

Periodontal pathogens affect the level of protease inhibitors in gingival crevicular fluid

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SUMMARY

In periodontitis, an effective host-response is primarily related to neutrophils loaded with serine proteases, including elastase (NE) and protease 3 (PR3), the extracellular activity of which is tightly controlled by endogenous inhibitors. *In vitro* these inhibitors are degraded by gingipains, cysteine proteases produced by *Porphyromonas gingivalis*. The purpose of this study was to determine the level of selected protease inhibitors in gingival crevicular fluid (GCF) in relation to periodontal infection. The GCF collected from 31 subjects (nine healthy controls, seven with gingivitis, five with aggressive periodontitis and 10 with chronic periodontitis) was analyzed for the levels of elafin and secretory leukocyte protease inhibitor (SLPI), two main tissue-derived inhibitors of neutrophil serine proteases. In parallel, activity of NE, PR3 and arginine-specific gingipains (Rgps) in GCF was measured. Finally loads of *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia* and *Treponema denticola* were determined. The highest values of elafin were found in aggressive periodontitis and the lowest in controls. The quantity of elafin correlated positively with the load of

P. gingivalis, *Ta. forsythia* and *Tr. denticola*, as well as with Rgps activity. In addition, NE activity was positively associated with the counts of those bacterial species, but not with the amount of elafin. In contrast, the highest concentrations of SLPI were found in periodontally healthy subjects whereas amounts of this inhibitor were significantly decreased in patients infected with *P. gingivalis*. Periodontopathogenic bacteria stimulate the release of NE and PR3, which activities escape the control through degradation of locally produced inhibitors (SLPI and elafin) by host-derived and bacteria-derived proteases.

INTRODUCTION

In many respects pathogenesis of periodontal diseases results from an interaction of certain periodontal pathogens with host immune responses. Among the bacterial species being strongly associated with periodontitis, *Aggregatibacter actinomycetemcomitans* and bacteria of the 'red complex' (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) seem to play a major role in disease initia-

tion and progression (American Academy of Periodontology, 1996; Borrell & Papapanou, 2005; Holt & Ebersole, 2005; Bamford *et al.*, 2010). *Porphyromonas gingivalis* is one of the major pathogens of severe chronic periodontitis (Lopez, 2000) but it can also be found in large numbers in patients with aggressive periodontitis (Takeuchi *et al.*, 2001; Miura *et al.*, 2005). Although a variety of virulence factors, including lipopolysaccharides, capsular material and fimbriae, are implicated in the pathogenicity of *P. gingivalis* (Sundqvist, 1993; Holt & Ebersole, 2005), proteases are central to the deterrence of host antimicrobial defenses by this bacterium (Potempa & Pike, 2009). Among several different types of proteolytic enzymes secreted by *P. gingivalis* (Potempa *et al.*, 2003), cysteine proteases, referred to as gingipains, are most important. The arginine-specific gingipains (RgpA and RgpB) are encoded by two genes (*rgpA* and *rgpB*), and a lysine-specific enzyme is a product of a single gene (*kgp*) (Potempa *et al.*, 2000). Acting alone or in concert gingipains are able to impair neutrophil function, manipulate the complement pathway, interfere with coagulation and kallikrein/kinin cascades, cleave immunoglobulins, inactivate endogenous protease inhibitors, as well as degrade the extracellular matrix proteins and bioactive peptides (Potempa & Pike, 2009; Guo *et al.*, 2010).

A primary host-response to bacteria colonizing the subgingival tooth surface is infiltration of the gingival tissue and sulcus by large numbers of neutrophils (Garant, 2003), which constitute the main source of proteolytic activity and antimicrobial peptides, including α -defensins 1–4 and hCAP18/LL-37 (Gallo *et al.*, 2002). The serine proteases, protease 3 (PR3), neutrophil elastase (NE) and cathepsin G are stored in primary granules and together with antimicrobial peptides are involved in non-oxidative killing of microorganisms (Korkmaz *et al.*, 2008; Pham, 2008). Moreover, they participate in inflammation and destruction of periodontal tissues. For example, both NE and PR3 are capable of increasing production of interleukin-8 and monocyte chemoattractant protein 1 in gingival fibroblasts (Uehara *et al.*, 2003), and NE degrades periodontal ligament (Ujii *et al.*, 2007).

To maintain homeostasis in tissues, the activity of neutrophil proteases has to be tightly regulated by blood plasma-derived and tissue-derived protease inhibitors. In gingiva the main serine protease

inhibitors produced locally include secretory leukocyte protease inhibitor (SLPI) and elafin (Williams *et al.*, 2006). Whereas SLPI inhibits mainly NE (Fritz *et al.*, 1978; Bergenfeldt *et al.*, 1992), elafin targets both NE and PR3 (Zani *et al.*, 2004). Recently, it has been shown that *P. gingivalis* induces the expression of protease inhibitors, including SPLI and elafin but at the same time it was noticed that these inhibitors could be degraded by proteolytic enzymes produced by this bacterium (Yin *et al.*, 2010). Indeed, in a more detailed study it was clearly shown that both RgpA and RgpB can efficiently abolish the ability of SLPI to inhibit NE (Into *et al.*, 2006). Furthermore, all three gingipains (RgpA, RgpB, Kgp) were found to cleave elafin and inactivate its inhibitory activity, with RgpB being far more effective than other gingipains (Kantyka *et al.*, 2009). Taken together these *in vitro* findings suggest that infections with *P. gingivalis* can exert a mutually opposite effect on the level of protease inhibitors in the inflamed gingival tissue. Therefore, the aim of this pilot study was to investigate a correlation between *P. gingivalis* counts in subgingival plaque as well as this bacterium-derived Rgps activity and the level of elafin and SLPI in gingival crevicular fluid (GCF) collected from periodontitis and gingivitis patients. Based on *in vitro* degradation of elafin and SLPI by Rgps (Into *et al.*, 2006; Kantyka *et al.*, 2009) we hypothesize that the level of the inhibitors in GCF will inversely correlate with the presence of *P. gingivalis* and Rgp activity. Furthermore, as the inhibitor presence should have a bearing on neutrophil protease activity, we have also determined the level of NE and PR3 activity in GCF.

METHODS

Subject recruitment

Thirty-one subjects were recruited from patients of the Department of Conservative Dentistry, University Hospital of Jena. The definition of aggressive and chronic periodontitis was based on the classification system of the 'International Workshop for a Classification System of Periodontal diseases and Conditions' from 1999 (Armitage, 1999). Subjects suffering from systemic disease (e.g. diabetes mellitus, cancer or coronary heart disease), or on antibiotic therapy within the last 6 months and pregnant or lactating females were excluded. A further exclusion criterion

was a periodontitis treatment within the last 2 years. Study was made in agreement with the guidelines of the Helsinki Declaration, revised in 2008. Ethical approval was obtained from the local ethics committee of the University of Jena. A written informed consent was obtained from each subject before participation.

Sampling of gingival crevicular fluid

Samples of GCF were collected in the morning, 2–3 h after breakfast from the deepest site per quadrant. The sites to be sampled were isolated with cotton rolls and gently air-dried. Crevicular washes were obtained using a previously described method (Sigusch *et al.*, 1992; Guentsch *et al.*, 2011). A capillary tip was carefully inserted into the crevice at a level of approximately 1 mm below the gingival margin. At each site, three sequential washes with 10 μ l of 0.9% sodium chloride were performed using a micropipette. The washes of one patient were pooled and transferred into a microcentrifuge tube, immediately frozen and kept at -20°C until analysis.

Enzyme activities of neutrophil elastase and proteinase 3

Enzyme activity of NE was determined by measuring the rate of release of *p*-nitroanilide (*p*-NA) from *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MeSuc-AAPV-*p*NA) used as substrate (Sigma, Munich, Germany). The assay was performed in a total volume of 150 μ l with 10 μ l of the GCF sample and 0.75 mM final substrate concentration in 50 mM Tris-HCl, pH 7.5. The rate of *p*NA released was recorded at 405 nm using a Spectromax 250 (Molecular Devices Corp., Sunnyvale, CA) for 30 min.

Activity of PR3 was determined using Abz-GVADn-VADYQ-Y(NO₂)-D as a substrate at a final concentration of 50 μ M in 0.1 M Tris-HCl, 5 mM EDTA, 0.15 M NaCl, 0.05% Tween-20, 5% dimethylformamide, pH 7.5, added to 10 μ l GCF. Substrate hydrolysis was measured as an increase of fluorescence at $\lambda_{\text{ex}} = 320$ nm and $\lambda_{\text{em}} = 420$ nm for 3 h at 37°C using a Spectramax GEMINI XS (Molecular Devices Corp.).

The activities of NE and PR3 in GCF are expressed as increase in absorbance per minute, or increase in relative fluorescence per minute, respectively.

Microbiology of periodontal pathogens

The DNA was extracted by using a DNA extraction system (A&A Biotechnology, Gdynia, Poland) from 5 μ l of the GCF wash according to the recommendations of the manufacturer. Periodontopathogens were determined by using micro-IDent[®] (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions. In short, polymerase chain reaction (PCR) amplification was carried out in a reaction volume of 25 μ l consisting of 2.5 μ l of template DNA and 22.5 μ l of reaction mixture containing 17.5 μ l of primer-nucleotide mix (micro-IDent[®], Hain Lifescience), 2.5 μ l 10 \times PCR buffer, 2.5 μ l 25 mM MgCl₂ and 1 U *Taq* polymerase (Fermentas Life Science, St. Leon-Rot, Germany). The PCR cycling was carried out in a Mastercycler (Eppendorf, Hamburg, Germany). The cycling conditions comprised an initial denaturation step at 95°C for 5 min, 10 cycles at 95°C for 30 s and at 60°C for 2 min, 20 cycles at 95°C for 10 s, at 55°C for 30 s and at 72°C for 30 s, and a final extension step at 72°C for 10 min. In the subsequent reverse hybridization, the biotinylated amplicons were denatured and incubated at 45°C with hybridization buffer and strips coated with two control lines and five species-specific probes. After PCR products were bound to their respective complementary probe, a highly specific washing step removed any not specifically bound DNA. Streptavidin-conjugated alkaline phosphatase was added, the samples were washed and hybridization products were visualized by adding a substrate for alkaline phosphatase. Then, the intensity of the band was measured as described recently (Eick *et al.*, 2011). In addition, real-time PCR was used to quantify the level of *P. gingivalis* by addition of primers described by Ashimoto *et al.* (1996) and the GoTaq(R) qPCR Master Mix (Promega AG, Dübendorf, Switzerland) and using the 7500 Real time PCR System (Applied Biosystem-Life Technologies, Carlsbad, CA). The positive control was *P. gingivalis* ATCC 33,277 in the range of 10^2 – 10^7 cells sample⁻¹.

Activity of arginine-specific gingipains

The activities of Rgps in the GCF were determined using the chromogenic substrate *N*-benzoyl-L-arginine-*p*-nitroanilide (BApNA) (Sigma, St. Louis, MO). Ten microliters of GCF samples were pre-incubated in 200 mM Tris-HCl, 100 mM NaCl, 5 mM CaCl₂, pH

7.6, supplemented with 10 mM cysteine, for 5 min at 37°C and assayed for amidase activity with 0.5 mM substrate in the total volume of 200 µl. The release of *p*-nitroanilide was monitored spectrophotometrically at 405 nm, as described above.

Determination of protease inhibitors

The amount of SLPI within the GCF was determined using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction, GCF samples were diluted 1 : 100 before being applied to the microplate. The detection level of the kit was 100 pg ml⁻¹.

For determination of elafin, GCF samples were diluted four times with sample buffer [0.125 mM Tris-HCl, 20% glycerol, 4% sodium dodecyl sulfate (SDS)], and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (15% acrylamide) using the Tris-Tricine discontinuous buffer system (Schagger *et al.*, 1988). As the reference, 20 pg elafin (kind gift from Proteo Biotech AG, Kiel, Germany) was always run on a gel. Western blot was performed by electro-transfer of SDS-PAGE resolved proteins onto a polyvinylidene fluoride membrane, followed by overnight blocking with 2% 0.22 µm-filtered bovine serum albumin in Tris-buffered saline with 0.05% Tween-20 (TTBS). Blocked membrane was incubated with primary antibody (1 : 500, biotinylated goat anti-human Trappin-2; BAF-1747; R&D, Wiesbaden-Nordenstadt, Germany), then washed three times with TTBS and streptavidin-horseradish peroxidase was applied for 1 h (1 : 20,000, RPN-1231; GE Healthcare, Munich, Germany). ECL+ (GE Healthcare) was used as the chemoluminescence substrate according to the manufacturer's instructions and membranes were exposed to X-ray films (Kodak: Eastman Kodak Company, Rochester, NY).

Data analysis

The clinical data were expressed as means ± standard deviation (SD). Laboratory variables are presented as median including quartiles. Groups were compared with Kruskal-Wallis and Mann-Whitney tests. The correlation between tested variables was made using Spearman test. Statistical software (PASW 18.0; SPSS, Chicago, IL) was used for all statistical analyses.

RESULTS

Clinical data

Nine periodontally healthy subjects, seven gingivitis patients, 10 patients with chronic periodontitis (CP) and five with aggressive periodontitis (AP) were recruited and participated in the study. The study group consisted of 15 women and 16 men; the mean age of all participants was 39.4 ± 10.2 years. Demographic and clinical data characterizing the patients' groups are summarized in Table 1. The patients in the AP group had a mean age of 39.4 ± 8.6 years, which is higher than normal for AP. All included patients had the generalized form of AP and the beginning of the disease was registered first before the age of 35 years. Periodontal destruction characterized by mean pocket depths of 4.40–4.67 mm was found in the periodontitis groups. A high bleeding on probing incidence as a sign of inflammation was also detected in both periodontitis and gingivitis groups.

Bacterial species associated with periodontitis

Selected bacterial species that are associated with periodontal inflammation (*P. gingivalis*, *Ta. forsythia*, *Tr. denticola*, *A. actinomycetemcomitans* and *Prevotella intermedia*) were not found in periodontally healthy controls (Table 2). High counts of *A. actinomycetemcomitans* were detectable in two cases (40%) of AP and in two cases (20%) of CP. In one case of AP the very high counts of *A. actinomycetemcomitans* were associated with none of the other four investigated pathogens. Whereas none of the pathogens was detectable in the control group, all pathogens had been detected in the gingivitis, CP and AP groups. In the groups showing signs of periodontal inflammation (AP, CP, gingivitis), *Tr. denticola* was prevalent (about 60% cases). *Tannerella forsythia* was detected in at least 80% of the cases but *Pr. intermedia* was only rarely present (Table 2).

Porphyromonas gingivalis was detected in the half of the samples from gingivitis patients and in more than 80% of the samples from periodontitis patients. Qualitatively, all samples found positive by using the hybridization-based strip-technology were shown containing a given pathogen using the real-time PCR technique. Similarly, negative samples with the first technique were confirmed negative with the other.

Table 1 Demographic and clinical data

	Control group (<i>n</i> = 9)	Gingivitis (<i>n</i> = 7)	Chronic periodontitis (<i>n</i> = 10)	Aggressive periodontitis (<i>n</i> = 5)
Age (years), mean ± SD	33.2 ± 9.8	38.6 ± 7.8	45.6 ± 12.9	39.4 ± 8.6
Gender, male : female	5 : 4	3 : 4	5 : 5	3 : 2
Probing depth (mm), mean ± SD	1.65 ± 0.37	2.23 ± 0.78	4.40 ± 0.75	4.67 ± 0.88
Bleeding on probing (%), mean ± SD	7.20 ± 10.65	80.34 ± 9.88	90.56 ± 18.05	89.67 ± 10.54
Teeth, mean ± SD	27.80 ± 2.23	28.33 ± 1.63	27.02 ± 2.36	27.31 ± 2.43

Table 2 Detection of periodontopathogens within groups by using semi-quantitative strip technology

	Controls (<i>n</i> = 9)	Gingivitis (<i>n</i> = 7)	Chronic periodontitis (<i>n</i> = 10)	Aggressive periodontitis (<i>n</i> = 5)
<i>Porphyromonas gingivalis</i>				
Positive	0 (0)	4 (57)	9 (90)	4 (80)
High load ¹	0 (0)	2 (29)	3 (30)	2 (40)
<i>Tannerella forsythia</i>				
Positive	0 (0)	6 (86)	8 (80)	4 (80)
High load ¹	0 (0)	3 (43)	0 (0)	1 (20)
<i>Treponema denticola</i>				
Positive	0 (0)	4 (57)	6 (60)	3 (60)
High load ¹	0 (0)	2 (29)	0 (0)	1 (20)
<i>Aggregatibacter actinomycetemcomitans</i>				
Positive	0 (0)	1 (14)	2 (20)	2 (40)
High load ¹	0 (0)	0 (0)	2 (20)	2 (40)
<i>Prevotella intermedia</i>				
Positive	0 (0)	1 (14)	1 (10)	1 (20)
High load ¹	0 (0)	0 (0)	0 (0)	0 (0)

¹High load was defined as an intensity of the band of ≥ 50% in relation to the hybridization control.

Quantitatively, the correlation coefficient *R* for detection of pathogens using both methods was 0.952 ($P < 0.001$). However, because only real-time PCR allows counting of bacterial cells within GCF, quantitative results obtained by this technique are discussed in the follow-up text. Accordingly, the highest numbers of *P. gingivalis* were determined in GCF samples from the AP group (median 1.07×10^6), followed by the CP group (median 2.27×10^5) and the gingivitis group (median 1.40×10^3 ; Fig. 1).

The arginine-specific amidolytic activity was highest in the CP group with a median of 0.18 U, a value significantly higher in comparison to the Rgp activity in controls ($P = 0.024$; Fig. 1).

Activities of serine-proteases

There was low variation in the level of the NE activity within individual groups of patients. Whereas the highest activity was detected in the gingivitis group, slightly lower activities have been determined in samples obtained from CP and AP subjects. Again, the

activity in periodontally healthy subjects was significantly ($P = 0.039$) lower than in gingivitis patients (Fig. 2).

The highest activity of PR3 was detected in gingivitis patients, followed by CP and AP patients. Periodontally healthy subjects showed a generally low activity, significantly lower ($P = 0.007$) than in gingivitis patients.

Levels of protease inhibitors

The SLPI was found to be present in very high concentrations in periodontally healthy subjects. The detectable amount of SLPI was lower in all patient groups characterized by gingival inflammation. The difference between periodontally healthy subjects and CP patients was statistically significant ($P = 0.043$) (Fig. 3).

The levels of elafin have been determined by using specific antibodies and the Western blot technique. As expected from the fact that *in vivo* elafin is secreted in the higher molecular form referred to as

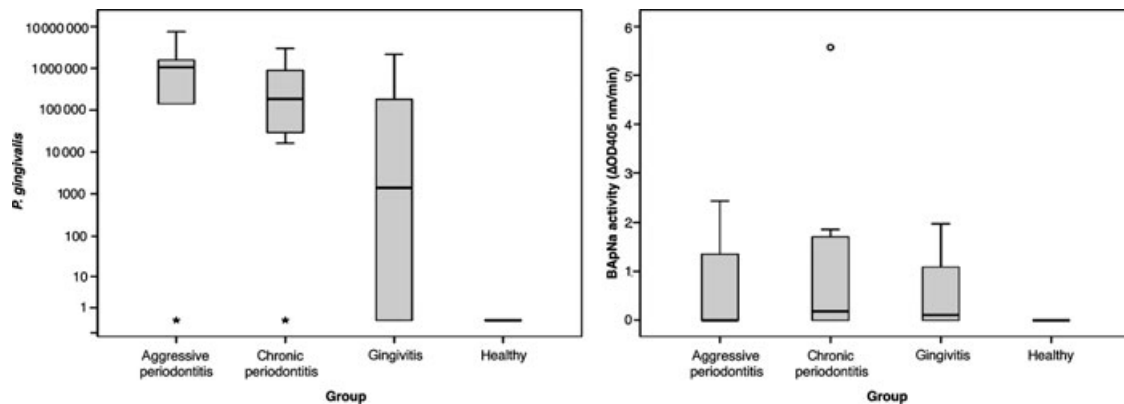


Figure 1 Counts of *Porphyromonas gingivalis* (median and 25th and 75th centiles) and Rgps activity (median and 25th and 75th centiles) determined by the release of *p*-nitroanilide from BAPNA in gingival crevicular fluid obtained from patients with aggressive periodontitis, chronic periodontitis, and gingivitis as well as from periodontally healthy subjects.

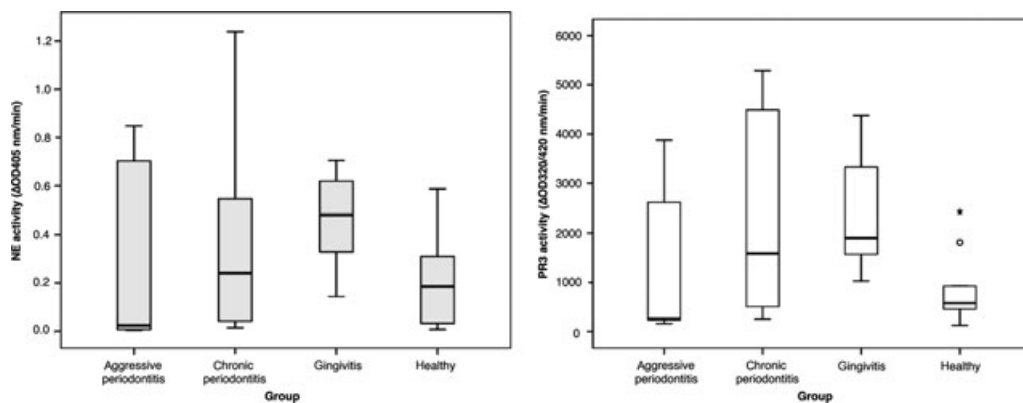


Figure 2 Activities of the neutrophil serine proteases, protease 3 (PR3) determined by using fluorogenic substrate (Abz-GVADnVADYQ-Y(NO₂)-D) and elastase (NE) by using chromogenic substrate (*N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide) in gingival crevicular fluid obtained from patients with aggressive periodontitis, chronic periodontitis, and gingivitis as well as from periodontally healthy subjects.

trappin and is usually covalently linked to connective tissue proteins (Guyot *et al.*, 2005), the molecular weight of the detected immunoreactive bands in GCF was higher than that of free elafin. This result confirms that also in the gingival tissue elafin occurs in the form linked to extracellular matrix components.

For quantification of elafin content in GCF all immunoreactive bands were scanned and their total intensity was used for calculation. The amount of elafin differed between the groups ($P = 0.001$). Elafin was found in significantly the highest quantities in AP patients compared with patients diagnosed with CP ($P = 0.040$) and gingivitis ($P = 0.042$). The most profound difference was observed between AP patients and healthy controls ($P = 0.001$). The level of elafin did not differ significantly between CP and gingivitis patients. In both

these groups quantities of the detected inhibitor were higher in comparison to control subjects ($P = 0.008$ and $P = 0.016$, respectively) (Fig. 4).

Associations between serine proteases, inhibitors of serine proteases and *P. gingivalis*

Out of a total of 31 analyzed GCF samples, 17 (54.8%) were tested positive for *P. gingivalis*. The neutrophil protease activities were higher in the *P. gingivalis*-positive group than in the *P. gingivalis*-negative group but the difference was statistically significant only for the PR3 activity ($P = 0.011$). Interestingly, the elafin level was significantly higher in *P. gingivalis*-infected patients in comparison to non-infected ones ($P = 0.002$) whereas SLPI levels were

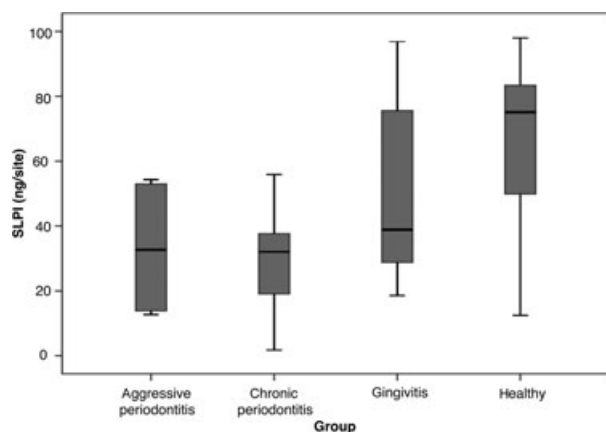


Figure 3 Level of secretory leukocyte protease inhibitor (SLPI) determined by using ELISA in gingival crevicular fluid obtained from patients with aggressive periodontitis, chronic periodontitis, and gingivitis as well as from periodontally healthy subjects.

inversely correlated ($P = 0.026$) with the *P. gingivalis* presence (Fig. 5).

These findings are further supported by the strong correlation between the counts of *P. gingivalis* and the Rgps activity in GCF. Furthermore, *P. gingivalis* counts and the arginine-specific amidolytic activity showed a positive correlation with activities of neutrophil serine proteases and, remarkably, also with elafin. The concentration of elafin was inversely correlated with the level of SLPI. Also, the association between the load of *P. gingivalis* and SLPI has a tendency to be inverse but without reaching significance. Finally, it should be noted that the activities of PR3 and NE strongly correlated one with another. In contrast, no association between the protease

activities with the levels of SLPI and elafin was registered (Table 3).

The activities of NE and PR3, as well as the level of elafin positively correlated with loads of *Ta. forsythia* and *Tr. denticola* (correlation coefficient between 0.406 and 0.714; $P < 0.05$ each). Conversely, *A. actinomycetemcomitans* was negatively correlated with SLPI ($R = -0.497$; $P = 0.004$).

DISCUSSION

In this pilot study four different groups of patients were included. Clinical parameters did not differ between the two periodontitis groups. In addition, bleeding on probing values, an accepted measure of gingival inflammation, were similar in gingivitis and periodontitis patients. Among analyzed groups the activity of neutrophil serine proteases was highest in gingivitis. The finding of the higher NE activity in gingivitis than in periodontitis patients contests the data presented in another report (Figueredo *et al.*, 2005). The discrepancy may result partially from different methods used to collect GCF, which were shown to affect NE recovery from the sampling device (Guentisch *et al.*, 2011). Alternatively, a design of this study as a pilot study with a relatively small number of subjects may weaken differences between the groups. Results of several studies suggested that the NE presence and/or activity in GCF from discrete periodontitis sites can be used to identify differences in disease severity within patients and to determine the success of periodontal treatment (Binder *et al.*, 1987; Lamster *et al.*, 1988; Yin *et al.*, 2010). The PR3

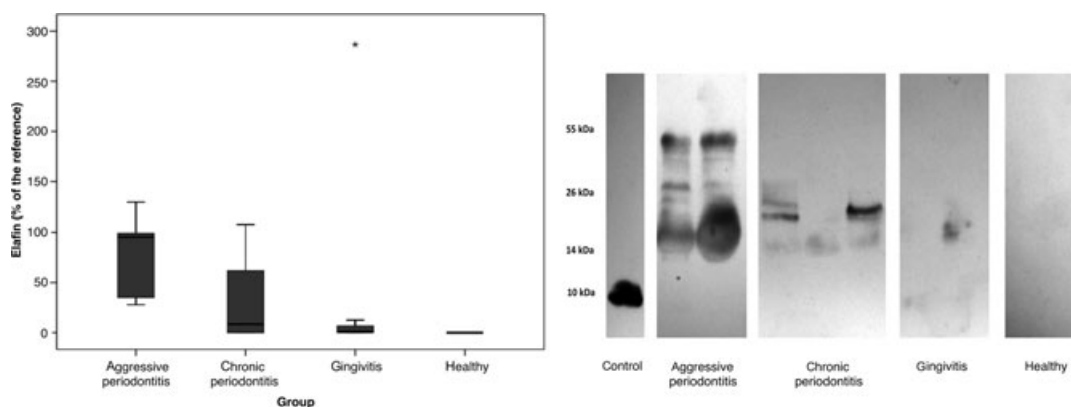


Figure 4 Level of elafin determined using densitometry after Western blot technique according to the reference (20 pg elafin) in gingival crevicular fluid obtained from patients with aggressive periodontitis, chronic periodontitis, and gingivitis as well as from periodontally healthy subjects and examples of Western blots.

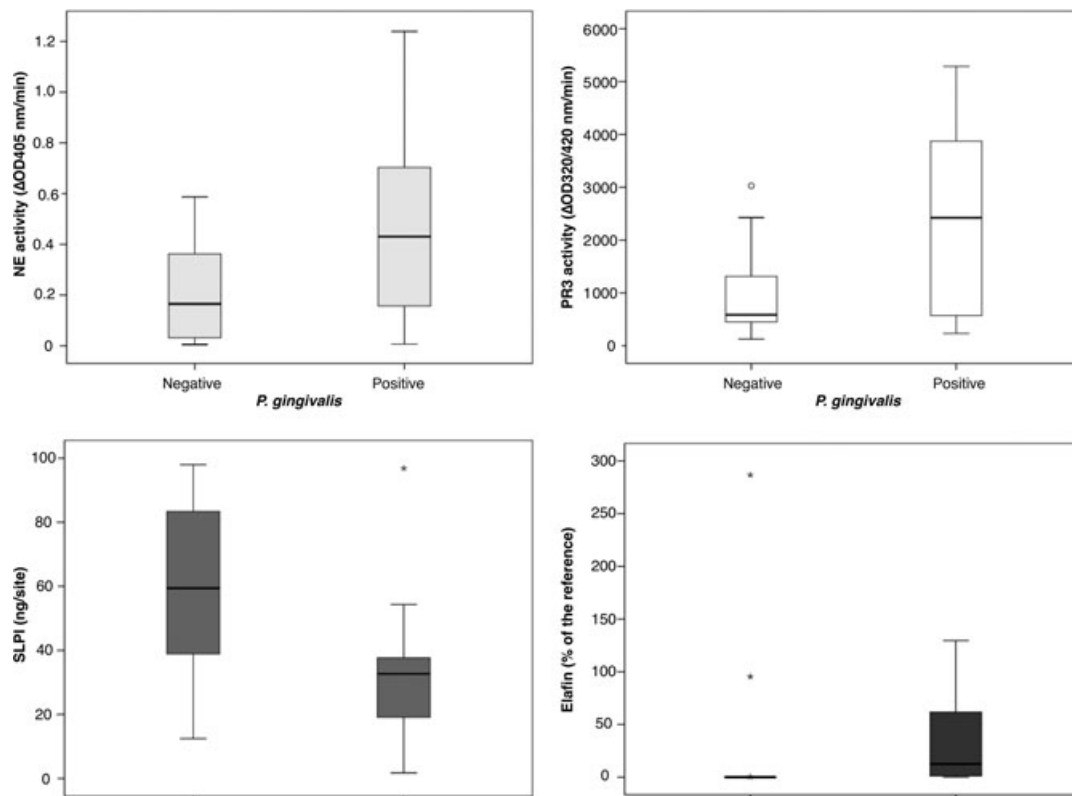


Figure 5 Activities of the neutrophil serine proteases, protease 3 (PR3) and elastase (NE), as well as the levels of the protease inhibitors secretory leukocyte protease inhibitor (SLPI) and elafin in gingival crevicular fluid obtained from patients being tested negatively and positively for *Porphyromonas gingivalis*.

Table 3 Significant correlations (Spearman) between different variables within gingival crevicular fluid

	Coefficient <i>R</i>	<i>P</i>
Elafin		
SLPI	-0.378	0.036
<i>Porphyromonas gingivalis</i>	0.618	< 0.001
BAPNA	0.643	< 0.001
Neutrophil elastase activity		
Proteinase 3 activity	0.790	< 0.001
<i>P. gingivalis</i>	0.493	0.013
BAPNA	0.678	< 0.001
Proteinase 3 activity		
<i>P. gingivalis</i>	0.496	0.005
BAPNA	0.825	< 0.001
<i>P. gingivalis</i>		
BAPNA	0.726	< 0.001

BAPNA, *N*-benzoyl-L-arginine-*p*-nitroanilide; SLPI, secretory leukocyte protease inhibitor.

activity has not yet been determined in gingival fluid. Therefore the finding that the PR3 activity correlates strongly with the NE activity suggests this enzyme as

a suitable biomarker for disease severity and progression. The release of enzymes caused by neutrophil lysis during GCF freezing is unlikely to contribute to this correlation because cathepsin G, NE and PR3 are stored in azurophilic granules in tight association with proteoglycans and their release requires high ionic strength (Reeves *et al.*, 2002).

In vivo activity of NE and PR3 escaped from neutrophils during these cells degranulation and NET formation or through frustrated phagocytosis and necrosis (Fox *et al.*, 2010) are instantly quenched by endogenous inhibitors. Elafin and SLPI are two inhibitors produced locally in tissues. Although SLPI is expressed in macrophages (Mihaila & Tremblay, 2001) this protein is predominantly made in epithelial cells (van Wetering *et al.*, 2000a), including gingival epithelial cells (Yin *et al.*, 2010). In epithelial cells, SLPI is constitutively expressed (Sallenave *et al.*, 1994), with the level of expression stimulated by lipopolysaccharide, lipoteichoic acid, interleukin-6, interleukin-10 (Jin *et al.*, 1998), interleukin-1 β , and tumor

necrosis factor- α (Sallenave *et al.*, 1994). Also, epithelia cell interaction with *P. gingivalis* enhances SLPI synthesis through an unknown mechanism (Yin *et al.*, 2010). At the same time it is known that Arg-gingipains are able to cleave SLPI (Into *et al.*, 2006; Yin *et al.*, 2010). *In vitro* at concentrations of 100 nM Rgps totally degraded SLPI whereas at 10 nM already eliminated SLPI ability to inhibit NE (Into *et al.*, 2006). In the light of recent findings that the GCF concentration of Arg-gingipains is up to 1500 nM, with a median about 58 nM at *P. gingivalis*-positive sites (Guentsch *et al.*, 2011), it is expected that SLPI can also be degraded or at least inactivated *in vivo*. In keeping, here we found a low level of SLPI in GCF from patients who were positive for *P. gingivalis*, in agreement with the previous finding (Into *et al.*, 2006). Unexpectedly this inverse correlation between SLPI and *P. gingivalis* was not as strong as that between *A. actinomycetemcomitans* counts and the SLPI level. It may be assumed that leukotoxin and cytolethal distending toxin produce by *A. actinomycetemcomitans* (Mayer *et al.*, 1999; Kachlany, 2010; Fong *et al.*, 2011) inhibit expression of SLPI. Finally, SLPI sensitivity to degradation by host-derived proteases such as cathepsins B, L and S (Taggart *et al.*, 2001) may compound the correlation. Indeed, it is known that active cathepsin B occurs at a high level in GCF of periodontitis patients (Ichimaru *et al.*, 1996). Taken together it is very likely that host-derived and pathogen-derived proteases, together with as yet unknown factors that may inhibit SLPI expression, contribute to significant depletion of this inhibitor in the infected periodontitis sites.

Similar to SLPI, elafin is also mainly expressed in epithelial cells (van Wetering *et al.*, 2000a,b; Yokota *et al.*, 2007; Lee *et al.*, 2009) in a manner significantly stimulated by chronic inflammatory conditions, e.g. chronic sinusitis (Lee *et al.*, 2009). Consistently, we detected high levels of elafin in patients with periodontitis and gingivitis. Interestingly, we found a strong positive correlation between elafin levels and *P. gingivalis* counts, as well as between elafin and the Arg-gingipain activity. Especially the latter correlation seems to be at odds with the ability of Rgps to cleave and efficiently inactivate elafin *in vitro* (Kantyka *et al.*, 2009).

Elafin is an extremely stable protein resistant to proteolytic degradation (Guyot *et al.*, 2010). Nevertheless, Rgps exert limited proteolysis of a single

peptide bond within the active site loop of elafin. The cleavage efficiently inactivates protein inhibitory activity but does not abolish its recognition by antibodies in Western blot analysis in non-reducing conditions (Kantyka *et al.*, 2009). Whereas proteolytic inactivation of elafin occurs at subnanomolar Rgps concentrations, visible degradation of the inhibitor requires 100 nM enzyme concentrations (Kantyka *et al.*, 2009). This inefficiency of elafin degradation correlates with the absence of elafin degradation products in analyzed GCF samples. On the other hand, taking into account Rgps concentration in GCF (Guentsch *et al.*, 2011) at least 100-fold higher than required to cleave the elafin's reactive loop, it is highly implausible that the inhibitor detected in GCF is active.

Our Western blot analysis has clearly shown that elafin in GCF occurs in high molecular mass forms, apparently representing elafin in the form of trappin-2 conjugated to fragments of extracellular matrix proteins (Guyot *et al.*, 2005; Baranger *et al.*, 2011). In this context it is important to reiterate that *in vivo* elafin occurs predominantly as trappin-2 immobilized in a meshwork of the extracellular matrix by the action of a type 2 transglutaminase (Guyot *et al.*, 2005). Immobilization of elafin prevents diffusion of the inhibitor from the location to where it is most needed to protect fragile matrix proteins from proteolytic degradation by the neutrophil-derived serine proteases.

Finally, the striking correlation between elafin and *P. gingivalis*/Rgps activity points at gingipains as the main sheddases of extracellular matrix-linked inhibitor. In keeping, gingipains can easily degrade fibronectin, the protein abundant in gingiva (Talonpoika, 1991; Figueredo & Gustafsson, 2000), which is the main anchorage extracellular matrix protein for trappin (Guyot *et al.*, 2005). It is also likely that elafin is released by fibronectin degradation by proteases of *Ta. forsythia* and *Tr. denticola* (Bamford *et al.*, 2010) as levels of these highly proteolytic species correlate with elafin levels in GCF. The contention that periodontal pathogen-derived proteases, including gingipains, can work as elafin sheddases is corroborated by the presence of fibronectin fragments of 40, 68 and 120 kDa in GCF, which quantity increased with disease severity (Huynh *et al.*, 2002).

Taken together based on our analysis of GCF content the following scenario can be suggested. Results of *ex vivo* studies (Yin *et al.*, 2010) strongly suggest

that in inflamed infected periodontal/gingival tissues expression of both SPLI and elafin is increased. While soluble SPLI is degraded *in situ* by bacteria and host-derived proteases, the ECM-conjugated elafin, although it resists proteolytic degradation, can be inactivated by limited proteolysis at the inhibitory reactive site loop (Kantyka *et al.*, 2009). The inhibitory capacity of any intact elafin and any intact SPLI is then saturated and neutrophil serine proteases are free to exert their broad range of biological activities. In addition, this will destroy other functions of inhibitors, such as mediation of wound healing by SLPI (Ashcroft *et al.*, 2000), chemoattractant and opsonin activity of elafin (Huynh *et al.*, 2002), and antibacterial activity of both inhibitors as well as their ability to suppress host response to lipopolysaccharide (Talponoika *et al.*, 1991; Hiemstra *et al.*, 1996; Samsom *et al.*, 2007; Baranger *et al.*, 2008). Apart from releasing neutrophil proteases from control, potential abrogation of the immunomodulatory functions of these inhibitors by bacterial proteases may profoundly contribute to severity and progression of the periodontal disease.

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