

Published in final edited form as:

*Mol Oral Microbiol.* 2012 February ; 27(1): 45–56. doi:10.1111/j.2041-1014.2011.00631.x.

## 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), which extracellular activity 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. GCF collected from 31 subjects (9 healthy controls, 7 with gingivitis, 5 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*, *T. forsythia*, and *T. denticola*, as well as with Rgps activity. In addition, NE-activity was positively associated with the counts of those bacterial species, but not with 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 due to degradation of locally produced inhibitors (SLPI and elafin) by host- and bacteria-derived proteases.

### Keywords

*Porphyromonas gingivalis*; periodontal disease; elafin; SLPI; neutrophil elastase; Proteinase 3

### 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

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The authors declare that there are no conflicts of interest in this study.

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 initiation and progression (1996; Borrell & Papapanou, 2005; Holt & Ebersole, 2005; Bamford *et al.*, 2010). *P. 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, 2005 #3; Holt & Ebersole, 2005), proteases are central to 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 of the main importance. While arginine-specific gingipains (RgpA and RgpB) are encoded by two genes (*rgpA* and *rgpB*), 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 (PMNs) (Garant, 2003), which constitute the main source of proteolytic activity and antimicrobial peptides, including  $\alpha$ -defensins 1–4 and hCAP18/LL-37 (Gallo *et al.*, 2002; RL Gallo; Murakami, 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. *E.g.*, both NE and PR3 are capable to increase production of interleukin-8 and monocyte chemoattractant protein 1 in gingival fibroblasts (Uehara *et al.*, 2003), NE degrades periodontal ligament (Ujii *et al.*, 2007).

To maintain homeostasis in tissues activity of neutrophil proteases has to be tightly regulated by blood plasma- and tissue-derived protease inhibitors. In gingiva the main serine protease inhibitors produced locally include SLPI (secretory leukocyte protease inhibitor) and elafin (Williams *et al.*, 2006). While SLPI inhibits mainly NE (Fritz *et al.*, 1978; Bergenfeldt *et al.*, 1992), elafin targets both NE and PR 3 (Zani *et al.*, 2004). Recently, it has been shown that *P. gingivalis* induces the expression of protease inhibitors, including SPLI and elafin but in the same time it was noticed that these inhibitors can be degraded by proteolytic enzymes produced by this bacterium (Yin *et al.*, 2010). Indeed, in more detailed study it was clearly shown that both RgpA and RgpB can efficiently abolish SLPI ability 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 GCF collected from periodontitis and gingivitis patients. Based *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, since 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 two years. Study was made in agreement with the guidelines of the Helsinki Declaration, revised in 2008. Ethical approval was obtained from local ethics committee of the University of Jena. A written informed consent was obtained from each subject prior to participation.

### Sampling of gingival crevicular fluid (GCF)

Samples 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  $\mu$ 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  $-20^{\circ}\text{C}$  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 total volume of 150  $\mu$ L with 10  $\mu$ 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, USA) for 30 min.

PR3 activity was determined using Abz-GVADnVADYQ-Y(NO<sub>2</sub>)-D as a substrate at final concentration of 50  $\mu$ 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  $\mu$ L GCF. Substrate hydrolysis was measured as an increase of fluorescence at  $\lambda_{\text{ex}} = 320\text{nm}$  and  $\lambda_{\text{em}} = 420\text{nm}$  for 3 h at  $37^{\circ}\text{C}$  using a Spectramax GEMINI XS (Molecular Devices Corp., Sunnyvale, USA).

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  $\mu$ L of the GCF wash according to recommendations of the manufacturer. Periodontopathogens were determined by using micro-IDent® (Hain Lifescience, Nehren, Germany) according to the manufacturer’s instruction. In short, PCR amplification was carried out in a reaction volume of 25  $\mu$ L consisting of 2.5  $\mu$ L of template DNA and 22.5  $\mu$ L of reaction mixture containing 17.5  $\mu$ L of primer–nucleotide mix (micro-IDent®), 2.5  $\mu$ L of  $10\times$  PCR buffer, 2.5  $\mu$ L of 25 mM MgCl<sub>2</sub> and 1 U Taq polymerase (Fermentas Life Science, St. Leon-Rot, Germany). PCR cycling was carried out in a Mastercycler (Eppendorf, Hamburg, Germany). The cycling conditions comprised an initial denaturation step at  $95^{\circ}\text{C}$  for 5 min, 10 cycles at  $95^{\circ}\text{C}$  for 30 s and at  $60^{\circ}\text{C}$  for 2 min, 20 cycles at  $95^{\circ}\text{C}$  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 non-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.* (Ashimoto *et al.*, 1996) and the GoTaq(R) qPCR Master Mix (Promega AG, Dübendorf, Switzerland) and using 7500 Real time PCR System (Applied Biosystem-Life Technologies, Carlsbad, CA, USA). The positive control was *P. gingivalis* ATCC 33277 in the range of  $10^2 - 10^7$  cells/sample.

### Activity of arginine specific gingipains (Rgps)

The activities of Rgps in the GCF were determined using the chromogenic substrate *N*-Benzoyl-L-Arginine-*p*-Nitroanilide (BApNA) (Sigma, St. Louis, MO, USA). Ten  $\mu$ L of GCF samples were pre-incubated in 200 mM Tris-HCl, 100 mM NaCl, 5 mM CaCl<sub>2</sub>, 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  $\mu$ L. 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 ELISA kit (R&D Systems, Minneapolis, MN, USA) 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.

For determination of elafin, GCF samples were diluted 4 times with sample buffer (0.125 mM Tris-HCl, 20% glycerol, 4% SDS), and resolved by SDS-PAGE (15% acrylamide) using the Tris-Tricine discontinuous buffer system (Schagger *et al.*, 1988). As the reference 20 pg elafin (kind gift of Proteo Biotech AG Kiel, Germany) was always run on a gel. Western blot was performed by electrotransfer of SDS-PAGE resolved proteins onto a PVDF membrane, followed by overnight blocking with 2% 0.22  $\mu$ m-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 3 times with TTBS and streptavidin-HRP 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 instruction and membranes were exposed to x-ray films (Kodak).

### Data analysis

The clinical data were expressed as means  $\pm$  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, USA) was used for all statistical analyses.

## RESULTS

### Clinical data

Nine periodontally healthy subjects, 7 gingivitis patients, 10 patients with chronic periodontitis (CP) and 5 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  $39.4 \pm 10.2$  years. Demographic and clinical data characterizing the patients' groups are summarized in Table 1. The patients in the aggressive periodontitis group were with a mean age of  $39.4 \pm 8.6$  years, which is higher than normal from 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 the mean pocket depths of 4.40 – 4.67 mm was found in the periodontitis groups. A high bleeding on probing (BoP) incidence as a sign of inflammation was also detected in both periodontitis and gingivitis groups.

### Bacterial species associated with periodontitis

Selected bacterial species, which are associated with periodontal inflammation (*P. gingivalis*, *T. forsythia*, *T. denticola*, *A. actinomycetemcomitans*, and *P. 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), *T. denticola* was prevalent (about 60% cases). *T. forsythia* was detected in at least 80% of the cases. *P. intermedia* was only rarely present (Table 2).

*P. gingivalis* was detected in the half of gingivitis patients and in more than 80% of the periodontitis patients-derived samples. 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. 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 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  $1.07 \times 10^6$ ), followed by the CP group (median  $2.27 \times 10^5$ ) and the gingivitis group (median  $1.40 \times 10^3$ ; Fig 1).

The arginine specific amidolytic activity was the highest in the CP group with a median of 0.18 U, the value significantly higher in comparison to the Rgps 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 lesser 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

SLPI was found to be present in very high concentrations in periodontally healthy subjects. The detectable amount of SLPI was lower in all patients' 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 Western blot technique. As expected from the fact that *in vivo* elafin is secreted in the higher molecular form referred to as 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 highest quantities in AP patients than in 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 total 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 *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 ( $p=0.002$ ) while SLPI levels were 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 the 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 *T. forsythia* and *T. 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 BoP values, an accepted

measure of gingival inflammation, were similar in gingivitis and periodontitis patients. Among analyzed groups the neutrophil serine proteases activity was the highest in gingivitis. The finding of the higher NE activity in gingivitis than in periodontitis patients contests the data presented in other 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 sampling device (Guentsch *et al.*, 2011). Alternatively, a design of this study as a pilot one with relatively low 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 success of periodontal treatment (Binder *et al.*, 1987; Lamster *et al.*, 1988; Yin *et al.*, 2010). The PR3 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 enzymes release due to neutrophil lysis during GCF freezing is rather unlikely to contribute to this correlation since 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 due to frustrated phagocytosis and necrosis (Fox *et al.*, 2010) are instantly quenched by endogenous inhibitors. SLPI and elafin 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.*, 2000), 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 (IL)-6, IL-10 (Jin *et al.*, 1998), IL-1 $\beta$ , tumor necrosis factor (TNF) $\alpha$  (Sallenave *et al.*, 1994). Also, epithelia cell interaction with *P. gingivalis* enhances SLPI synthesis through yet 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 the 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 being 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 the high level in GCF of periodontitis patients (Ichimaru *et al.*, 1996). Taken together it is very likely that host and pathogen derived proteases, together with 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.*, 2000; 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 elafin and the Arg-gingipain activity. Especially the latter correlation seems to be at odds with Rgps ability to cleave and efficiently inactivate elafin *in vitro* (Kantyka *et al.*, 2009).

Elafin is the 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). While 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 (ECM) by the action of a type 2 transglutaminase (Guyot *et al.*, 2005). Immobilization of elafin prevents diffusion of the inhibitor from the location 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 a main sheddases of ECM-linked inhibitor. In keeping, gingipains can easily degrade fibronectin, the protein abundant in gingiva (Talonpoika, 1991; Figueredo & Gustafsson, 2000), which is the main anchorage ECM protein for trappin (Guyot *et al.*, 2005). It is also likely that elafin is released by fibronectin degradation by proteases of *T. forsythia* and *T. denticola* (Bamford *et al.*, 2010) since loads of these highly proteolytic species correlates 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 kDa, 68 kDa 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 resists proteolytic degradation, it 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 both inhibitors antibacterial activity as well as ability to suppresses host response to LPS (Talonpoika *et al.*, 1991; Samsom *et al.*, 2007); (Hiemstra *et al.*, 1996; 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.

## Acknowledgments

Most the study was founded by the participating departments. The authors acknowledge the support of Oliver Laugisch by the German Academic Exchange Service (grant No: 314-D/08/48763) and a grant for a foreign training by the German Society of Periodontology. In addition, this study was partially supported by grants from European Community (FP7-HEALTH-2010-261460 "Gums&Joints" and Marie Curie ITN-290246 "RAPID"), Foundation



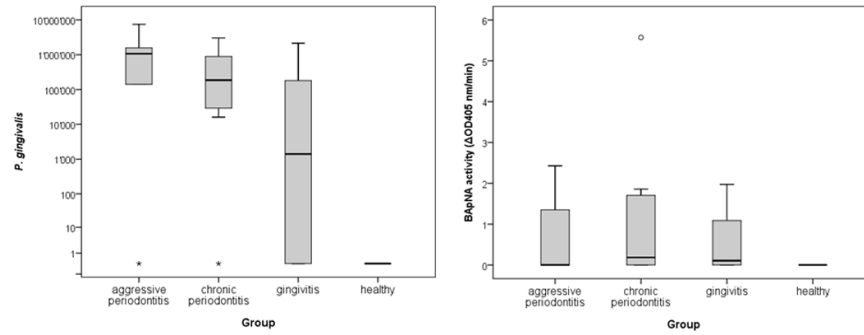
for Polish Science (TEAM project DPS/424-329/10), and the National Institutes of Health, USA (Grant DE 09761). The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a beneficiary of the structural funds from the European Union (grant No: POIG.02.01.00-12-064/08 – “*Molecular biotechnology for health*”).

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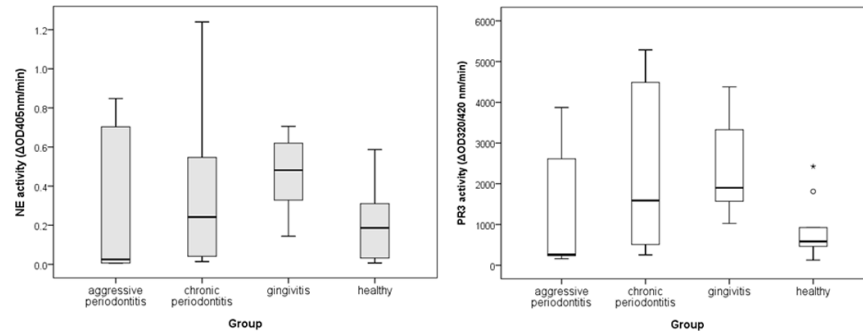
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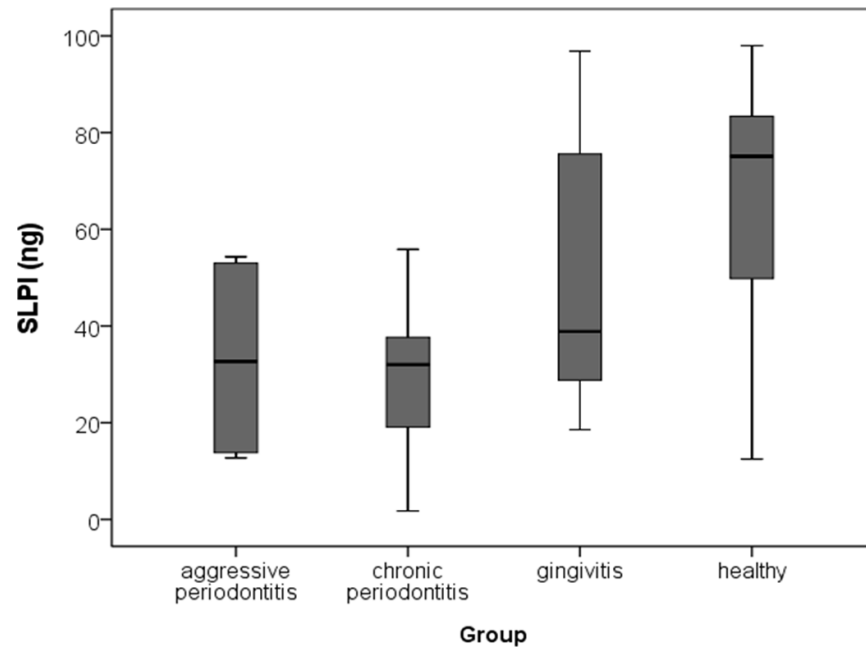
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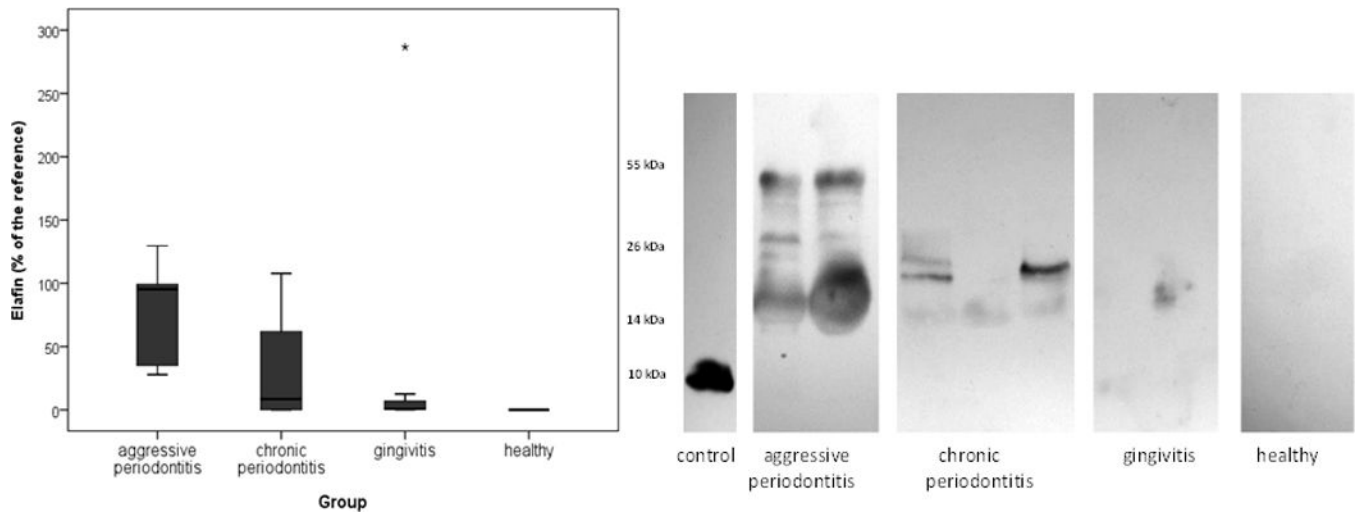
**Figure 1.** Counts of *P. gingivalis* (median and 25 and 75 percentiles) and Rgps activity (median and 25 and 75 percentiles) determined by the release of *p*-nitroanalide from BApNA in GCF obtained from patients with AP (aggressive periodontitis), (CP) chronic periodontitis, and gingivitis as well as from periodontally healthy subjects



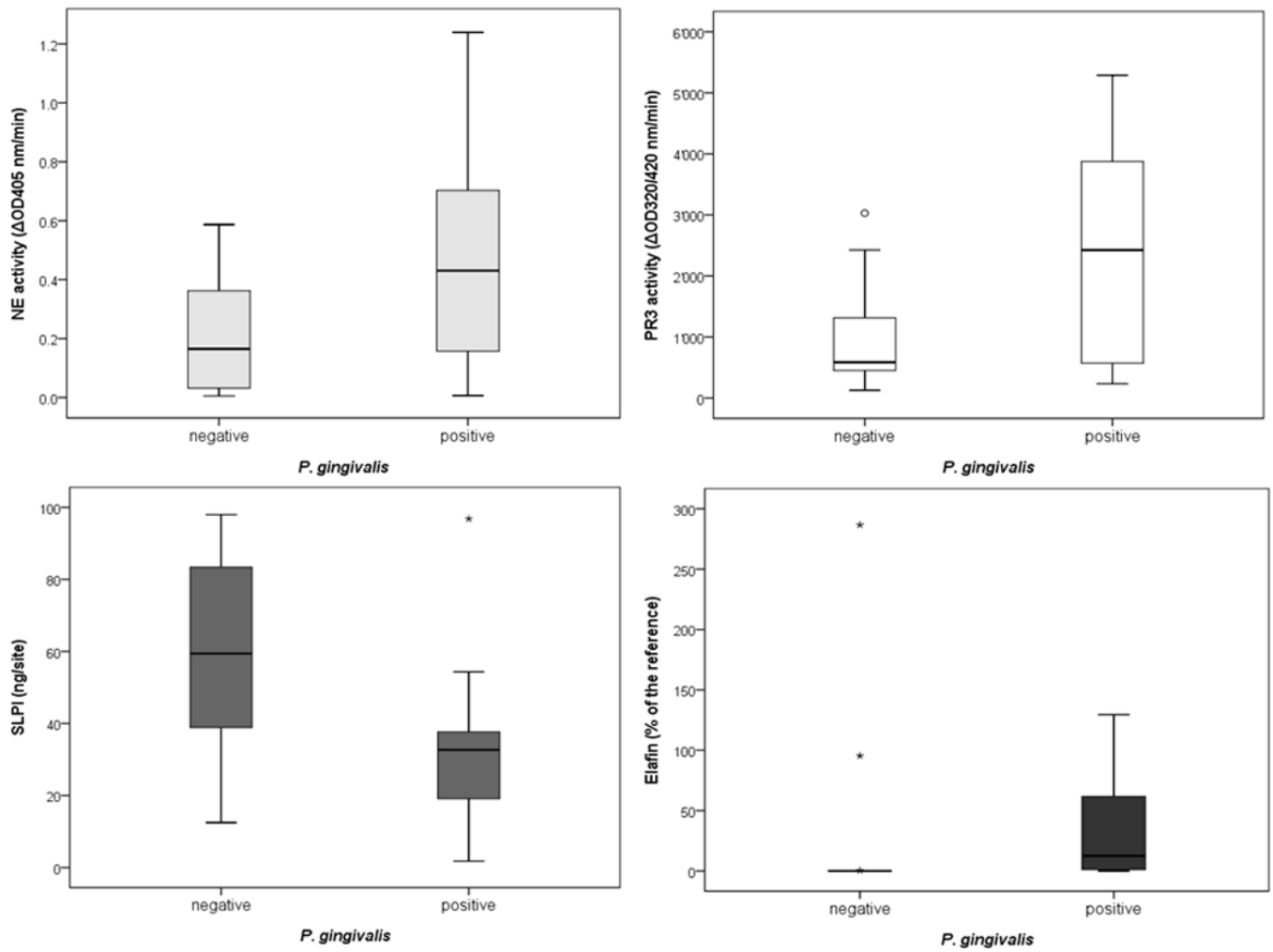
**Figure 2.** Activities of the neutrophil serine proteases, PR3 determined by using fluorogenic substrate (Abz-GVADnVADYQ-Y(NO<sub>2</sub>)-D) and NE by using chromogenic substrate (N-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide) in GCF obtained from patients with AP (aggressive periodontitis), CP (chronic periodontitis), and gingivitis as well as from periodontally healthy subjects



**Figure 3.** Level of SLPI determined by using ELISA in GCF obtained from patients with AP (aggressive periodontitis), CP (chronic periodontitis), and gingivitis as well as from periodontally healthy subjects.



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



**Figure 5.** Activities of the neutrophil serine proteases, PR3 and NE, as well as the levels of the protease inhibitors SLPI and elafin in GCF obtained from patients being tested negatively and positively for *P. gingivalis*.



**Table 1**

## Demographic and clinical data

	<b>Control Group n=9</b>	<b>Gingivitis n=7</b>	<b>Chronic Periodontitis n=10</b>	<b>Aggressive Periodontitis n=5</b>
Age (mean $\pm$ SD)				
(years)	33.2 $\pm$ 9.8	38.6 $\pm$ 7.8	45.6 $\pm$ 12.9	39.4 $\pm$ 8.6
Gender (m:f)	5:4	3:4	5:5	3:2
PD (mean $\pm$ SD) (mm)	1.65 $\pm$ 0.37	2.23 $\pm$ 0.78	4.40 $\pm$ 0.75	4.67 $\pm$ 0.88
BoP (mean $\pm$ SD) (%)	7.20 $\pm$ 10.65	80.34 $\pm$ 9.88	90.56 $\pm$ 18.05	89.67 $\pm$ 10.54
Teeth (mean $\pm$ SD)	27.80 $\pm$ 2.23	28.33 $\pm$ 1.63	27.02 $\pm$ 2.36	27.31 $\pm$ 2.43

**Table 2**

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

	Controls n=9	Gingivitis n=7	Chronic Periodontitis n=10	Aggressive Periodontitis n=5
<i>P.gingivalis</i>				
Positive	0 (0%)	4 (57%)	9 (90%)	4 (80%)
High load*	0 (0%)	2 (29%)	3 (30%)	2 (40%)
<i>T. forsythia</i>				
Positive	0 (0%)	6 (86%)	8 (80%)	4 (80%)
High load*	0 (0%)	3 (43%)	0 (0%)	1 (20%)
<i>T. denticola</i>				
Positive	0 (0%)	4 (57%)	6 (60%)	3 (60%)
High load*	0 (0%)	2 (29%)	0 (0%)	1 (20%)
<i>A. actinomycetemcomitans</i>				
Positive	0 (0%)	1 (14%)	2 (20%)	2 (40%)
High load*	0 (0%)	0 (0%)	2 (20%)	2 (40%)
<i>P. intermedia</i>				
Positive	0 (0%)	1 (14%)	1 (10%)	1 (20%)
High load*	0 (0%)	0 (0%)	0 (0%)	0 (0%)

\* high load was defined as an intensity of the band of  $\geq 50\%$  in relation to the hybridization control

**Table 3**

Significant Correlations (Spearman) between different variables within GCF

	Coefficient R	P
Elafin		
SLPI	-0.378	0.036
<i>P. 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