

# The Varied Proportion of *Filifactor alocis* in Periodontal Health and Disease in the South Indian Subpopulation

## Abstract

**Background and Aim:** The periodontal microbiome being complex, this study was aimed to detect and quantify the prevalence of *Filifactor alocis* in various stages of periodontitis and to evaluate its prospect as a diagnostic marker for periodontal disease. **Settings and Design:** Sixty subjects were selected (20 healthy controls, 20 with chronic periodontitis, and 20 with aggressive periodontitis) for the study. **Materials and Methods:** Clinical parameters probing depth and the level of clinical attachment was recorded, subgingival plaque samples were collected. The *F. alocis* 16srDNA was cloned, sequenced, and used as the standard for real-time quantification of bacterial load using SYBR green chemistry. **Statistical Analysis:** Clinical, microbiological, and quantitative polymerase chain reaction (PCR) data were analyzed using ANOVA and Pearson's coefficient correlation. **Results:** (a) Real-time PCR analysis showed the highest average *F. alocis* count in chronic periodontitis subjects (32,409.85), which was followed by count in healthy controls (3046.15) and the least count in aggressive periodontitis subjects (939.84). The bacterial count was statistically significant at  $P = 0.005$ . (b) An intra-group comparison reveals that there was a statistically significant increase in the bacterial count with age and mean probing pocket depth at  $P = 0.0005$ . **Conclusion:** *F. alocis* population in aggressive periodontitis was lower compared to chronic periodontitis and healthy controls. The *F. alocis* population surge in healthy controls may be due to geographical variations and the ethnicity of the subjects. A higher population of *F. alocis* in chronic periodontitis proves its high pathogenic potential to invade the host tissues to aid in further periodontal destruction.

**Keywords:** Chronic periodontitis, *Filifactor alocis*, microbial load, real-time quantitative polymerase chain reaction

## Introduction

Periodontitis is an intricate interplay of bacteria-induced infection and host counteraction, modified by systemic factors that prompt the obliteration of the encompassing periodontal structures.<sup>[1]</sup> The three keystone pathogens *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* initiate the disease.<sup>[2-5]</sup> *P. gingivalis* is the most extensively studied organism, but *P. gingivalis* alone does not suffice to cause infection.<sup>[6]</sup> Pathobiont is a critical factor in infection and disease establishment; it exist in the normal oral microbiota and act opportunistically during inflammation.<sup>[7]</sup> Pathobionts ameliorate the microenvironment for other periodontal pathogens and aid in host immunity manipulation.<sup>[7]</sup> *F. alocis*,

a periodontal pathogen identified through novel culture-independent techniques such as polymerase chain reaction (PCR), Next-Gen Sequencing, DNA hybridization is emerging as a critical pathobiont due to its increased presence in periodontally diseased patients.<sup>[7]</sup> *F. alocis* is obligately anaerobic, fastidious, Gram-positive asaccharolytic bacilli that are slow-growing and difficult to culture.<sup>[8]</sup>

*F. alocis* is found along with *P. gingivalis* qualifying as a microbial diagnostic marker.<sup>[8]</sup>

Technological advances such as immunoassays, biochemical tests, and nucleic acid hybridization have been used for microbial examination. Since bacterial culture is a prerequisite, the above techniques are mostly laborious and time-consuming. Advanced molecular biology techniques have largely evolved

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over the past few years to engage in culture-independent methods; PCR is a robust, rapid, and sensitive technique for the identification of periodontal pathogens. The 16S RNA (16SrDNA) gene of the small ribosomal subunit is often used as a target for PCR for its highly conserved regions through evolution and species-specific variable sequences. The conserved sequences can offer PCR primers for amplification of 16SrDNA from all the bacterial species and species-specific regions for the identification of unique bacterial species.<sup>[9]</sup>

*F. alocis* is emerging as a pathobiont, gaining more relevance in the world of periodontal microbiology. There is very little done to report *F. alocis* prevalence in periodontitis in the Indian ethnic population. This study aims to detect the presence and estimate the absolute count of *F. alocis* in periodontal health and disease in the South Indian ethnic population.

## Materials and Methods

### Study samples

Sixty systemically healthy individuals visiting the Department of Periodontics underwent a comprehensive periodontal examination that included probing depth (PD) and level of clinical attachment (CAL). The subjects were assigned into three groups: periodontally healthy (Group A), aggressive periodontitis (Group B), and chronic periodontitis (Group C) [AAP 1999] based on the following inclusion criteria: group A-Clinically healthy gingiva without bleeding on probing, CAL and PD  $\leq 3$  mm [Figure 1a], Group B-aggressive periodontitis, at least four sites showing  $\geq 3$  mm CAL and PD  $\geq 5$  mm including rapid attachment loss and bone destruction, radiographically visible vertical or arc-shaped bone loss, particularly in central incisors and first molars, amount of destruction does not commensurate with local factors [Figure 1c] and Group C-Chronic periodontitis, at least four sites showing  $\geq 3$  mm CAL and probing pocket depth (PPD)  $\geq 5$  mm, amount of destruction



**Figure 1:** Representation of the various clinical specimens involved in the study. (a) Healthy periodontium, (b) chronic periodontitis, (c) aggressive periodontitis, and (d) sample collection using paper points

consistent with local factors, radiographically visible bone loss in the horizontal direction, frequent detection of subgingival plaque and calculus [Figure 1b].<sup>[10]</sup> The patients who had any systemic diseases like diabetes mellitus, HSV or HIV infection, patients with a history of either smoking or chewing tobacco, and patients who underwent periodontal treatment or antimicrobial therapy in the duration of the past 6 months were excluded from the study.

### Sample collection

The plaque samples were collected the next day following clinical examination to avoid contamination of the samples by bleeding induced by probing. The samples were collected from four deep pockets using sterile paper points of size 35, pooled and transferred immediately to Eppendorf tubes containing 1 mL of Tris-EDTA buffer [Figure 1d].<sup>[11]</sup> This ensures high sensitivity and accurate representation of the subject. The samples were freeze stored at  $-80^{\circ}\text{C}$  until the bacterial count.

### 16srDNA cloning and sequencing

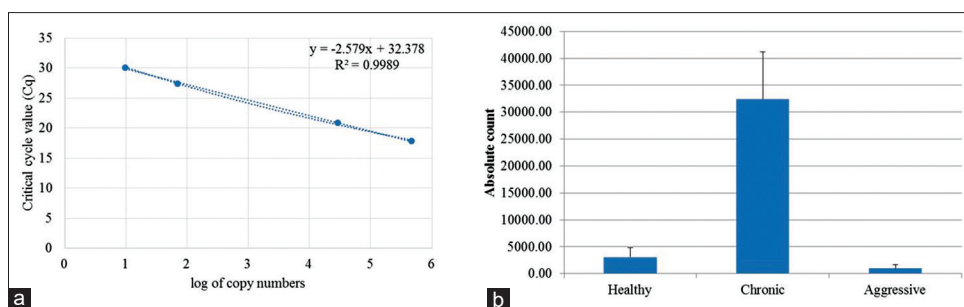
From the total sample collected, 1  $\mu\text{l}$  of the sample was used to amplify 594 bp of 16srDNA region of *F. alocis* by PCR using the primer pair FP-CTAATACCGCATAACGTCCTAAG/RP-CTACTAAGCAATCAAGTTGCC. The PCR conditions used were  $95^{\circ}\text{C}$  for 3 min (initial denaturation) followed by 35 cycles of  $95^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 30 s and a final extension at  $72^{\circ}\text{C}$  for 3 min. The PCR product was gel purified, cloned into the pGEM-T easy vector, transformed into Top10 cells and confirmed by DNA sequencing. The plasmid was used as a standard for the absolute quantification of *F. alocis*.

### Absolute quantification by quantitative polymerase chain reaction

A 20  $\mu\text{l}$  reaction was set using 2.5 pm each of primers, with known concentrations of standard plasmid DNA. Absolute quantification was performed using the following program:  $95^{\circ}\text{C}$ , 7 min, 35 cycles of  $95^{\circ}\text{C}$  for 20 s,  $55^{\circ}\text{C}$  for 20 s, and  $72^{\circ}\text{C}$  for 20 s, melt analysis consisting of  $95^{\circ}\text{C}$  for 1 min,  $-65^{\circ}\text{C}$  for 1 min,  $-95^{\circ}\text{C}$  continuous acquisition. Fluorescence data was captured during the extension stage of each cycle. The plasmid containing the 16srDNA fragment was used as a standard [Figure 2a]. A standard graph was computed using log of the copy number of the plasmid against Cq value.

### Statistical analysis

The data were analyzed with IBM. SPSS statistics software 23.0 Version (SPSS statistics software produced by SPSS Inc, Chicago, Illinois USA). Descriptive statistics were performed using mean and standard deviation, while



**Figure 2: Absolute quantification of *Filifactor alocis* in healthy, chronic and aggressive subjects of periodontitis. The 16srDNA cloned plasmid was used as a standard to quantify *Filifactor alocis* in the subjects (a). The average absolute count of the bacteria in the various subjects were estimated by pooling of paper points of individual subjects and absolute count quantified in triplicates using quantitative polymerase chain reaction (b)**

multivariate analysis was computed using one-way ANOVA with Tukey's Post-Hoc test at  $P = 0.05$ . To assess the relationship between the variables, Pearson's Correlation was used.

## Results

### PPD and CAL correspond to the severity of periodontitis

The mean age of Group A, Group B, and Group C was  $36.85 \pm 8.55$ ,  $26.20 \pm 3.32$ , and  $43.05 \pm 9.29$ , respectively [Supplementary Table 1]; Group B subjects were younger than Group C and Group A subjects. In addition, there was no significant difference in gender-wise distribution among the three groups. Periodontal examination revealed that PPD and CAL were high in chronic subjects, but was maximum in aggressive subjects. The mean PPD for Group A, Group B, and Group C was  $2.45 \pm 0.65$ ,  $6.75 \pm 0.75$ , and  $5.71 \pm 0.98$ , respectively [Supplementary Table 1]. The mean CAL for Group A, Group B, and Group C was  $0$ ,  $7.28 \pm 1.47$  and  $6.23 \pm 1.23$ , respectively [Supplementary Table 1]. The mean PPD and mean CAL were statistically significant at  $P \leq 0.05$  ( $0.0005$ ) [Supplementary Table 2].

### Cloning and absolute quantification of *Filifactor alocis*

The 16srDNA of *F. alocis* was PCR amplified, cloned, and sequence confirmed (MT573543) for use as a standard to quantify the pathogen in healthy, aggressive, and chronic patient groups. The standard curve was prepared with varying copy numbers of the 16srDNA containing plasmid ( $r^2 = 0.9989$ ), and the bacteria were quantified in all three groups [Figure 2a].

### *Filifactor alocis* colonization and growth depends on the periodontal microbiome

Absolute count of *F. alocis* was determined using real-time quantitative PCR (qPCR). The average bacterial count in Group A (healthy) was 3046.15; 40% of the samples showed more than the average bacterial count, whereas the average bacterial count in Group B (aggressive) was only 939.84; 40% of the samples showed more than the average. The mean bacterial count in Group C (chronic) was 32,409.85, with 15% of the samples having an absolute count more than the group's average count [Figure 2b].

### Inter- and intra-group comparison for clinical parameters and absolute count

Analysis of variance showed a significant difference between age, PPD, CAL, and the count of *F. alocis* in healthy, aggressive, and chronic patients [Supplementary Table 1]. The correlation within the groups for the absolute bacterial count with age, mean PPD, and mean CAL was studied using intra-group Pearson's coefficient relation [Supplementary Tables 2-5 and Figure 3]. An intra-group comparison across the three groups for the bacterial count showed a significant increase with age, mean PPD, and mean CAL increased in healthy, aggressive, and chronic periodontitis groups ( $P \leq 0.01$ ).

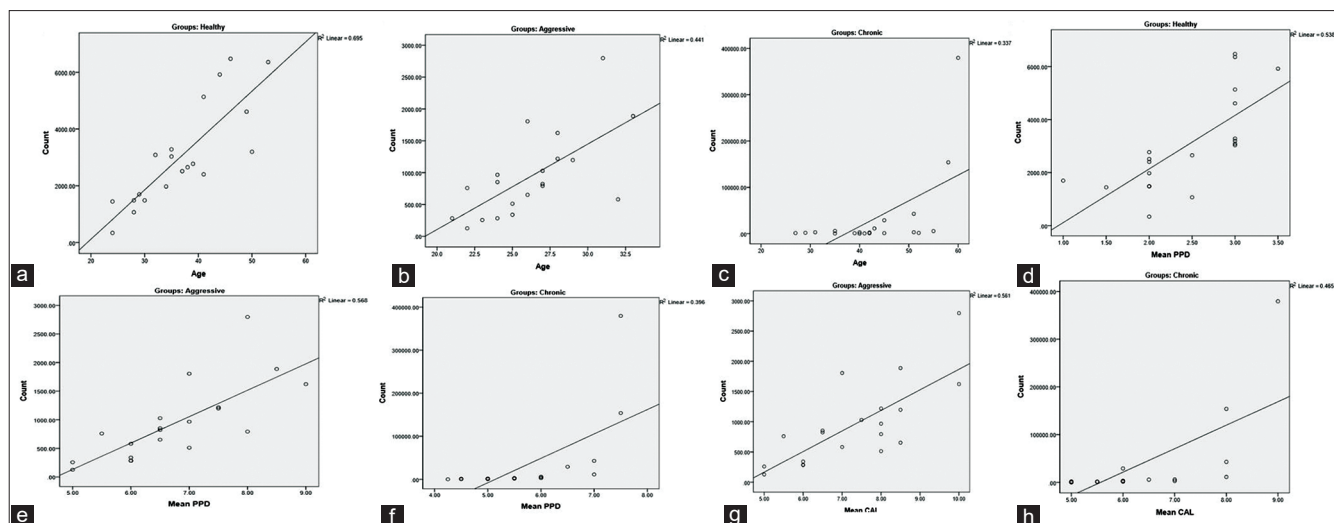
## Discussion

Periodontitis is characterized by chronic inflammation of the surrounding periodontal structures leading to alveolar bone loss and destruction of gingival and periodontal ligament attachments to the teeth.<sup>[12-14]</sup>

Advances in technology have redefined the oral microbiome into a synergistic and dysbiotic microbial community rather than by select "periopathogens," such as the "redcomplex."<sup>[5]</sup> Certain newer organisms, such as *F. alocis*, TM7, *Selenomonas*, *Desulfobulbus*, and *Synergistes*, have been implicated in periodontal pathogenesis. Culturing *F. alocis*, the third-most prevalent pathogen in aggressive periodontitis, the second most prevalent in chronic periodontitis is difficult owing to its fastidious and anaerobic nature, prevalent at mild to moderate infections in healthy controls.<sup>[15-17]</sup>

Here, we have used qPCR to quantify *F. alocis* in clinically categorized healthy, aggressive and chronic periodontal subjects; the pathogen was detected in all the samples analyzed. The prevalence of *F. alocis* is high in periodontitis compared to gingivitis and healthy; more common in sites with periodontitis than in healthy sites.<sup>[18,19]</sup> We observed the highest average bacterial count in the chronic subjects. The prevalence of *F. alocis* vary from 14%,<sup>[17]</sup> 20%,<sup>[16]</sup> 30%,<sup>[20]</sup> 44%<sup>[21]</sup> and up to 60%<sup>[15]</sup> in chronic periodontitis. In some other parts of the world, it varies from 30% in Sweden,<sup>[18]</sup> 66.7% in Germany<sup>[22]</sup> to





**Figure 3:** The correlation between age and bacterial count in Group A, Group B, and Group C shows a linear increase in the count as age increases with a positive linear association of 0.695 (a), 0.441 (b), and 0.337 (c) respectively. The correlation between mean probing pocket depth and bacterial count in Group A, Group B, and Group C shows a linear increase in the count as mean probing pocket depth increases with a positive linear association of 0.538 (d), 0.568 (e), and 0.396 (f). The correlation between mean clinical attachment and bacterial count in Group B and Group C shows a linear increase in the count as mean clinical attachment increases with a positive linear association of 0.581 (g), and 0.465 (h) respectively

83% and 36%, respectively, in deep and shallow sites of periodontal region in Korea.<sup>[23]</sup>

Periodontal therapy reduced *F. alocis* occurrence in patients with chronic periodontitis conditions in the Thai population;<sup>[24]</sup> however, in another study, non-surgical periodontal treatment failed to reduce *F. alocis*.<sup>[25]</sup> Therefore, we believe that the microbiome is complex and might be region or population specific with more factors implicated in the constitution of the microbiome. Furthermore, the prevalence and virulence of one bacteria influence other members of the microbiota. *F. alocis* and *P. gingivalis* co-exist together and have a symbiotic relationship; *P. gingivalis* is more dominant in chronic periodontitis, it can enhance the multiplication and virulence potential of *F. alocis*. Coexistence and resistance to oxidative stress might be the critical cause for the increased incidence of *F. alocis* in chronic periodontitis.<sup>[26]</sup>

The *F. alocis* prevail at low to moderate levels in healthy controls.<sup>[16,17,22,23,27,28]</sup> In our study, about 40% of the healthy controls showed more than the average bacterial count of 3046. Although the incidence of *F. alocis* varied among healthy controls, a maximum of 29% has been detected in healthy controls.<sup>[15]</sup> The variation in the incidence of *F. alocis* may be due to strain variability and difference in PD; less virulent strain of *F. alocis* ATCC 35896 was shown to be less invasive than the isolate D-62D.<sup>[26]</sup> Since only PCR was used to detect *F. alocis*, the magnitude of prevalence was unknown, our report is the first to report real-time detection and quantification of *F. alocis* in various subjects.

In our study, the average estimate of *F. alocis* in the aggressive subjects was low (939.84), the tissue invasiveness and lack of subgingival plaque might be a compelling factor for the lower incidence. Furthermore,

*F. alocis* prevail in consortium with *Aggregatibacter actinomycetemcomitans* and *Streptococcus parasanguinis* before the bone loss in localized aggressive periodontitis patients.<sup>[29]</sup> The incidence and prevalence of *F. alocis* are complicated by the age of the patient, personal habits, population type, and country of origin.<sup>[30,31]</sup> Biologically, the population of *F. alocis* is dependent on the periodontal microbiome; the microbiota varies significantly between chronic and aggressive patients. In an *in vitro* community model study, *Streptococcus gordonii*, Gram-negative bacteria of aggressive periodontitis microbiota was antagonistic to *F. alocis*, and *A. actinomycetemcomitans* interacted with *F. alocis* in a strain-specific manner.<sup>[32]</sup>

The present study observed a statistically significant association between age, clinical periodontal parameters such as PPD and CAL, and the presence of the organism. Intra-group correlation of the age with bacterial count was substantial in all the three groups ( $P \leq 0.01$ ). Similarly, intra-group correlation reveals that there was a positive correlation between PPD and CAL with the bacterial population in all three groups ( $P \leq 0.01$ ). Geographical locations, ethnicity, food habits, smoking habits influence the distribution, prevalence, and virulence of the oral microbiome; variations prevail in the subgingival microbiota worldwide.<sup>[33]</sup>

## Conclusion

*F. alocis* is a novel microorganism gaining more relevance in periodontal pathogenesis. It has been implicated to be the second- and third-most prevalent organism in chronic and aggressive periodontitis, respectively. The highest bacterial count was observed in chronic periodontitis patients (32,409.85), followed by healthy controls (3046.15) and least in aggressive periodontitis patients (939.84). There

was a statistically significant difference between the bacterial count of three groups with  $P$  value at 0.005 ( $P \leq 0.05$ ). On intra-group comparison, there was a statistically significant increase in the bacterial count as the age and mean PPD increases with  $P$  value at 0.0005 ( $P \leq 0.05$ ). The higher prevalence of *F. alocis* in chronic periodontitis patients in the Indian population indicates that it can be a candidate marker for chronic periodontitis; however, to qualify it as a marker, validation with more longitudinal studies in a larger study group is warranted.

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### Conflicts of interest

There are no conflicts of interest.

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**Supplementary Table 1: Summarises the mean, standard deviation, maximum and the minimum values of count, age, PPD and clinical attachment level in each group**

	Mean	SD	SE	Minimum	Maximum
<b>Count</b>					
Aggressive	939.84	670.03	149.82	127.37	2796.95
Chronic	32,409.85	88,735.34	19,841.83	96.23	379,451.37
Healthy	3046.15	1784.16	398.95	337.07	6473.92
Total	12,131.95	52408.83	6765.95	96.23	379,451.37
<b>Age</b>					
Aggressive	26.20	3.32	0.74	21.00	33.00
Chronic	43.05	9.29	2.08	27.00	60.00
Healthy	36.85	8.55	1.91	24.00	53.00
Total	35.37	10.20	1.32	21.00	60.00
<b>Mean PPD</b>					
Aggressive	6.75	1.09	0.24	5.00	9.00
Chronic	5.71	0.98	0.22	4.25	7.50
Healthy	2.45	0.65	0.14	1.00	3.50
Total	4.97	2.06	0.27	1.00	9.00
<b>Mean CAL</b>					
Aggressive	7.28	1.47	0.33	5.00	10.00
Chronic	6.23	1.23	0.28	5.00	9.00
Healthy	0.00	0.00	0.00	0.00	0.00
Total	4.50	3.42	0.44	0.00	10.00

*n* denotes the number of subjects in each group (20). CAL: Clinical attachment level; PPD: Probing pocket depth; SD: Standard deviation; SE: Standard error

**Supplementary Table 2: Intergroup and intragroup comparison of count, age, mean probing pocket depth, and mean clinical attachment level using analysis of variance with *P* value at a significance of 0.05**

	Sum of squares	df	Mean square	<i>F</i>	Significant
<b>Count</b>					
Between groups	12,380,164,074	2	6,190,082,037	2.357	0.104
Within groups	149,674,263,552	57	2,625,864,273		
Total	162,054,427,626	59			
<b>Age</b>					
Between groups	2905	2	1453	25.566	0.0005
Within groups	3238.700	57	56.819		
Total	6143.933	59			
<b>Mean PPD</b>					
Between groups	201.402	2	100.701	116.881	0.0005
Within groups	49.109	57	0.862		
Total	250.511	59			
<b>Mean CAL</b>					
Between groups	618.525	2	309.263	251.918	0.0005
Within groups	69.975	57	1.228		
Total	688.500	59			

*P* Highly significant at  $P \leq 0.01$

*P* Not significant at  $P > 0.05$

Intergroup comparison of the bacterial count shows that it is statistically not significant. A large *F* value indicates a varied proportion of mean squares among the three groups. ANOVA: Analysis of variance; PPD: Probing pocket depth; CAL: Clinical attachment level

**Supplementary Table 3: Intragroup correlation of count with age and mean probing pocket depth in Group A by Pearson coefficient correlation with *P* value significant at  $\leq 0.01$**

Correlations <sup>A</sup>		
Count	Age	Mean PPD
Pearson correlation	0.834**	0.733**
Significant (two-tailed)	0.0005	0.0005
<i>n</i>	20	20
<i>P</i>	**Highly significant at $P \leq 0.01$	

\*\*Correlation is significant at the 0.01 level (two-tailed).

Group A: Healthy; PPD: Probing pocket depth

**Supplementary Table 4: Intragroup correlation of count with age, mean probing pocket depth, and mean clinical attachment level in Group B by Pearson coefficient correlation with *P* value significant at  $\leq 0.01$**

Correlations <sup>B</sup>			
Count	Age	Mean PPD	Mean CAL
Pearson correlation	0.664**	0.754**	0.749**
Significant (two-tailed)	0.001	0.0005	0.0005
<i>n</i>	20	20	20
<i>P</i>	**Highly significant at $P \leq 0.01$		

\*\*Correlation is significant at the 0.01 level (two-tailed).

Group B: Aggressive; PPD: Probing pocket depth; CAL: Clinical attachment level

**Supplementary Table 5: Intragroup correlation of count with age, mean probing pocket depth, and mean clinical attachment level in Group C by Pearson coefficient correlation with *P* value significant at  $\leq 0.01$**

Correlations <sup>C</sup>			
Count	Age	Mean PPD	Mean CAL
Pearson correlation	0.580**	0.629**	0.682**
Significant (two-tailed)	0.007	0.003	0.001
<i>n</i>	20	20	20
<i>P</i>	**Highly significant at $P \leq 0.01$		

\*\*Correlation is significant at the 0.01 level (two-tailed). Group C: Chronic; PPD: Probing pocket depth; CAL: Clinical attachment level