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Protease Inhibitor Levels in Periodontal Health and Disease

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Abstract

Background and Objectives—Our previous study showed protease inhibitors were attenuated by periopathogen *Porphyromonas gingivalis* in cultured gingival epithelial cells. We hypothesize fewer protease inhibitors would be present in more advanced periodontal sites where the level of *P. gingivalis* may be high. The goal of this study was to investigate the relationship between the protease inhibitor (SLPI, ELAFIN, SCCA) levels in gingival crevicular fluid (GCF) and the number of *P. gingivalis* in subgingival plaque.

Materials and Methods—Plaque samples from subjects without (n=18) and with moderate to advanced periodontitis (n=41) were used to quantify *P. gingivalis* using real-time PCR. Protease inhibitor levels in the GCF of all the subjects were determined by ELISA.

Results—*P. gingivalis* was detected in 68.3% of subjects with periodontitis, while 16.7% of patients without periodontitis had detectable level of *P. gingivalis*. Subjects with periodontitis and *P. gingivalis* in their plaque exhibited lower SLPI and ELAFIN levels (p<0.001) compare to control subjects without periodontitis. SLPI was also reduced (p<0.05) in GCF of periodontal patients without detectable level of *P. gingivalis*. Periodontal patients with high vs. low levels of *P. gingivalis* exhibited reciprocal mean levels of SLPI and ELAFIN concentrations.

Conclusion—The reduced concentrations of SLPI and ELAFIN may contribute to the loss of host protective capacity and increase susceptibility to breakdown from chronic infection. The work of this investigation may aid in finding diagnostic and prognostic markers in periodontal health and disease and may further help in finding pharmacological targets directed against periodontal inflammation.

Keywords

protease inhibitors; periodontitis; *Porphyromonas gingivalis*; gingival crevicular fluid

INTRODUCTION

Periodontitis is a chronic inflammatory disease characterized by apical migration of epithelial attachment accompanied by loss of connective tissue and alveolar bone. It is classified based on different clinical measures including the presence and severity of periodontal pockets, clinical attachment loss and extent of alveolar bone loss. Bacteria associated with the initiation of periodontal disease are predominantly Gram-negative anaerobic bacteria and spirochetes, notably *Porphyromonas gingivalis*, *Tannerella forsythia*

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and *Treponema denticola* (1) which belong to the red complex within subgingival biofilms (2). In particular, *P. gingivalis* plays a significant role in the progression of chronic periodontitis (3). It has a wide repertoire of virulence factors, including fimbriae (4), hemagglutinins (3) and proteases (gingipains) (5). The list of host proteins which are degraded by gingipains *in vitro* include extracellular matrix components such as laminin, fibronectin, and collagen type III, IV, and V (6). These proteases have been reported to account for at least 85% of the general proteolytic activity displayed by *P. gingivalis* (7). According to Imamura and Potempa (8) the protease gingipain R is able to promote plasma clotting by activating coagulation factor IX, X and prothrombin. Once the proteolytic cascade pathway is activated, thrombin not only plays a role in hemostasis by converting fibrinogen to fibrin, but also enhances vascular permeability (9) and induces leukocyte chemotaxis (10). At this point the content in gingival crevicular fluid increases significantly, and polymorphonuclear neutrophils (PMN) accumulate in actively resorbing periodontitis sites. Neutrophils have been implicated as playing a destructive role in the periodontal tissue breakdown process due to high levels of lysosomal enzymes, generation of superoxides and reactive oxygen derivatives (11). One of these enzymes, namely the neutrophil elastase, degrades important molecules of the periodontium during an inflammatory process. Its presence in gingival crevicular fluid (GCF) is associated with periodontal inflammation and may be an indicator for the severity of inflammation at individual sites (12).

Concomitant with the increase in proinflammatory enzymes in GCF, epithelial cells start secreting protease inhibitors to protect the tissue from excessive damage. These protease inhibitors include secretory leukocyte protease inhibitor (SLPI), elastase-specific inhibitor (ELAFIN) and squamous cell carcinoma antigen (SCCA). SLPI regulates the activity of neutrophil elastase and therefore can protect local tissues from destruction during an inflammatory response (13). It is found in a variety of mucous secretions, including in GCF from sites of periodontal disease (14). ELAFIN, also known as skin-derived antileukoprotease (SKALP), is thought to play a regulatory role in inflammation and is expressed in human epithelia of the tongue, palate, lingual tonsils, pharynx and gingiva (15). ELAFIN is directed against PMN-derived elastase and proteinase 3, and thus is involved in the maintenance of epithelial integrity by protecting tissues against excessive proteolysis (16). SCCA belongs to a group of serine protease inhibitors (serpins) and may serve as a marker for certain inflammatory conditions. Co-expression of SCCA1 and 2 in the squamous epithelium of mucous membranes and other areas lined with squamous epithelium plays an important role in the coordinated regulation of certain serine and cysteine proteases associated with both normal and transformed cells (17). SCCA1 inhibits papain-like cysteine proteinases, whereas SCCA2 inhibits chymotrypsin-like serine proteinases (18, 19).

Our previous *in vitro* study found that SLPI, ELAFIN, SCCA1 and 2 were induced in gingival epithelial cells in the presence of the common oral bacterium *Fusobacterium nucleatum* (20). We have also found that the secretion of SLPI and ELAFIN was significantly reduced in response to *P. gingivalis*, and that *P. gingivalis* supernatants digested recombinant SLPI, ELAFIN, SCCA1 and 2, suggesting degradation of protease inhibitors by *P. gingivalis* may result in decreased host protective capacity and higher susceptibility to *P. gingivalis* infection (21). This is in agreement with a study by another group that reported *P. gingivalis* gingipains, in particular RgpB, digested ELAFIN *in vitro* (22). Thus, we hypothesize that *P. gingivalis* in subgingival plaque of patients with chronic periodontitis decreases the amount of protease inhibitors in gingival crevicular fluid. Our aim in this investigation is to follow up the recent *in vitro* findings with *in vivo* studies to compare the level of protease inhibitors with the presence of *P. gingivalis* in periodontitis patients.

MATERIALS AND METHODS

Study population

Human GCF and dental plaque samples were obtained from subjects without periodontitis and from patients with generalized chronic periodontitis in accordance with the approval by University of Washington Institutional Review Board for human subjects research. Periodontally diseased subjects had at least 2 teeth with probing depth greater or equal to 5 mm, bleeding upon probing and attachment loss of greater or equal to 4 mm. Subjects without periodontitis had probing depth less than 4 mm without bleeding upon probing. Subjects were excluded from the study if they suffered from diabetes mellitus, were younger than 30 years of age, had their most recent periodontal maintenance treatment within the last 3 months, or they were treated with antibiotics for any medical or dental conditions within the last few months. Samples were collected from two teeth per subject, and the samples from periodontitis group were later sub-divided into *P. gingivalis*-positive or *P. gingivalis*-negative groups according to the presence or absence of detectable level of *P. gingivalis* in the plaque sample. Documented clinical data included probing depth (PD), attachment level (AL: the amount of attachment loss), plaque and gingival index (PI and GI) (23, 24), bleeding on probing (BOP), and suppuration. PD was defined as the distance from the coronal-most margin of the free gingival margin to the most apical penetration of the periodontal probe (PCP-UNC 15, Hu-Friedy, Chicago, IL) and was noted to the nearest mm. AL was defined as the distance from the cemento-enamel junction to the most apical penetration of the Marquis periodontal probe, which is marked every 3 mm between 3–12 mm. The presence of supragingival plaque was classified according to the Plaque Index defined by Loe (23). The severity of inflammation was classified according to the Gingival Index by Silness and Loe (24). BOP and suppuration within 20 s were recorded dichotomously.

Harvest of GCF and dental plaque

For the harvest of GCF, two paper strips per site were used. First, supragingival plaque was removed with a curette. Samples from subjects with periodontitis were selected from the deepest sites, while those from healthy patients were mainly from premolars and canines. Because not all patients were fully dentate, we were unable to select one particular tooth for everyone. However, both mesiobuccal and distobuccal sides were considered for sampling depending on tooth position and access. Each paper strip was placed into the gingival sulcus for 30 s according to the intracrevicular, superficial method by Loe and Holm-Pederson (25). The paper strips were then stored in small tubes and frozen at -80°C . Dental plaque was harvested using one paper tip ISO 50 (SybronEndo, Glendora, CA) per site. Each paper tip was held into the gingival sulcus/periodontal pocket for 10 s, removed and put in 500 μl sterile water. The samples were centrifuged at 1000 rpm, the paper tip was removed and the liquid was centrifuged at 14,000 rpm for 10 min. The plaque pellicle obtained from centrifugation was kept at -80°C until DNA was extracted using Tris-EDTA buffer (26).

Quantitative real-time PCR for quantitation of bacteria in dental plaque

The amount of overall bacterial load and of *P. gingivalis* in plaque samples was determined using quantitative real-time PCR (QRT-PCR). The oligonucleotide primer sequences for *P. gingivalis* and the TaqMan probe were previously described by Morillo (26). The universal primer sequences for bacterial load and the probe were described by Nadkarni (27). QRT-PCR was conducted with the TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA). The concentration used was 20 μl containing 13.2 μl of Master Mix and 6.8 μl extracted plaque DNA in sterile water. The amplification conditions were initial denaturation at 94°C for 10 minutes, followed by denaturation at 94°C for 60 s, annealing at 60°C for 60 s and elongation at 60°C for 45 s.

ELISA to determine the levels of protease inhibitors in GCF

Each GCF sample was gently thawed on ice prior to the elution process. For the analysis of SLPI and ELAFIN, we obtained total of 400 μ l elution by 2 wash procedures, each with a 20-min rotation at 4°C in the elution buffer (200 μ l of 0.1% BSA PBS). For the analysis of SCCA, we obtained a total of 250 μ l elution by 2 wash procedures, each with 125 μ l of 0.1% BSA PBS. The difference in the total elution volume was necessary because the optimal concentration range for each ELISA kit differed based on our preliminary tests. All samples were centrifuged at 1000 rpm for 5 min at 4°C between wash procedures. The following ELISA kits were used for analyses of protease inhibitors: Human SLPI Quantikine Immunoassay (R & D Systems, Minneapolis, MN); Human Trappin-2/ELAFIN DuoSet ELISA Development kit (R & D Systems); SCCA1 ELISA kit (Accurate Chemical & Scientific Corp., Westbury, NY).

Classification of samples

Samples were classified as either without periodontitis or with periodontitis according to their clinical parameters described in the inclusion criteria. After the quantity of *P. gingivalis* has been determined with QRT-PCR, the samples were further divided into a diseased group with or without the presence of *P. gingivalis* based on the correlation between the standard curve for predicted bacterial number and threshold cycle number (C_T) generated from QRT-PCR (26). The division into the three groups not only allowed the investigation of different protease inhibitor levels between healthy and *P. gingivalis*-infected sites, but also allowed the comparison of protease inhibitor levels between periodontally involved sites with and without *P. gingivalis*.

Data analysis

All experiments were performed in duplicate. Values are shown as the Mean $\pm$ SEM (standard error of the mean). Within groups defined by periodontal status and *P. gingivalis*, protease inhibitor levels were non-normally distributed and therefore were analyzed using Mann-Whitney U tests. These tests were pre-planned to compare the healthy control subjects with each of the two groups with periodontitis defined by the presence vs. absence of *P. gingivalis*. Within the two groups defined in terms of low vs. high levels of *P. gingivalis*, protease inhibitor levels were normally distributed and were analyzed using Student's t-tests. Analyses were performed using PASW (v. 18, IBM, Somers, NY). Statistical significance was established at $\alpha < 0.05$, two-tailed.

RESULTS

The amounts of total bacteria and of *P. gingivalis* in plaque samples of subjects with vs. without periodontitis

The study group consisted of 14 males providing 21 sample sites and 23 females providing 38 sample sites. Among them, 18 were without periodontitis and 41 were periodontally diseased. Mean age of patients was 57.41 ± 1.34 . The clinical parameters of the group without periodontitis differed significantly ($p < 0.05$) from periodontitis group (Table 1). The mean probing depth in the group without periodontitis was 2.94 ± 0.05 mm, the mean attachment level was 1.94 ± 0.06 mm, and the mean plaque and gingival index were 0.17 ± 0.09 and 0.06 ± 0.06 , respectively. This group did not have detectable amount of *P. gingivalis* in its plaque. Among the periodontitis group, 25 had detectable level of *P. gingivalis*, while 16 did not. The mean probing depth for the diseased group without detectable *P. gingivalis* in its plaque was 6.5 ± 0.27 mm, the mean attachment level was 7.44 ± 0.49 mm, and the mean plaque and gingival index were 1.44 ± 0.24 and 0.88 ± 0.15 , respectively. For the periodontitis group with *P. gingivalis* in its plaque, the mean probing

depth for these sites was 6.4 ± 0.23 mm. The mean attachment level was 6.68 ± 0.37 mm, and the mean plaque and gingival index were 1.64 ± 0.19 and 1.56 ± 0.14 , respectively. Among the diseased groups, periodontal patients with *P. gingivalis* had higher numbers of total bacteria compared to the ones without *P. gingivalis* (Figure 1). The difference in total bacterial count was statistically significant between the group without periodontitis vs. *P. gingivalis*-positive group ($p < 0.001$) and between the two diseased groups ($p < 0.001$) (Figure 1).

ELAFIN concentration decreased in *P. gingivalis*-positive group

We examined the concentration of protease inhibitor ELAFIN in GCF samples of the three different groups utilizing commercially available ELISA kits. The ELAFIN concentration ranged from 0 to 349.33 pg/ml, the mean concentration being 75.72 ± 92.05 pg/ml. Mann-Whitney U tests indicated that the distribution of ELAFIN concentration of the diseased group with *P. gingivalis* (30.0 pg/ml) differed significantly from that of the healthy control subjects (86.21 pg/ml) ($p < 0.001$) (Figure 2). The ELAFIN concentration of the diseased group without *P. gingivalis* (44.0 pg/ml) was also decreased in comparison to the healthy control subjects and the difference in this case approached significance ($p = 0.058$) (Figure 3). These results suggest the level of the protease inhibitor ELAFIN is decreased in GCF of periodontitis subjects, and in particular, of *P. gingivalis*-infected subjects.

SLPI concentration decreased in periodontitis group

Next we examined the concentrations of protease inhibitor SLPI amongst different study groups. The overall SLPI concentration ranged from 202 pg/ml to 5,289 pg/ml with a mean concentration of 1237.01 ± 1105.17 pg/ml. Both diseased groups showed significantly reduced concentration of SLPI (755.52 pg/ml for non-*P. gingivalis*-infected group and 623.13 pg/ml for the *P. gingivalis*-infected group) in GCF compared to the group without periodontitis (1617.17 pg/ml) ($p < 0.05$ and $p < 0.001$, respectively) (Figure 3). These results suggest that the concentration of SLPI is significantly reduced in sites with periodontitis regardless of the presence or absence of *P. gingivalis*.

SCCA1 concentration showed little difference between study groups

Subsequently, we examined the concentration of the protease inhibitor SCCA1 using ELISA. The overall concentration ranged from 107.94 pg/ml to 1575.17 pg/ml with a mean concentration of 596.25 ± 451.96 pg/ml. The difference between the medians of 396.5 pg/ml for the healthy group, 479.78 pg/ml for the diseased group without *P. gingivalis* and 492.7 pg/ml for the diseased group with *P. gingivalis* was statistically not significant ($p = 0.108$) (Figure 4). The results of this experiment suggest that the concentration of the protease inhibitor SCCA1 is neither affected by the disease status nor by the presence or absence of *P. gingivalis*.

Concentrations of SLPI and ELAFIN in GCF are negatively associated with *P. gingivalis* counts in dental plaque

Since ELAFIN and SLPI levels were decreased in GCF of periodontal subjects with *P. gingivalis*, we performed analyses to determine if the concentration of these protease inhibitors differed between periodontal patients with high vs. low levels of *P. gingivalis*. Subdivision of the *P. gingivalis*-infected sites into two groups with high and low levels of *P. gingivalis* (cut-off point was C_T of 39) revealed lower mean values of protease inhibitors in the high *P. gingivalis* group vs. the low *P. gingivalis* group. For SLPI the mean concentrations were 861.76 ± 139.42 pg/ml and 1219.81 ± 234.63 pg/ml, respectively, for high and low *P. gingivalis* groups ($p < 0.05$) (Figure 5a). For ELAFIN level the mean concentrations were 16.95 ± 18.37 pg/ml in the high *P. gingivalis* level group vs. $37.56 \pm$

16.34 pg/ml in the low *P. gingivalis* level group ($p < 0.05$) (Figure 5b). These data show the amounts of *P. gingivalis* in dental plaque and the levels of protease inhibitors in GCF are negatively associated.

DISCUSSION

Our findings showed that host-derived protease inhibitors, which are released as a response to environmental and microbial stimuli, are decreased in concentration in periodontal pockets with *P. gingivalis*. Additionally, our study also showed that SLPI and ELAFIN were also reduced in periodontitis patients that were not infected with *P. gingivalis*, suggesting factors other than *P. gingivalis* may also play a role in the regulation of protease inhibitors, reflecting the complex features of the periodontal biofilm. Within periodontal biofilms, Socransky described 5 different complexes, of which some were closely related to periodontal inflammation and periodontal destruction (2). Socransky suggests that the orange complex precedes the red complex in the subgingival plaque formation and that both complexes are closely associated. Contrary to the majority of other microbial species, all species of the red complex and most of the species of the orange complex show a significant increase in count with increasing pocket depth (28). In our previous *in vitro* study, exposure to *F. nucleatum*, a member of the orange complex, induced the expression of protease inhibitors, while *P. gingivalis*, a member of the red complex, degraded these protease inhibitors (21). The dominance of *P. gingivalis* in the degradation of protease inhibitors is important to note, since during the formation of dental plaque, protease inhibitors may be induced as a host protective mechanism by the presence of bridging organisms such as *F. nucleatum*, but may be rendered ineffective once protease-secreting pathogens are established.

In an ecological community attempting to grow, such as dental plaque biofilm, virulence factors of different species take on a different significance (29). *P. gingivalis* proteases may have the most potent activity in tissue destruction (30), and as shown in our *in vitro* studies, are able to degrade protease inhibitors most effectively (21). However, less potent trypsin-like proteases of certain *Capnocytophaga* species and *T. denticola* might play a secondary role in the environment of a microbiological network. A significant correlation between the increased total bacterial load and deeper pocket depth found in our study reinforces a notion that the biofilm influences the protease inhibitor concentration. Our data showed an inverse correlation between the number of *P. gingivalis* and the amount of SLPI and ELAFIN secreted. Our data also showed the amount of these protease inhibitors was reduced in the periodontitis group without the presence of *P. gingivalis* but with clinical parameters of severe periodontitis, compared to the control group without periodontitis. Our previous report of *in vitro* studies using cultured gingival epithelial cells showed *P. gingivalis* supernatant was able to degrade ELAFIN and SLPI, and that *P. gingivalis* mutants lacking proteases had decreased capacity to degrade recombinant protease inhibitors (21). However, we acknowledge that there may be additional factors other than *P. gingivalis* proteases involved in the decreased level of protease inhibitors in GCF. These additional factors might include host-derived proteases such as cathepsins and neutrophil elastase. SLPI and elastase levels have been previously reported to be inversely correlated in periopatients (31). The same study also reported SLPI and Cathepsin B are also inversely correlated, although this correlation was statistically not significant (31). Taken together, our data suggest that other factors in addition to *P. gingivalis* gingipains are involved in the reduction of SLPI and ELAFIN, supporting the notion of the complexity, quantity and the bacterial interrelationship in subgingival biofilm.

An earlier study compared SLPI concentrations in GCF from active periodontitis patients and periodontitis patients in maintenance (5). Both study groups were *P. gingivalis*-positive,

but its quantity differed. SLPI was significantly reduced in the group with high amount of *P. gingivalis*. The authors attributed the finding to the proteolytic activity of *P. gingivalis* gingipain isoform RgpA. The overall bacterial load in these samples was not specified, but this is in agreement with our previous study utilizing *P. gingivalis* mutant strains lacking various gingipains (21). Although RgpA may contribute to the degradation of SLPI, the bacterial biofilm may play a role as well. Particularly, increased neutrophil elastase level in the high *P. gingivalis* group in the Into study could be a reflection of a higher bacterial load of the respective plaque samples.

Aggressive periodontitis is associated with neutrophil dysfunction such as adhesion deficiency, reduced chemotaxis and phagocytosis activity (32, 33). In addition, hyper-functional neutrophils in a primed status may lead to abnormal tissue injury due to abnormal signal transduction mechanisms (34). In that case, hyper-responsive neutrophils could defeat the protease inhibitors and render the host more susceptible to periodontal breakdown. Guentsch (35) as well as Giannopoulou (36) showed in their clinical studies that patients with aggressive and chronic periodontitis released significantly more elastase from granulocytes as compared to healthy control patients. Since we know that SLPI, ELAFIN and SCCA are susceptible to bacterial proteases, particularly to the different isoforms of *P. gingivalis* gingipains (21), there might also be a dose-dependent susceptibility to neutrophil elastase, their actual target. An *in vitro* study design would help in understanding the effect of neutrophil elastase on these protease inhibitors. A study has shown that ELAFIN is cleaved by its cognate enzyme neutrophil elastase at excessive concentrations in sputum from individuals with cystic fibrosis, and that ELAFIN was degraded more quickly in *Pseudomonas aeruginosa*-infected sputum than in *P. aeruginosa*-negative sputum (37). According to Joseph (38), *P. aeruginosa* is able to induce the expression of chemoattractant IL-8 and therefore increase recruitment and activation of neutrophils at inflammatory sites. Assuming the twofold interaction between serine protease inhibitors and neutrophil elastase, two contributing factors might be involved in the reduced amount of protease inhibitors in our two periodontally involved groups. On one hand, bacterial infection with its biofilm leads to the release of proinflammatory cytokines, which results in vast recruitment of potent neutrophils. On the other hand, neutrophils in a primed status are hyper-functional (34) and contribute to excessive neutrophil elastase in sites of periodontal disease. Thus the two contributors, host-derived neutrophil elastase and *P. gingivalis*-derived gingipains, might be involved in the reduction of host protease inhibitors. This reflects the various degrees of reduction of host protease inhibitors between the diseased group with and without *P. gingivalis* infection. The lower concentration of SLPI and ELAFIN found in the GCF of periodontitis patients with *P. gingivalis* infection suggests that two contributors might be responsible for this reduction.

Whereas SLPI and ELAFIN concentrations in GCF presented a similar pattern in periodontal disease, SCCA1 concentration was unaffected by the presence of bacterial biofilm and therefore not significantly different between the three groups tested. Unlike ELAFIN and SLPI whose activities tend to be inhibitory in inflammation, SCCA's activity may be more pronounced in tissue repair and cell differentiation. Kato (39) described an interaction of SCCA with cathepsin L as a function to indirectly protect against infection in the skin via inhibition of the final differentiation of the epidermis. Katagiri et al. (40) proposed a novel UV protection mechanism in human skin due to a SCCA1-mediated blockade of UV-induced keratinocyte apoptosis. SCCA was found to be up-regulated in psoriatic skin (41), in atopic dermatitis (42) and bronchial asthma (43). Interestingly, these non-infectious inflammatory diseases are associated with type I hypersensitivity immune reactions with involvement of IgE, Th2 helper cells and mediating cytokines IL-4 and IL-10. In contrast, the inflammatory cell infiltrates in periodontal disease are composed of a mixed Th1 and Th2 cell profile, with respective predominance at different phases of the disease

(44). Therefore, SCCA's role in inhibition and regulation of infectious inflammation and in host defense might not be pronounced but instead may be directed toward tissue differentiation and protection. Furthermore, SCCAs have been reported to be intracellularly localized as serine protease inhibitors, not like other protease inhibitors (45). Therefore, SCCA1 level might be integrated with multiple factors *in vivo*. However, further studies are needed to better understand the mechanisms behind the regulation of SCCAs.

In conclusion, we showed that the concentrations of serine protease inhibitors SLPI and ELAFIN are reduced in periodontitis, and *P. gingivalis* plays a role in the reduction of these protease inhibitors, although does not appear to be the sole cause. In contrast to SLPI and ELAFIN, the concentration of SCCA1 was unaffected by the periodontal pathogen and the associated host response and therefore might not be as involved in the innate immunity process in periodontal disease. The work of this investigation may aid in finding diagnostic and prognostic markers in periodontal health and disease and may further help finding pharmacological targets to support the host immune responses directed against periodontal inflammation.

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Figure 1.

The total bacterial count in plaque samples of the three groups. The top and bottom of each box indicate the 75th and 25th percentile, respectively. The band inside each box indicates the median bacterial count. The top and the bottom ends of the whiskers represent 98th and 2nd percentile, respectively. Planned comparisons of both diseased groups with the healthy group were conducted using non-parametric analysis. The amount of total bacteria in plaque is the lowest in the healthy group and increased in the diseased groups. Periodontal patients with *P. gingivalis* show higher number of total bacteria compared to the ones without *P. gingivalis*.

Figure 2.

Boxplots of ELAFIN concentration in GCF of the three groups. The top and bottom of each box indicate the 75th and 25th percentile, respectively. The band inside each box indicates the median concentration. The top and the bottom ends of the whiskers represent 98th and 2nd percentile, respectively. Significance levels indicate results of pre-planned Mann-Whitney U tests of both diseased groups with the group without periodontitis.

Figure 3. Boxplots of SLPI concentration in GCF of the three groups. Interpretation and significance levels are as per the Figure 2 legend.

Figure 4.

Boxplots of SCCA1 concentration in GCF of the three groups. Interpretation is as per the Figure 2 legend. No significant differences were found between the three groups.

Figure 5. Groups defined by high vs. low levels of *P. gingivalis* exhibited reciprocal mean levels of both SLPI (a) and ELAFIN (b) concentrations. The cut-off point for high vs. low levels of *P. gingivalis* was set at C_T of 39. P-values were obtained using Student's t-tests.

Table 1

Clinical parameter of different subject groups

Group		Age	PI	GI	Probing depth	Recession	AL
Healthy (N=18)	Mean	59.33	0.17	0.06	2.94	0.00	1.94
	SEM	1.85	0.09	0.06	0.05	0.00	0.06
Diseased w/o Pg (N = 16)	Mean	54.63	1.44**	0.88**	6.5**	1.31*	7.44**
	SEM	3.02	0.24	0.15	0.27	0.41	0.49
Diseased w/Pg (N = 25)	Mean	57.8	1.64**	1.56**	6.4**	0.8*	6.68**
	SEM	2.14	0.19	0.14	0.23	0.2	0.37
Total (N = 59)	Mean	57.41	1.14	0.92	5.37	0.69	5.44
	SEM	1.34	0.14	0.11	0.24	0.15	0.37

p < 0.001*
p < 0.05