ISSN (O) : 2582-3271
PubMed-National Library of Medicine - ID: 101766917

*International Journal of Dental Science and Clinical Research (IJDSCR)*

*Dr. Angel Vaidic Publication*

*Available Online at: http://www.ijdscr.org*

*Volume 3, Issue 4, July - 2021, Page No. : 01 - 13*

# Effects of Initial Periodontal Therapy on the Gingival Crevicular Fluid Levels of NlRP3 & IL-1β in Chronic Periodontitis Subjects

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**Citation of this Article:** Dr. Nikhila Chandramohan, Dr. Swetha A, Dr. Niranjan R J, "Effects of Initial Periodontal Therapy on the Gingival Crevicular Fluid Levels of NlRP3 & IL-1β in Chronic Periodontitis Subjects." IJDSCR – July - 2021, Vol. – 3, Issue - 4, P. No. 01-13.



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**Type of Publication:** Original Research Article

**Conflicts of Interest:** Nil

## Abstract

Periodontal disease of the oral cavity is a plaque-induced inflammation of the periodontal tissues involving the up-regulation of various pro-inflammatory cytokines like IL-1β. Their unbalanced production appears to mediate periodontal tissue destruction. Processing of IL-1β is regulated by intracellular innate immune response system called ***NLRP3 inflammasome complex*** which is activated by a number of microbial components like Lipopolysacchrides, etc. The inflammasome complex formation leads to caspase-1 activation and thereby maturation of IL-1 family cytokines. Studies have shown increased mRNA expression of NLRP3 in periodontitis patients similar to the elevation of various inflammatory cytokines like IL-1, IL-6,INF-α etc. The periodontally diseased subjects in this study underwent comprehensive nonsurgical periodontal therapy including deep scaling and root planing (SRP) under local anesthesia. It has been established that with SRP alone there was an exorbitant improvement in clinical parameters and diminishment of inflammatory mediators. In this view, the present study is structured to estimate NLRP3 & IL-1β levels in Gingival crevicular fluid (GCF) of Chronic periodontitis (CP) patients pre & post SRP and to correlate them with the clinical periodontal parameters. Present study revealed that the mean concentrations of both in GCF

significantly reduced in CP patients after SRP. In addition we observed a positive correlation between NLRP3 & IL-1β levels in GCF with clinical periodontal parameters and with each other. In conclusion the present study throws light on the emerging role of NLRP3 inflammasome in periodontal disease.

**Keywords**

NLRP3 inflammasome; IL-1β; chronic periodontitis; crevicular fluid;SRP;

## Introduction

Periodontal disease is dependent on the host response to pathogenic bacteria that colonizes the tooth surface and alters the host defense mechanism involving the activation of a broad axis of innate immunity, specifically by up-regulation of various pro-inflammatory cytokines like TNF-γ, Interleukin 1, IL-6, IL-18, etc, and their unbalanced production appears to cause periodontal tissue destruction[8] . As a first line, the innate immune system relies heavily on the presence of evolutionarily conserved *Pattern Recognition Receptors (PRRs),* the best characterized of which include Membrane-Bound *Toll Like Receptors (TLRs), Nod-Like Receptors (NLRs), C-type Lectin Receptors (CLRs) and RIG Like Receptors (RLRs),* to recognize various pathogenic components[32] . These special receptors, can sense 'Pathogen Associated Molecular Patterns' *(PAMPs)* and also respond to 'Danger-Associated Molecular Patterns' *(DAMPs)* which may be host or environmentally derived and are expressed by many cell types. The activation of PRRs by PAMPs and their post-receptor signaling via stimulation with DAMPs can ultimately drive the recruitment of 'inflammasome' complexes and play a crucial role in the activation of specific inflammatory cascades [22,36] .

The inflammasome complex is classically composed of a Nucleotide Binding domain Leucine-Rich Repeat (NLR) protein, the Adaptor molecule apoptosis associated Speck-like protein containing a CARD (ASC) domain, and pro-caspase-1. This oligomeric assembly of multiprotein complexes functions as a molecular platform that triggers the maturation and secretion of pro-inflammatory cytokines, such as IL-1β and IL-18 in response to microbial products or stress signals, which are known to be potent immunomodulators. The NLRP3 is the most immensely studied due to a wide array of activators of microbial and non microbial origin [4] . The inflamed gingival periodontal tissues are characterized by an influx of inflammatory cells. These cells have been shown to express the NLRP3 inflammasome complex, sensing and responding to bacterial challenge. A range of studies on NLRP3 inflammasome implicates an increased expression in obesity, gout, type 2 diabetes mellitus, metabolic liver disease, atherosclerosis, alzheimer's disease, cancer, COPD and rheumatoid arthritis.

NLRP3 expression was shown to be higher by 7·4- fold in the gingival tissue samples of aggressive periodontitis, 4·3-fold in chronic periodontitis and 7·7 -fold in gingivitis subjects compared to healthy controls [6] . Another in vitro study showed an increased inflammasome transcription in response to supragingival biofilms is commensurate with early inflammatory events in periodontal disease, whereas decreased transcription in response to subgingival biofilms corroborates the dampening of host immune responses, in favour of pathogen survival and persistence[7] . Infection with P. gingivalis triggered the activation of NLRP3 and AIM2 inflammasomes via TLR2 and TLR4 signaling, leading to IL-1 secretion and pyroptic cell death[26]. A significant high levels of NLRP3 salivary concentration was elucidated in the periodontitis group along with significant positive correlation with clinical

periodontal parameters [14] , but impact of periodontal treatment on GCF concentration of NLRP3, has not been investigated yet.

Of the humoral factors influencing immuno-modulatory activities within periodontal tissues, a pivotal role is played by IL-1β and IL-8. The effects IL-1β, a vital cytokine secreted by monocytes and macrophages, increases the inflammatory response in the periodontium that subsequently causes degradation of periodontal ligament and alveolar bone loss. Its effects range from regulation of inflammatory mediators to catabolic effects on osteoclasts and activation of matrix metalloproteinases that degrade extracellular matrix proteins. The spectrum of immune-stimulating activities and lipopolysaccharide elicited production made IL-1, a very attractive candidate, as an important host factor in periodontal diseases. Some of its biological effects include stimulation of fibroblast proliferation, prostaglandin E2 production by monocytes and fibroblasts & affects neutrophil chemotaxis and activation [24] . It has been proved that IL-1β levels are higher in GCF at periodontally diseased sites [21, 34] compared to healthy sites and statistically significant reduction in levels post SRP [18].

There are several treatment modalities available for the treatment of periodontal diseases but SRP still remains to be the gold standard for periodontal therapy. Numerous studies have elucidated a significant improvement after SRP, in clinical periodontal parameters and marked reduction of various cytokines. This study was taken up to explore this role of NLRP3 inflammasome and correlate it with IL-1β levels in GCF of chronic periodontitis patients before and after SRP.

## Materials and Methods

### A. Patients & Samples

The periodontitis subjects belonging to both the sex were selected. This was an interventional study which extended for a period of 1 year. Out of 75 subjects, 60 agreed to participate in the study and 30 subjects met the inclusion criteria.

Subject groups - Group I consisted of 30 subjects (14 males & 16 females) with generalized moderate to severe chronic peridodontitis [33] who were within the age group of 25-55 years with ≥20 teeth excluding the third molars, who had not received any periodontal treatment in the previous six months. GROUP II consisted of group 1 subjects who underwent scaling and root planing. Criteria for periodontitis were gingival index ≥1, presence of bleeding on probing, presence of at least 8 teeth showing ≥3mm of clinical attachment loss (CAL) and ≥5mm of probing pocket depth (PD). Criteria for exclusion from the study is as follows : History of underlying systemic disease which may affect the periodontium. Pregnant and lactating women, History of undergoing periodontal treatment in the past 6 months, Under antibiotic treatment, NSAIDs, steroids or any other medication that can alter the course of periodontal disease, History of Tobacco chewing, Current and former smoker. Approval from the Ethical Committee of M.R. Ambedkar Dental College and Hospital was obtained. The nature and purpose of the study was explained to the individuals and a written informed consent was taken from all the patients

### B. Clinical Examination

All patients underwent full mouth periodontal examination that included Plaque index [19] , Bleeding index[2], Gingival index [30] , Clinical probing depth (PD) and Clinical attachment level (with CEJ as reference). Probing pocket depths were measured from the gingival margin to the base of the clinical pocket with the probe tip parallel to the long axis of the tooth. Measurements were made to the nearest millimeter and where any

doubt existed the lower value was scored. The parameters were recorded for the patients at baseline and at 12 weeks after completion of SRP. All clinical parameters were assessed by one examiner using a University of north Carolina-15 probe.

### C. Gingival Crevicular Fluid Sampling

In each subject, one site with periodontitis was selected for GCF collection on the day of examination and the patient was recalled the following day for GCF sample collection to avoid contamination of the sample with blood after screening. The subjects were asked to rinse their mouth with water to cleanse the teeth of loosely adherent debris. Samples of GCF were obtained from these pre-determined sites by placing color coded, calibrated, volumetric, micro-capillary pipettes with 5µl range, at baseline and at 12 weeks after completion of scaling and root planing. Sites with deepest probing depth was selected and isolated with cotton rolls and a saliva ejector was used to decrease the risk of salivary contamination. Volumetric micro-pipettes were placed extra-crevicularly at the entrance of the gingival crevice and standardized volume of 3µl GCF samples were collected from each patient. The pipettes contaminated with blood or saliva was discarded.

### D. Sample Storage

The samples were transferred from the pipettes into eppendorf tubes and immediately stored at -80°C, until analyzed for NLRP3 and IL-1β using commercially available ELISA kits.

### E. Treatment Protocol

Following the collection of gingival crevicular fluid and clinical data prior to treatment, chronic periodontitis patients received full-mouth scaling and root planing with curettes and ultrasonic instruments under local anesthesia. Prior to scaling and root planing, pre procedural wash with 1% povidone iodine was done. Full mouth scaling was done using ultrasonic scalers, and root planing was performed using specific Gracey curettes in 2-3 appointments over a period of 2 weeks. Oral hygiene instruction was provided. Patients were recalled every 4 weeks till 3 months to evaluate their oral hygiene status.

### F. Enzyme Linked Immunosorbent Assay of IL-1β

The assay was performed using IL-1β ELISA test kit (Ray Biotech Inc, USA).The manufacturer's instructions were carefully adhered to and each plate was checked before use to ensure the calibration curve measured IL-1β standards within the stated limits of the assay. The kit made use of biotinylated anti- IL-1β antibody and streptavidin-horse- radish peroxidase. The substrate color reaction was measured at 405 nm by an ELISA reader (Molecular Dynamics, Sunnyvale, CA, USA). The optical density values obtained with the known samples were used to calculate the quantity of IL-1β in the other samples.

### G. NLRP3 Assay

The assay was performed using Human NACHT, LRR and PYD domains-containing protein 3 (NLRP3/C1orf7/CIAS1/NLRP3 /PYPAF1) ELISA Kit (My Biosource Inc, USA).The manufacturer's instructions were carefully adhered to and each plate was checked before use to ensure the calibration curve measured NLRP3 inflammasome standards within the stated limits of the assay. The kit used the reaction between Biotin-antibody and TMB substrate. The substrate color reaction was measured at 405 nm by an ELISA reader (Molecular Dynamics, Sunnyvale, CA, USA). The optical density values obtained with the known samples were used to calculate the quantity of NLRP3 in the other samples.

### H. Statistical Analysis

Descriptive statistical analysis was carried out in the present study. Data are presented as means and SD. Results on continuous measurement are presented on Mean ± SD (min-max) and results on categorical measurements are presented in number (%). Significance is assessed at 5% level of significance. Paired student't' test has been used to find the significance of clinical periodontal parameters before and after treatment in the same group. Multiple linear regression analysis has been used to find the relationship between clinical periodontal parameters with NLRP3 inflammasome and IL-1β levels in GCF with CAL as the dependent factor. Correlation between clinical periodontal parameters and the two biomarkers & also between the two biomarkers was evaluated using Pearson's correlation analysis.

## Results

### A. Effect of SRP on Clinical Parameters

The ELISA testing of GCF samples showed that initial periodontal therapy led to a statistical improvement in all examined clinical periodontal parameters. Difference between the two groups with respect to clinical attachment loss, pocket depth and gingival index reached $p < 0.001$(*Table 2)*. The Mean plaque index was 1.160±0.128 at baseline and after SRP it showed a significant decrease to 0.989±0.288 (p=0.003). Mean bleeding index and Mean gingival index as well showed a decreasing trend from 89.786±5.217 to 70.984±7.305 and 2.373±0.351 to 0.389±0.189 respectively which was statistically significant (p <0.001). The Mean probing depth in Group I was 3.589±0.244 and group II was 1.899±0.401 and the Mean clinical attachment level was 2.398±0.510 in group I and in group II was 1.207±0.305, both the parameters showed a statistically significant reduction after SRP (p<0.001). It should be mentioned that the post-SRP healing was uneventful in all cases and no complications, such as abscesses or infections, were observed throughout the study period.

### B. Effect of SRP on Amounts of NLRP3 and IL-1B in GCF.

Mean NLRP3 levels in GCF in Group I was 1.842±0.242 ng/ml and in group II was 1.335±0.246 ng/ml while IL-1β levels s in GCF was 95.72±7.41 pg/ml in group I and 31.29±11.91 pg/ml in group II (*Table 3)*. In group II i.e. after SRP, both GCF constituents (*NLRP3 & IL-1β*) showed a statistically significant decrease from baseline to 12 weeks.

### C. Correlation Analysis

Pearson's correlation analysis between clinical periodontal parameters including BI, PI, GI, PD, CAL and NLRP3 levels and with IL-1β levels of GCF showed a positive correlation in both groups (*Table 4 & 5, Fig 1 & 2*). All the above correlations are statically significant except of NLRP3 inflammasome with PI in group II (p=0.15, r=0.21) and of IL-1β with PI in group II (p=0.17. r=0.26) which were not statistically significant. Pearson correlation analysis done to assess the relationship between the NLRP3 inflammasome levels in GCF in pre-treatment and post treatment time periods, showed a positive correlation at p <0.001. A similar positive correlation was also observed between the GCF levels of IL-1β in group I and Group II at p <0.001 (*Table 6)*. The correlation analysis between GCF levels of NLRP3 inflammasome & IL-1β in group I and group II also shows a positive correlation at p< 0.001 (*Table 7, Fig 3 & 4)*.

Multiple linear regression analysis was used to evaluate the joint effects of all the predictors namely the age, BI, PI, GI, PD , NLRP3 inflammasome levels and IL-1β levels on CAL in group I and group II (*Table 8 &*

9). GCF levels of NLRP3 and IL-1β and the clinical parameters namely BI, PI, GI, PD showed a positive correlation with CAL indicating that, an increase in any of the independent variables resulted in an increase in CAL in group I and group II. The adjusted R squared value of 0.45 and 0.32 suggests that after adjusting 45% and 32% of CAL is explained by the predictors in group I & II respectively. Overall the model was statistically significant for group I & II with p value = 0.002 and p = 0.004 respectively.

## Discussion

IL-1β is one of the most consistently discovered potent signature innate cytokine associated with periodontitis. It has been revealed recently that the mechanism by which IL-1β is induced involves the activation of a molecular platform, NLRP3 inflammasome by PAMPs and DAMPs. Increased mRNA expression of NLRP3 and NLRP2 in gingival tissues affected by periodontal disease was established using real time PCR technique [6] . Increased levels of IL-1β are frequently detected in the saliva and GCF of patients with deeper pocket depths and more severe bleeding on probing (BOP) compared with healthy controls [17,27,28] . Indeed, a functional relationship of NLRP3 with its downstream processing targets, IL-1β in periodontal diseases can be anticipated. In this line, some studies have provided novel insights regarding the differential expression of inflammasome-related proteins across the spectrum of periodontal disease, and compelling evidence suggests that oral biofilms may concomitantly regulate the expression of inflammasomes and their associated cytokine targets [35] . Recently, a significantly high levels of NLRP3, ASC, and IL-1β salivary concentrations in the periodontitis group was elucidated along with significant positive correlation with clinical periodontal parameters [14] . Although from these studies it has become evident that the inflammasome and its constituents are possibly critical for the onset of periodontal disease, little is known about the significance GCF concentration of NLRP3 with regard to the periodontal clinical status.

It is well established that various host cells are activated to release IL-1β in the presence of LPS and endotoxin derived from Gram-negative microorganisms. IL-1β is the known to be the most potent inducer of bone demineralization and induces major alterations in the connective tissue matrix composition, stimulates MMPs and affects neutrophil functions [12]. This study is the first of its kind to have been designed with the intention of comparing the quantitative levels of NLRP3 inflammasome & IL-1β in the GCF of patients with generalized chronic periodontitis before and after scaling & root planning.

This study shows that with increase in inflammation and tissue destruction there was increase GCF NLRP3 levels. And with down regulation of inflammatory burden post SRP, there was a decline in the same. It was shown that the concentration of IL-1β in the GCF significantly reduced post SRP (at the end of 12 weeks) ($P < 0.001$), in agreement with previous studies which demonstrated that the total levels of numerous cytokines including IL-1β, IL-1α, IL-6, etc decreased significantly in disease sites in response to therapy suggesting a relationship between active disease and cytokine production [10,18] . Positive correlation of NLRP3 and IL-1β GCF levels with most of the clinical parameters was established. This was in line with studies that stated the total amounts of IL-1β were positively correlated with gingival index [11] , as well as pocket depth[13].

The exact mechanism of NLRP3 inflammasome activation in periodontal diseases has not been fully

elucidated. While some authors hypothesizes that NLRP3 expression is down regulated by subgingival biofilm[5] , other observations suggest that the expression of the NLRP3 inflammasome components, namely NLRP3 and ASC, are increased in response to microbial infection [16] . In our study, the positive correlation between NLRP3 and IL-1 β GCF concentrations and each of them with clinical parameters before and after SRP was statistically significant (P<0.001). The results presented, can be attributed to that fact that inflammasomes are protein platforms that mediates the activation of pro-caspase-1, which is responsible for processing & release of IL-1β. The pathogenic microorganisms and their virulence factors function by targeting inflammasomes and modulating IL-1β processing, as well as its shedding from monocytes/macrophages and activated neutrophils within the periodontal microenvironment which, all together, leads to development and progression of periodontal diseases.

This study provides a mechanistic insight to the host immune responses involved in the pathogenesis of periodontal disease by demonstrating the modulation of this cytokine-signaling pathway by a bacterial challenge as well as the benefit of mechanical intervention. Hence, NLRP3 inflammasome can be used as a diagnostic biomarker in the field of periodontics. Thus, further longitudinal studies with larger sample sizes maybe required in the similar direction.

**Table 1:** Demographic Data

| Age & Gender distributions among study subjects | | | |
|---|---|---|---|
| Variables | Categories | n | % |
| Age Groups | 25-35 yrs | 5 | 16.7% |
| | 36-45 yrs | 8 | 26.7% |
| | 46-55 yrs | 13 | 43.3% |
| | > 55 yrs | 4 | 13.3% |
| Sex | Males | 14 | 46.7% |
| | Females | 16 | 53.3% |

**Table 2:** Comparision of Mean of Clinical Parameters among Groupi & Groupii

| Parameters | Pre-Rx | | Post Rx | | t | P |
|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | | |
| PI | 1.160 | 0.128 | 0.989 | 0.288 | 3.194 | 0.003* |
| BI | 89.786 | 5.217 | 70.984 | 7.305 | 22.426 | <0.001* |
| GI | 2.373 | 0.351 | 0.389 | 0.189 | 30.141 | <0.001* |
| PD | 3.589 | 0.244 | 1.899 | 0.401 | 20.323 | <0.001* |
| CAL | 2.398 | 0.510 | 1.207 | 0.305 | 10.819 | <0.001* |

**Table 3:** Comparison of Mean Values of IL-1β & NLRP3 Inflammasome Levels Among Group I and II in GCF

| Parameters | Pre-Rx | | Post Rx | | t | P |
|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | | |
| NLRP3 | 1.842 | 0.242 | 1.335 | 0.246 | 24.755 | <0.001* |
| IL-1B Conc | 95.72 | 7.41 | 31.29 | 11.91 | 68.034 | <0.001* |

Table 4 - Pearson correlation to assess the relationship of periodontal parameters with NLRP3 & IL-1B levels s in GCF during Pre-Rx Period

| Parameters | Values | Age | PI | BI | GI | PD | CAL |
|---|---|---|---|---|---|---|---|
| NLRP-3 | r | 0.63 | 0.67 | 0.77 | 0.60 | 0.65 | 0.61 |
| | P-Value | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| IL-1B | r | 0.62 | 0.66 | 0.78 | 0.61 | 0.66 | 0.63 |
| | P-Value | <0.001* | <0.001* | <0.001* | 0.001* | <0.001* | <0.001* |

Table 5: Pearson correlation to assess the relationship of periodontal parameters with NLRP3 & IL-1B levels s in GCF during Post-Rx Period

| Parameters | Values | Age | PI | BI | GI | PD | CAL |
|---|---|---|---|---|---|---|---|
| NLRP-3 | r | 0.55 | 0.21 | 0.81 | 0.60 | 0.60 | 0.54 |
| | P-Value | 0.001* | 0.15 | <0.001* | <0.001* | 0.001* | 0.006* |
| IL-1B | r | 0.61 | 0.26 | 0.90 | 0.57 | 0.58 | 0.53 |
| | P-Value | 0.001* | 0.17 | <0.001* | 0.001* | 0.002* | 0.003* |

**Table 6:** Pearson Correlation to Assess the Relationship B/W Pre-Rx & Post-Rx Time Periods of NLRP3 & IL-1β Conc. in GCF

| Parameters | Values | Pre Rx | Post Rx |
|---|---|---|---|
| NLRP3 | r | 1 | 0.89 |
| | P-Value | | <0.001* |
| IL-1B | r | 1 | 0.96 |
| | P-Value | | <0.001* |

**Table 7:** Pearson Correlation Test to Estimate the Relationship between NLRP3 & IL-1β at Pre & Post Treatment Periods

| Time Period | Values | NLRP3 | IL1B |
|---|---|---|---|
| Pre Rx | r | 1 | 0.99 |
| | P-Value | | <0.001* |
| Post Rx | r | 1 | 0.96 |
| | P-Value | | <0.001* |

Table 8: Multiple Linear Regression of Chronic Periodontitis group with CAL as the dependent factor during Pre Rx Period

| Multiple Linear Regression - Regression Statistics | | | | |
|---|---|---|---|---|
| R | $R^2$ | Adj. $R^2$ | F | P-Value |
| 0.75 | 0.56 | 0.45 | 4.858 | 0.002* |

| Variable | β-Coeffic | S.E. | t | P-value |
|---|---|---|---|---|
| PI | 0.47 | 0.15 | 3.846 | 0.002* |
| BI | 0.65 | 0.22 | 3.751 | 0.005* |
| GI | 0.41 | 0.15 | 2.759 | 0.01* |
| PD | 0.56 | 0.26 | 3.307 | 0.006* |
| IL-1B | 0.05 | 0.02 | 2.906 | 0.008* |
| NLRP3 | 0.95 | 0.63 | 2.868 | 0.008* |
| Constant | 2.57 | 1.14 | 2.573 | 0.01* |

TABLE 9: Multiple Linear Regression of Chronic Periodontitis group with CAL as the dependent factor during Post Rx Period

| Multiple Linear Regression - Regression Statistics | | | | |
|---|---|---|---|---|
| R | $R^2$ | Adj. $R^2$ | F | P-Value |
| 0.67 | 0.46 | 0.32 | 4.109 | 0.004* |

| Variable | β-Coeffic | S.E. | T | P-value |
|---|---|---|---|---|
| PI | 0.35 | 0.14 | 3.632 | 0.003* |
| BI | 0.43 | 0.12 | 3.587 | 0.003* |
| GI | 0.37 | 0.15 | 2.635 | 0.01* |
| PD | 0.52 | 0.21 | 2.815 | 0.008* |
| IL-1B | 0.03 | 0.01 | 2.942 | 0.007* |
| NLRP3 | 0.87 | 0.55 | 2.837 | 0.008* |
| Constant | 2.38 | 1.01 | 2.533 | 0.01* |

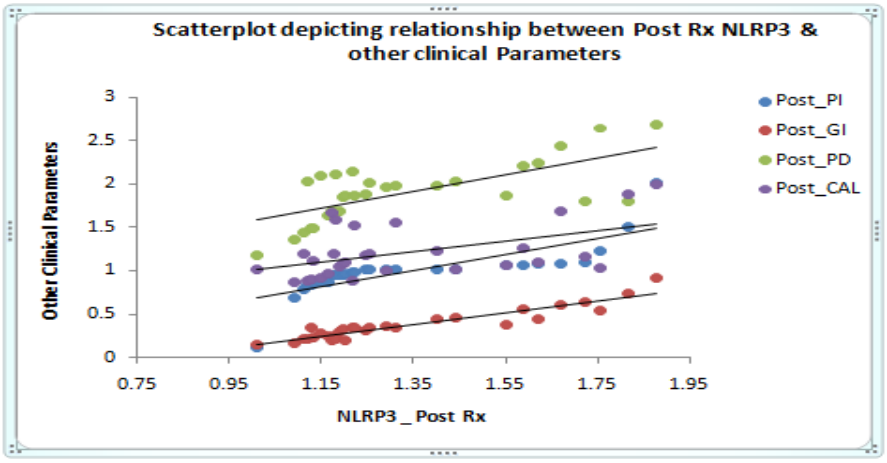


**Figure 1:** Pearson's Correlation to Assess the Relationship of PI, GI, PD, CAL with CONC. of NLRP3 Inflammasome in GCF in Group 2

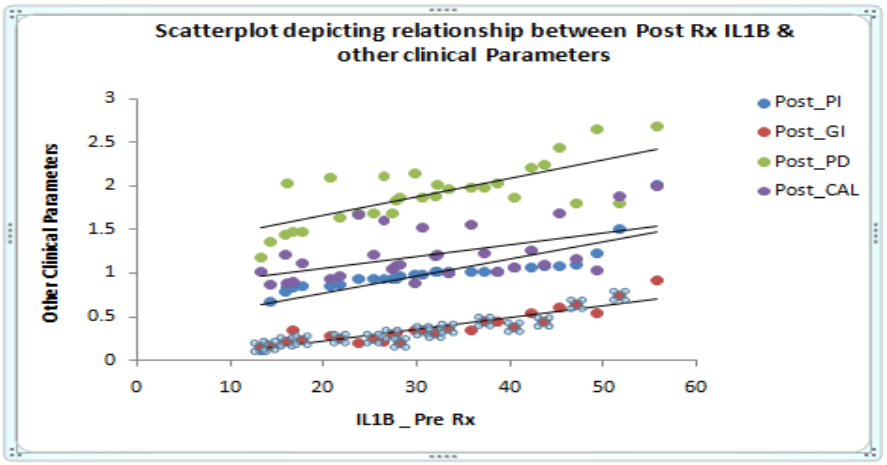


**Figure 2**: Pearson's Correlation to Assess the Relationship of PI, GI, PD, CAL with CONC. of IL-1β in GCF in Group 2

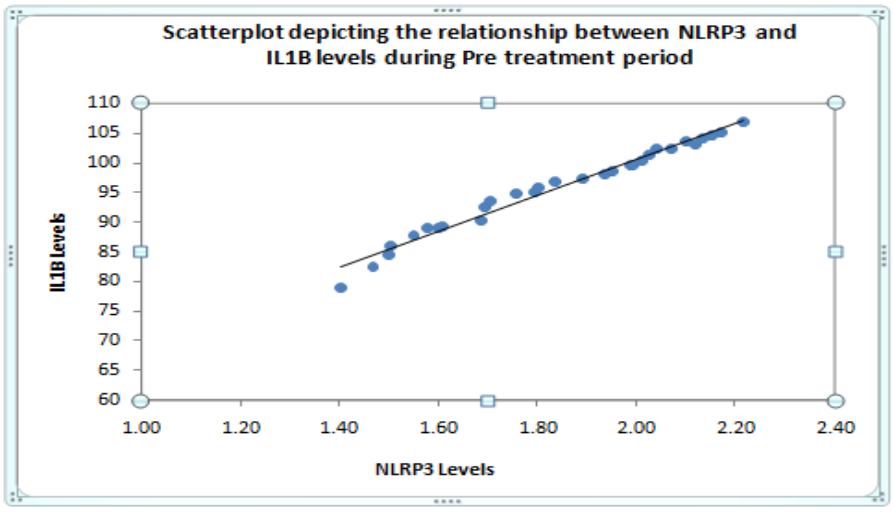


**Figure 3:** Pearson's Correlation to Assess the Relationship of CONC. of NLRP3 Inflammasome with CONC. of IL-1β in GCF in Group 1

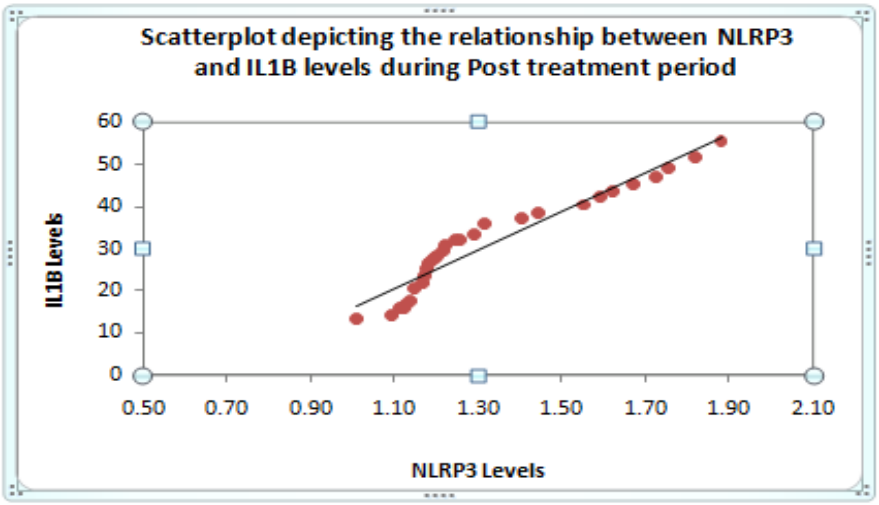


**Figure 4:** Pearson's Correlation to Assess the Relationship of CONC. of NLRP3 Inflammasome with CONC. of IL-1β in GCF in Group 2

## Conclusion

In conclusion, the present study demonstrated a significant reduction in crevicular expression of the NLRP3 inflammasome complex & IL-1β in generalized chronic periodontitis patients, 12 weeks after SRP. This cytokine-signaling pathway therefore is crucial in the regulatory control of inflammatory responses in periodontal diseases. Hence they may act as independent indicators of the amount and extent of periodontal breakdown in CP and could potentially be used for the prevention and therapy of these inflammatory diseases.

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