group IIIA; $n$ = 20). GCF samples collected from each individual at baseline and 6 weeks after NSPT for Group III individuals (Group IIIB; $n$ = 20) were quantified for IL-35 levels using enzyme-linked immunosorbent assay. **Statistical Analysis:** All analyses were performed using Shapiro–Wilk test, analysis of variance, Tukey's honestly significant difference *post hoc* test, and multiple regression analysis. **Results:** The mean IL-35 concentration in GCF was significantly high ($P < 0.05$) for Group IIIA (70.26 ± 4.0 pg/ml), as compared to Group I (54.81 ± 22.3 pg/ml) and Group IIIB (55.72 ± 10.2 pg/ml). **Conclusion:** In the present study, GCF and serum IL-35 concentration among CP individuals was highest among all the groups. Individuals receiving NSPT showed a significant reduction in IL-35 levels as compared to CP individuals.

**Keywords:** *Cytokines, gingival crevicular fluid, nonsurgical periodontal therapy, periodontitis*

## Introduction

Periodontal disease is the most common form of bone pathology worldwide. Being a chronic inflammatory disorder, it causes destruction of tooth-supporting structures and also acts as a modifying factor of the systemic health of patients.[1] Although the initiation of the disease process is due to periodontopathogens present in the dental plaque,[2] the tissue destruction is mainly caused by the host response to the etiologic microorganism through a variety of inflammatory cytokines.[2-5]

Cytokines are a broad group of soluble factors that function in an autocrine or paracrine manner.[6] They play pivotal roles in coordinating activities of diverse immune cell types by coupling extracellular stimuli to intracellular signal transduction networks and mediate multiple physiological processes including differentiation, cell growth, and development of target cells.[6] They are produced by host immune-inflammatory cells such as T-helper (Th) cells and macrophages in response to the endotoxins produced by the pathogens. These Th cells are mainly Th1 and Th2 cells.[7] Cytokines produced by Th1 cells (interleukin [IL]-2, interferon gamma, IL-12, tumor necrosis factor-alpha) induce cell-mediated responses while cytokines produced by Th2 cells (IL-4, IL-13) induce antibody-mediated responses.[7-9]

IL-35 is a newer generation of signal molecule belonging to IL-12 cytokine family. It is produced by T-regulatory cells (Treg) consisting of α chain (p35) and β chain (Ebi3).[10] It is characterized by sharing of three α (p19, p28, p35) and



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***Address for correspondence:***
*Dr. Subash Chandra Raj, Department of Periodontics, Sriram Chandra Bhanja Dental College and Hospital, Cuttack - 753 007, Odisha, India.*
*E-mail: 31stodc@gmail.com*

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two β (p40 and Ebi3) subunits.[10] Recent studies have shown that IL-35 is an anti-inflammatory cytokine which suppresses the immune response through the expansion of Treg and suppression of Th17 cell development.[11,12] This is suggestive of a possible role of IL-35 in chronic inflammatory disorder such as periodontitis. However, there is limited information regarding the exact mechanism.

In periodontal diagnostics, recently, there has been a steady growth in trends to develop tools for monitoring periodontitis. So far, diagnosis of periodontal disease relies primarily on clinical and radiographic parameters. These measures are useful in detecting evidence of past disease, or verifying periodontal health, but provide only limited information about patients and sites at risk for future periodontal breakdown. Numerous biomarkers in the saliva, gingival crevicular fluid (GCF) have been proposed and used as diagnostic tests for periodontal disease.

The aim of the present study was to estimate and compare the IL-35 levels in GCF and serum among healthy, gingivitis, and chronic periodontitis (CP) individuals and also to evaluate the effect of nonsurgical periodontal treatment (NSPT) on IL-35 levels among patients with CP to explore the possibility of using IL-35 as a biomarker for periodontal disease activity.

## Materials and Methods

This was a comparative study conducted over a period of 6 months (October 2015 to March 2016); the study participants were selected from the outpatient Department of Periodontology, Srirama Chandra Bhanja Dental College and Hospital, Cuttack, India. A total of 60 subjects (31 males and 29 females, aged 25–60 years) were divided into three clinical groups, based on the periodontal parameters.

CP patients were diagnosed based on the criteria of American Academy of Periodontology classification of periodontal diseases (1999). The subjects for sampling were selected at random from individuals scheduled for a routine oral examination. Periodontal evaluation included recording of full-mouth gingival index (GI) score (Loe and Silness, 1963), probing pocket depth (PPD), and clinical attachment loss (CAL) using a graduated periodontal probe (University of North Carolina [UNC]-15 periodontal probe, Hu-Friedy, Chicago, IL, USA). All clinical measurements were performed by a single examiner (SCR). For intra-examiner calibration, 20 sites were examined twice, 24 h apart, before the commencement of the study. Calibration was accepted when 95% of differences were within 1.0 mm.

On the basis of GI score, PPD, and CAL measurements, participants were initially categorized into three groups each having 20 individuals ($n = 20$).

- Group I: healthy individuals (GI = 0, PD < 3 mm, and CAL = 0)
- Group II: Gingivitis (GI > 1, PD < 3 mm, and CAL = 0 mm)
- Group III: CP (GI > 1, PD > 3 mm, CAL > 3 mm).

Subjects in Group III underwent NSPT, i.e., scaling and root planing using periodontal curettes (Gracey curettes; Hu-Friedy, Chicago, IL, USA), completed in two sessions within 24 h in accordance with the Quirynen's one-stage, full-mouth debridement protocol.[13]

### Site selection and gingival crevicular fluid collection

In Group I, GCF was collected from multiple sites (3–5 sites per subject) to ensure the collection of an adequate amount of sample. For Group II and Group III, only one site per subject was selected having the greatest GI score or CAL, respectively, determined with the help of UNC-15 probe.

Sterile cotton rolls were used to clean and isolate the selected site. The supragingival plaque was removed gently using a Gracey curette (Universal Gracey curette #4R/4 L, Hu-Friedy, Chicago, IL, USA) so as to avoid contamination of the site. Three microliters of GCF was then collected using color-coded 1–5-μL calibrated volumetric microcapillary pipettes (Sigma-Aldrich Chemical Co. Ltd, St Louis, MO, USA) from the predetermined sites by intracrevicular method. The GCF collected was immediately transferred to a plastic vial and stored at −70°C, until the assay was performed. Microcapillary pipettes contaminated with blood and saliva were discarded.

For the subjects of Group III, sample collection was repeated 6 weeks after performing NSPT. For ease of understanding, the subjects of Group III have been referred to as Group IIIA at baseline and IIIB for recording data 6 weeks after the NSPT. The site selection and sample collection were done by an independent examiner (SMP).

### Blood collection

Two milliliters of blood was collected from the antecubital fossa by venipuncture using a 20-gauge needle with 2-ml syringe and immediately transferred to the laboratory. The serum was prepared from the blood sample after 1 h when the blood was allowed to clot by centrifuging at 3000 × g for 5 min. The serum was immediately transferred to a plastic vial and stored at −70°C until the time of assay.[14]

### Human interleukin-35 analysis

The GCF and serum samples were then assayed for IL-35 using enzyme-linked immunosorbent assay kit (Wuhan Fine Biological Technology Co., Ltd, China) according to the manufacturer's instructions by an examiner who was blinded to the groups allotted and was not involved in sample collection (KP). The concentrations of IL-35 in the tested samples were estimated using the reference calibrated standard curve, plotted using the optical density values of the standards (provided with the kit).

### Statistical analysis

The statistical analysis was performed using statistical software (SPSS version 16). The level of significance was set to 0.05 for all statistical inferences. All continuous data were tested for normality by Shapiro–Wilk test before using inferential statistics. For comparison of two means, Student's *t*-test was used. For comparison of more than two means, analysis of variance (ANOVA) was used for normal data followed by Tukey's honestly significant difference *post hoc* test for results which was found to be significant. A multiple regression analysis was carried out to determine the strength of intergroup difference of IL-35 levels.

## Results

Of 247 patients screened for the study, 84 participants were recruited, and finally, data of 60 participants were subjected to statistical analysis [Figure 1]. Table 1 shows the descriptive statistics (mean ± standard deviation) of the study population. It was found that the mean IL-35 concentration in GCF as well as serum was highest in Group IIIA and least in Group I. Table 2 shows the results of ANOVA test carried out to find the equality of means of IL-35 concentration of GCF and serum between healthy, gingivitis, and CP individuals (Group I, II, and IIIA). It was seen that difference in mean was statistically significant among three groups only for the IL-35 concentration in GCF ($P$ = 0.03). Subsequently, *post hoc* analysis was performed for pairwise comparisons between groups for IL-35 concentrations in GCF [Table 3]. It was found that IL-35 levels in GCF were significantly higher in Group IIIA as compared to Group I ($P$ = 0.002). Multiple regression analysis was performed to find the strength of association for IL-35 levels in GCF among the four groups [Table 4]. It was found that individuals having periodontal disease are likely to have 17.283 times the level of IL-35 than healthy controls. Table 5 shows the results of Student's *t*-test (paired) carried out to compare the GCF and serum IL-35 concentration of CP group before and after NSPT (Group IIIA and Group IIIB). Difference in mean between these two group was statistically significant only for IL-35 levels in GCF concentration ($P$ = 0.001).

## Discussion

Biomarkers are defined as objective, quantifiable characteristics of biological process. They may not correspond to patient's clinical condition or his/her perception of the state of health.[15] However, they assess the underlying disease process with more accuracy. Hence, it is a commonplace in basic and clinical research to use biomarkers as a diagnostic tool and as well as for assessing prognosis. Such biomarkers, are of more relevance for inflammatory conditions like periodontitis as they assess the current disease activity and not just the cumulative tissue destruction.[16]

IL-35 is one such inflammatory biomarker. It is a suppressive cytokine integral to the negative feedback loops and tolerance-promoting pathways.[17] Its role in inflammatory disorders is being explored widely in recent times.

Role of IL-35 in periodontitis is a relatively unexplored area. One of the pioneering studies was done by Kalburgi *et al.* in 2013.[18] They compared IL-35 levels in gingival

**Table 1: Descriptive data for study population (mean±standard deviation)**

| Study group | Group I (*n*=20) | Group II (*n*=20) | Group IIIA (*n*=20) | Group IIIB (*n*=20) |
|---|---|---|---|---|
| Age (years) | 40.10±7.9 | 38.60±5.0 | 45.10±7.7 | 45.10±7.7 |
| Sex (male/female) | 10/10 | 11/9 | 10/10 | 10/10 |
| GI | 0.95±0.6 | 2.04±0.5 | 2.17±0.33 | 0.72±0.31 |
| PD (mm) | 2.10±0.74 | 2.75±0.64 | 6.40±1.1 | 4.75±2.17 |
| CAL (mm) | 0 | 0 | 5.71±1.00 | 3.95±1.9 |
| GCF (IL-35) (pg/ml) | 54.81±22.3 | 62.36±7.3 | 70.26±4.0 | 55.72±10.2 |
| Serum (IL-35) (pg/ml) | 63.41±15.9 | 64.90±16.6 | 72.60±5.1 | 64.69±14.7 |

GCF: Gingival crevicular fluid; GI: Gingival index; PD: Pocket depth; CAL: Clinical attachment loss; IL-35: Interleukin-35

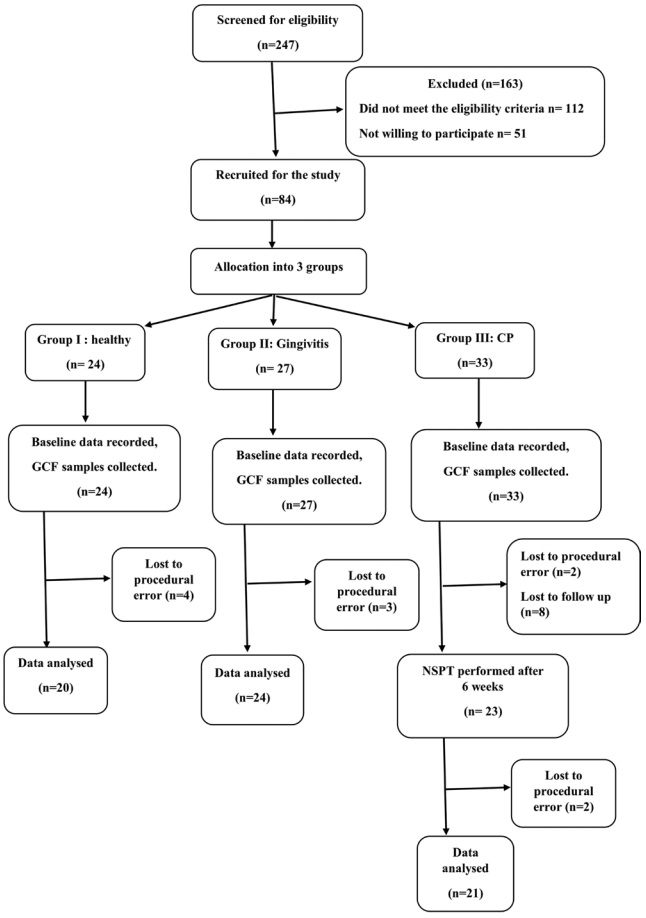


**Figure 1: Flow diagram following patient recruitment and follow-up**

**Table 2: Comparison of mean gingival crevicular fluid interleukin-35 concentration (pg/ml) among study groups**

| | Study groups | Mean±SD | *F* | *P* |
|---|---|---|---|---|
| GCF | Group I | 54.81±22.3 | 6.92 | 0.003* |
| | Group II | 62.36±7.3 | | |
| | Group IIIA | 70.26±4.0 | | |
| Serum | Group I | 63.41±15.9 | 2.62 | 0.081 |
| | Group II | 64.90±16.6 | | |
| | Group IIIA | 72.60±5.1 | | |

*ANOVA, significant at $P<0.05$. ANOVA: Analysis of variance; SD: Standard deviation; GCF: Gingival crevicular fluid

**Table 3: *Post hoc* analysis for comparison of mean gingival crevicular fluid Interleukin-35 concentration**

| | Study groups | Mean difference | *P* |
|---|---|---|---|
| GCF | Group I and Group II | −7.54 | 0.202 |
| | Group I and Group IIIA | −15.45 | 0.002* |
| | Group II and Group IIIA | −7.90 | 0.174 |

**Post hoc* Tukey test, significant at $P<0.05$. GCF: Gingival crevicular fluid

**Table 4: Regression coefficients for effect of different study groups, age and gender on GCF (IL 35)**

| | GCF IL 35 | |
|---|---|---|
| | β(SE) | *P* |
| Constant | 68.90 (8.72) | <0.001* |
| Group II | 7.05 (4.08) | 0.08 |
| Group III A | 17.28 (4.20) | <0.001* |
| Group III B | 2.74 (4.10) | 0.82 |
| Age | -0.36 (0.21) | 0.08 |
| Sex | 1.21 (2.90) | 0.68 |

* Significant at $P < 0.05$. GCF: Gingival crevicular fluid; SE: Standard error; IL-35: Interleukin-35

**Table 5: Comparison of level of interleukin-35 in gingival crevicular fluid and plasma (pg/ml)**

| | Group | Mean±SD | Mean difference | *t* | *P* |
|---|---|---|---|---|---|
| GCF | Group IIIA | 70.26±4.0 | 14.54±10.8 | 5.97 | 0.001* |
| | Group IIIB | 55.72±10.2 | | | |
| Serum | Group IIIA | 72.60±5.1 | 7.91±16.9 | 2.08 | 0.051 |
| | Group IIIB | 64.69±14.7 | | | |

*Student's *t*-test (paired), significant at $P<0.05$. GCF: Gingival crevicular fluid; SD: Standard deviation

tissues of healthy controls and CP and aggressive periodontitis patients. The result showed that IL-35 mRNA was expressed in gingival tissues of all the three groups; CP exhibited highest IL-35 expression followed by aggressive periodontitis and healthy patients.[18]

In our study, we assessed the IL-35 levels in GCF of healthy controls and gingivitis and CP subjects; the results showed that the mean IL-35 concentration in GCF among CP subjects was highest followed by gingivitis and healthy subjects, while the posttreatment concentration was similar to healthy subjects.

In another study by Mitani *et al*., IL-35 production in GCF and their gene expression levels in human gingival tissue were investigated.[19] They reported that GCF from CP subjects had significantly higher IL-35 levels as compared to healthy participants, and the gene expression of EBI3, IL-12A mRNA in inflamed gingival tissue was significantly higher in healthy control tissues.[19]

A recent study by Köseoğlu *et al*. aimed to compare the expression of IL-35 in plasma, saliva, and GCF among healthy, gingivitis, and CP subjects.[20] The CP subjects exhibited significantly higher volume of IL-35 in GCF as compared to gingivitis and healthy group. However, on comparing the concentration, it was found that levels of IL-35 in GCF were higher in healthy subjects than gingivitis and CP group. This discrepancy was attributed to the fact that GCF was collected with periopaper strips inserted in the gingival sulcus for 30 s. The amount of GCF collected in healthy subjects in the time interval was less than that in gingivitis and CP group. In our study, a standardized volume of GCF was collected from the subjects in all four groups as it can be a better indicator of the concentration of the cytokine in the GCF.[20]

Exact role of IL-35 in etiopathogenesis of periodontal disease is not clear, but it is reported to be secreted from Treg cells as negative feedback regulation.[17] According to Nakajima *et al*., there is an increase in expression of Treg cell in CP as compared to gingivitis and healthy subjects because these cells are recruited by immune system for containing the disease and arresting tissue destruction.[21] Therefore, reduction in inflammatory load reduces the expression of Treg cells; hence, IL-35 levels in GCF also decrease. This could explain the significant reduction in GCF IL-35 levels in CP patients 6 weeks after NSPT was performed in our study.

The mean plasma IL-35 concentration was found to be 63.4–72.6 pg/ml, which was similar to earlier reports.[20,22,23] In our study, the mean plasma concentration of IL-35 was highest in CP followed by gingivitis and healthy subjects; however, the difference was statistically not significant ($P = 0.08$). Further, the mean plasma concentration of IL-35 in CP subjects after NSPT was less compared to CP group, but the difference was not statistically significant ($P = 0.051$). This is similar to earlier reports by Köseoğlu *et al*. They reported that periodontitis and gingivitis do not affect the plasma level of IL-35.[20]

In the present study, we found a predictable association between concentration of IL-35 and presence of periodontal inflammation. Multiple regression analysis showed a significant beta coefficient even after adjusting for age and gender, suggesting that GCF IL-35 can be considered as a predictable biomarker of established periodontal disease.

However, the difference between mean GCF and serum IL-35 was not significant for gingivitis subjects, which suggests that IL-35 may not be a predictable marker in initial stages of periodontal disease.

This was the first study of its kind to estimate level of GCF and serum IL-35 in CP subjects after NSPT. However, IL-35 is relatively a newcomer among suppressive cytokines.[17] Estimation of other established cytokines such as transforming growth factor-β along with IL-35 may give a clearer picture of its position in etiopathogenesis of periodontitis. Further multicenter, longitudinal prospective studies should be carried out to affirm these findings and further validate the role of IL-35 in periodontal inflammation.

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### Conflicts of interest

There are no conflicts of interest.

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