In vivo expression of proteases and protease inhibitor, a serpin, by periodontal pathogens at teeth and implants

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SUMMARY

Porphyromonas gingivalis and *Tannerella forsythia* secrete proteases, gingipains and KLIKK-proteases. In addition, *T. forsythia* produces a serpin (miropin) with broad inhibitory spectrum. The aim of this pilot study was to determine the level of expression of miropin and individual proteases *in vivo* in periodontal and peri-implant health and disease conditions. Biofilm and gingival/peri-implant crevicular fluid (GCF or PISF respectively) samples from healthy tooth and implant sites (n=10), gingivitis and mucositis sites (n=12), and periodontitis and peri-implantitis sites (n=10). Concentration of interleukins (IL)-8, IL-1β and IL-10 in GCF was determined by ELISA. Loads of *P. gingivalis* and *T. forsythia* and the presence of proteases and miropin genes were assessed in biofilm by qPCR, while genes expression was estimated by qRT-PCR.

Presence of *P. gingivalis* and *T. forsythia,* as well as the level of IL-8 and IL-1β, were associated with disease severity in the periodontal and peri-implant tissues. In biofilm samples harboring *T. forsythia* genes encoding proteases were found to be present at 72.4% for *karilysin* and 100% for other KLIKK-proteases genes and *miropin.* At the same time, detectable mRNA expression of individual genes was in the range from 20.7% to 58.6% samples (for *forsylisin* and *miropsin-1*, respectively). In comparison to the *T. forsythia* proteases, *miropin* and the gingipains were highly expressed. The level of expression of gingipains was associated with those of *miropin* and certain *T. forsythia* proteases around teeth but not implants. Cumulatively, KLIKK-proteases and especially miropin might play a role in pathogenesis of both periodontal and peri-implant diseases.

INTRODUCTION

According to an emerging paradigm, pathogenesis of periodontal disease is considered as a result of disturbed homeostasis within tooth-supporting tissues by dysbiotic microbiota leading to inflammation and slowly eroding periodontal tissues.[1] Using murine model of diseases it was shown that the presence of certain bacteria might influence the entire microbiome around the tooth by modulating the immune response, thus leading to a shift from a symbiotic into a dysbiotic microbiota.[1] In particular, *P. gingivalis* was designated as a keystone pathogen in this transition.[2] The virulence of *P. gingivalis* heavily relies on its arginine- and lysine-specific cysteine proteases, called gingipains.[3, 4] The arginine-specific gingipains (RgpA and RgpB) are encoded by two genes, *rgpA* and *rgpB*. The lysine-specific gingipain (Kgp) originates from a single gene, *kgp*.[5] Gingipains can skew the immune response by cleaving immunoglobulin G1, interfering with the complement system, and disassembling cytokine cross-talk networks.[6, 7]

Apart from *P. gingivalis* other bacterial species such as *T. forsythia* may play a similar key role. *T. forsythia*, together with *P. gingivalis,* is a part of the so called red complex,[8] and they are very often found together in sites with periodontal destruction.[9, 10] For example, *T. forsythia* was detected in approximately 50% of young adult patients with gingivitis,[11] while in 85% of cases in subjects with chronic periodontitis often (50% cases) in association with *P. gingivalis.*[10] However, little is still known regarding the virulence factors of *T. forsythia*. The genome of *T. forsythia* contains many sequences that apparently encode proteases.[12] In particular, 6 enzymes with a KLIKK sequential motive at the C-terminus and therefore referred to as KLIKK-proteases are of interest as putative virulence factors.[12] Interestingly, *T. forsythia* also secretes miropin, a potent protease inhibitor belonging to the serpin superfamily, which may contribute to pathogenicity. The unique feature of miropin is the ability to inhibit a broad range of target proteases, including neutrophil-derived cathepsin G and elastase important for antibacterial activity of these phagocytes.[13, 14]

During the last 2-3 decades, replacement of teeth with implants became a predictable and widely used therapy. However, peri-implant diseases are now becoming increasingly prevalent and negatively impacting oral health. In a recent systematic review, the weighted mean prevalence of peri-implant mucositis and peri-implantitis among individuals with implants was 43% and 22%, respectively.[15] Despite major similarities in the pathogenesis of peri-implant diseases with that of periodontal diseases, there are apparent differences in the host response to the bacterial challenge in these diseases.[16] For example, *T. forsythia* appears to play a greater role in pathogenesis of peri-implant disease than in periodontitis. Ten years after placement of implants, greater numbers of *T. forsythia* were found at implant

sites than at the adjacent teeth in correlation with disease severity.[17] The tissue destruction in peri-implantitis seems to be more progressive and extensive than in periodontitis.[18] So far, however, it is not known which *T. forsythia* putative virulence factors are expressed *in vivo* and whether there are differences in their expression between periodontal and peri-implant diseases and/or disease severity.

Thus, the aim of this pilot study was to investigate expression of *T. forsythia* miropin and KLIKK-proteases *in vivo* in periodontal and peri-implant diseases, correlate differences in their expression with the disease entity, and determine a possible association between expression of *T. forsythia* and *P. gingivalis* proteases in sites infected with both pathogens.

MATERIALS AND METHODS

Subject recruitment

Thirty-two subjects were recruited from patients attending the specialty clinic at the Department of Periodontology, Faculty of Odontology, Malmö University, Malmö, Sweden. These subjects contributed with periodontally healthy sites and healthy implants (n=10), with gingivitis and mucositis sites (n=12), as well as with periodontitis and peri-implantitis sites (n=10).

Periodontal diseases (gingivitis, periodontitis) were defined based on the classification system established in 1999,[19] while peri-implant diseases (mucositis and peri-implantitis) were defined according to Zitzmann and Berglundh.[20] An ethical approval was granted by the Regional Ethics Committee in Lund, Sweden (Dr. nr.; 2014/700), and all patients signed an informed written consent prior to entering the study.

Sampling

Samples were obtained from the site of the tooth or implant with the deepest pocket depth, according to existing dental record registrations, using paper points and paper strips. The periodontal or peri-implant probing depths were < 4 mm in periodontal health, 3 – 5 mm in gingivitis, 3-6 mm in peri-implant-health or mucositis, 5 - 9 mm in case of periodontitis and 6 – 9 mm in peri-implantitis lesions. For collecting gingival crevicular fluid (GCF) or peri-implant sulcular fluid (PISF) the site was gently air dried and isolated by cotton rolls; then paper strips were inserted in the entrance of the sulcus (superficial method)[21] for 30 s. This procedure was immediately followed by collection of subgingival biofilm. For this, endodontic paper points were inserted into pockets until resistance from the base of the pocket was felt and were kept in place for 30 s. After sampling, paper strips and points were stored in sterile

tubes. The tubes for storing paper points also contained 300 µl of RNAlater (RNAlater®, Sigma-Aldrich, St. Louis, USA). Paper points and strips were stored shortly after collection at -80°C until processed.

Levels of GCF/PISF biomarkers

GCF/PISF samples were eluted in 750 µl phosphate-buffered saline (PBS) overnight at 4°C. The levels of interleukin (IL)-8, IL-1β and IL-10 in the GCF/PISF were determined by using commercially available ELISA kits (DuoSet® ELISA Development Systems kits (R&D Systems, Inc., Minneapolis, Minnesota, USA) according to the manufacturer's instructions. The detection levels were 1 pg/site each.

DNA/RNA extraction

DNA and RNA were extracted simultaneously from paper points using a DNA/RNA extraction kit (innuPREP DNA/RNA Mini Kit, Analytik Jena, Jena, Germany) according to the manufacturer's instructions.

Determination of bacterial counts

To determine counts of bacteria being associated with periodontitis (*P. gingivalis*, *T. forsythia*, *Treponema denticola*, *Aggregatibacter actinomycetemcomitans*), the real-time polymerase chain reaction (PCR) was performed using the 7500 Real-time PCR System (Applied Biosystems™, Foster City, USA) and reference strains as described previously.[22] The detection level was determined as 10^3 bacteria per sample.

Expression of bacterial proteases and miropin

To determine the *in vivo* expression of *T. forsythia* proteases *miropsin-1*, *miropsin-2*, *mirolysin*, *mirolase*, *karilysin* and *forsylisin*-1, the protease inhibitor *miropin,* and the gingipains *rgpA*, *rgpB* and *kgp*, cDNA was amplified from total RNA obtained from the paper points. The RNA was first treated with DNase I, RNase-free (ThermoFisher Scientific, Waltham, Massachusetts, USA) and thereafter cDNA was generated using RevertAid Reverse Transcriptase (ThermoFisher Scientific) according to the manufacturer's instructions.

Real-time PCR using GoTaq® qPCR Master Mix (Promega) and the respective primers (Table 1) was performed for different bacterial proteases and miropin according to the

manufacturer's instructions. Quantification was made related to the *sod* gene expression of respective bacteria.

Negative controls contained 1:5 diluted RNA (before generation of cDNA) and as positive controls cDNA generated from RNA of 24 h-cultures of *P. gingivalis* ATCC 33277 as well as of *T. forsythia* ATCC 43037 were used.

Statistics

Statistical analysis was performed using non-parametric tests (Kruskal-Wallis, Mann-Whitney, Friedman tests) for continuous variables and Chi^2-test for dichotomized variables, with SPSS 23.0 (IBM, Chicago, IL, USA).

RESULTS

Presence of bacteria and biomarkers levels in gingival / peri-implant fluid

There were no statistically significant differences between biofilms collected from the teeth and implants in terms of frequency of detection and numbers of the various bacteria at health and at the various disease severities. In both cases bacterial presence increased from health to disease in correlation with severity of periodontal and peri-implant destruction (Table 2). The differences between healthy and diseased sites were statistically significant for *T. forsythia* and *T. denticola,* both, at the teeth (p=0.024; p=0.007) and at implants (p=0.011; p=0.004).

In GCF collected from periodontal sulci or pockets around teeth, the level of IL-8 correlated with the severity of periodontal disease (p=0.048), and it was significantly higher in periodontitis than in gingivitis (p=0.036) or in health (p=0.029). Amount of IL-1β in GCF also varied in a manner dependent on the periodontal disease severity (p=0.010). At teeth, levels of IL-1β were higher in periodontitis than in gingivitis (p=0.004), whereas at implants there was no statistically significant difference between mucositis and peri-implantitis but only between the health and mucosistis sites (p=0.025) and between the health and peri-implantitis sites (p=0.043). Comparing teeth with implants, the levels of IL-1β in periodontitis exceeded those in peri-implantitis (p=0.035). The anti-inflammatory IL-10 was lower in periodontitis and peri-implantitis in comparison with gingivitis (p=0.021) and mucositis (p<0.001). Results with statistically significant differences are presented in Figure 1.

Porphyromonas gingivalis and expression of gingipains

Expression of gingipains genes was standardized on expression of the housekeeping gene *sod*. In samples with *P. gingivalis* counts less than 10^5 we sometime failed to detect the *sod* transcript therefore we have compared gingipain expression levels only in samples with ≥10^5 bacteria per site. In this subset of samples expression of each gingipain was always detectable, with *kgp* expression being the highest, followed by *rgpA* and *rgpB* ($p<0.001$) (Table 3).

Tannerella forsythia and expression of its proteases and miropin

The part of samples bearing less than 10^5 *T. forsythia* were excluded from quantitative analysis because no mRNA for the *sod* gene could be detected (Table 4). In the subset of biofilm samples with bacterial load >10^5 the detection of the protease genes varied from 72.4% (*karilysin*) to 100% (*miropsin-2*, *mirolysin*, *mirolase*, *miropin*) of the samples. Detectable amount of mRNA of protease genes transcripts was found at lower frequency ranging from 20.7% (*forsylisin-1*) to 58.6% (*miropsin-1*) of the samples. Quantitatively except for one peri-implantitis specimen having high level of *miropsin-2*, in all other samples expression of any protease gene was always low (in comparison to *sod*).

In *T. forsythia*-positive samples (bacterial count over 10^5) there was no statistically significant difference in detection of protease gene transcripts in samples collected from the tooth and implant sites (Table 5). Exception was *miropsin-2* showing higher levels of expression in periodontitis or peri-implantitis than in periodontal health/gingivitis or peri-implant health/mucositis (teeth: $p=0.015$; implants: $p=0.044$).

Contrary to the proteases, *miropin* was highly expressed. In all samples positive for *T. forsythia*, miropin was detected on the mRNA level. In samples with ≥10^5 *T. forsythia* expression was in median 2.34 related to *sod* (Table 4).

Correlation of proteases expressions

Expression of the three gingipains was highly correlated, both at teeth ($r=0.890$ up to $r=0.972$, each $p<0.001$) and implants biofilms ($r=0.996$ up to $r=0.999$, each $p<0.001$). Also expression of *T. forsythia* proteases was correlated, with the strongest correlation seen for *karilysin* and *forsylisin-1* ($r=0.856$, $p<0.001$), then *miropsin-1* and *mirolase* ($r=0.769$, $p<0.001$) in biofilm derived for the subgingival teeth surface. In biofilm collected from the implant sites the strongest correlation was found between *mirolase* and *miropsin-2* ($r=0.796$, $p<0.001$) and then between *mirolase* and *miropsin-1* ($r=0.790$, $p<0.001$). In biofilm collected from teeth also

the *miropin* expression was found to correlate with those of *miropsin-1* (r=0.740, p<0001) and *miropsin-2* (r=0.784, p<0.001). In case of the biofilm from implants the correlation factor r did not exceed 0.6 for any combination of *T. forsythia* protease genes expression.

In biofilm collected from tooth sites, gingipains mRNA levels correlated with expression of the *T. forsythia* proteases. The strongest correlation was found between *rgpB* or *kgp* with *miropsin-2* (r=0.523, p=0.002; r=503, p=0.003, respectively) and between *kgp* and *mirolase* (r=0.501, p=0.003). There was also a positive correlation between gingipains and *miropin* expressions (*rgpA*: r=0.536, p=0.002; *rgpB*: r=0.429, p=0.014, *kgp*: r=0.550, p=0.001).

In biofilm from implant sites, no significant correlation between gingipains and any of *T. forsythia* proteases was detected (r was always below 0.5). The same was observed for relation between the *miropin* and gingipain expression.

In samples collected from teeth sites, gingipain expression in biofilm did not correlate with the levels of biomarkers in GCF. In contrast, the expression of certain *T. forsythia* proteases correlated positively with the IL-1β GCF level. This was found for *miropsin*-1 (r=0.417; p=0.018) and *karilysin* (r=0.406, p=0.021). In samples from implant sites, expression of *miropsin*-1 was positively associated with levels of IL-1β (r=0.417, p=0.018) and negatively associated with those of IL-10 (r=-0.430, p=0.014). Also the expression of *miropsin*-2 (r=-0.391, p=0.027) and *mirolase* (r=-0.510, p=0.003) were inversely correlated with IL-10 levels in PISF. No association of the *miropin* expression with the biomarker levels was observed.

Found correlations were underlined by a principal components analysis, where components with more than 20% variance were considered (Table 6). Principal components analysis is a multivariate statistical method used to find hidden complex, and possible relationships between features in a data set. Correlated features are converted by means of the principal axes transformation into new features, the so-called 'principal components' where the importance of a factor (variable) is expressed by its loading. The principal components themselves are uncorrelated.[23]

The clear association between certain *T. forsythia* proteases is visible both in the teeth- and implants-derived biofilms. In this analysis, *miropin* has only a relevant loading at teeth, and biomarkers (IL-8, IL-1β) are linked with *karilysin* expression also in the teeth-surface biofilm.

DISCUSSION

In this study, the presence and expression of *P. gingivalis and T. forsythia* proteases and the bacterial protease inhibitor, miropin, was assessed in biofilm samples derived from teeth and implants, in health and disease. Of note, instead the 16S RNA gene known to differ in number of copies even within one species[24] we chose *sod* as a reference gene. Being aware

that *sod* expression depends on oxygen tension[25] we assumed that sampled sites were rather uniform with respect to anaerobic conditions and small variations in oxygen tension should not significantly affect local *sod* expression. The above threshold level of mRNA of bacterial *sod* was reliably and quantitatively measured in all samples having a bacterial load equal or higher than 10^5 bacteria per site. Groups were differentiated related to the degree of inflammation without alveolar bone loss and showing different levels of pathological bone destruction. Both pro-inflammatory biomarkers, IL-8 and IL-1β increased from health to disease, and with disease severity, i.e. from gingivitis/mucositis to periodontitis/peri-implantitis. On the other hand, the level of the anti-inflammatory IL-10 was high at inflamed sites and low at sites exhibiting bone destruction, both at implants and at the teeth. The finding of higher levels of IL-1β and lower levels of IL-10 at peri-implantitis sites is in accordance with the observation by Casado *et al*.[26] Interestingly, in contradiction to the observation by Hultin *et al.*[27] showing similar levels of this cytokine in sites with peri-implantitis and periodontitis collected from the same patient, we have found lower levels of IL-1β at peri-implantitis sites than in GCF collected from periodontitis sites. This discrepancy suggests that more detailed studies need to be performed to differentiate importance of cytokines in the pathology of peri-implantitis and periodontitis.

As expected, disease severity was associated with an increased prevalence of certain bacterial species, i.e. a significantly larger numbers of *T. forsythia* and *T. denticola* were found at periodontitis/peri-implantitis sites compared to gingivitis/mucositis sites, and in gingivitis/mucositis sites they were more abundant than in healthy sites. However, there was never a difference between the bacterial counts at the teeth and implants, collected from sites of the comparable severity, i.e. gingivitis versus peri-implant mucositis, and periodontitis versus peri-implantitis. In general, our finding of correlation between the pathogens load and the disease severity is in accordance with results reported by Cortelli *et al*. [28] In this study authors counted periodontal pathogens collected from around teeth and implants from two independent groups of individuals. They found lower amount of *T. denticola* and *T. forsythia* at implants in comparison with the teeth.[28] This observation contradicts our finding but the discrepancy must be due to different experimental set-up. On the other hand, the fact that *T. denticola* and *T. forsythia* can easily transmit from a periodontally diseased tooth to an implant[29-31] and probably vice versa, explains the comparable load of these periodontal pathogens around the diseased teeth and implants herein.

P. gingivalis cysteine proteases (gingipains) were always expressed both at implants and at teeth, which corroborates with results of our recent *in vitro* study using titanium and dentine disks.[32] The highest mRNA expression related to *sod* was found for *kgp,* followed by *rgpA*

and *rgpB*. Level of Kgp was determined as being up to 10 nM in periodontium,[33] those of Arg-gingipains up to 1.5 µM.[34] Unfortunately, there is still a little data on gingipains level at peri-implantitis sites. DNA of *rgpA*, *rgpB* and *kgp* was used as vaccines in an animal model with *kgp* being most efficiently blocking bone loss in experimental peri-implantitis whereas *rgpB* was ineffective.[35] Gingpains are able to cleave adherence junctions of epithelial cells,[36] a property which may impair epithelial barrier around implants. Moreover, gingipain-dependent manipulations of recruitment and functions of neutrophils,[37] exploitation of the complement system, activation of the kinin system leading to activation of prostaglandin in osteoblasts and extravasation,[6] might be of importance both at the teeth and implants.

Expression of *T. forsythia* proteases was found at lower level than that of gingipains. Protease genes transcripts were determined in 20 – 59% of the samples with *T. forsythia* load exceeding $\geq 10^5$ bacterial cells. This result confirms findings of our recent study on patients with periodontitis, in which the *in vivo* expression of *karilysin* and *mirolysin* was experimentally verified in a small set of samples.[38, 39] In the present study, mainly transcripts (mRNA) of *miropsin-1*, *miropsin-2* and *mirolase* were found. In general, expression of *T. forsythia* proteases, in particular *miropsin-2* was associated with periodontal or peri-implant destruction supporting a potential role of the KLIKK-proteases in disease progression. There is little known about miropsin-1 and miropsin-2 up to now. Miropsin-1 and miropsin-2 are proteolytically active when using casein or gelatine as substrates, however their activity was lower when compared to other KLIKK proteases.[40] Nevertheless, they may specifically target molecules important for local homeostasis in periodontal tissues. In contrast to other KLIKK proteases, miropsin-2 is not auto-processing into lower molecular mass forms.[40] Finally, mirolase was characterized as a calcium-dependent serine protease with the ability to degrade fibrinogen and hemoglobin.[12]

Karilysin and *mirolysin* expression was found in about 40% of the samples with more than 10^5 *T. forsythia*. Karilysin was characterized as a matrix metalloprotease-like enzyme able to degrade elastin, fibrinogen and fibronectin.[41] The pathogenic potential of karylisin to interfere with innate immunity is manifested by its ability to inactivate the antimicrobial peptide LL-37[42] and to induce expression of TNFα in macrophages, and then shedding it from the macrophage surface thus leading to an increased release of this proinflammatory cytokine.[43] Similar to karilysin, the metalloprotease mirolysin can also inactivate LL-37.[44] Both karilysin and mirolysin inhibit all pathways of the complement system.[38, 39] Expression of *T. forsythia* proteases was highly correlated at the teeth and furthermore associated also with gingipains expression. Synergism between gingipains and *T. forsythia* has been investigated. Gingipains are involved in a synergistic increase of IL-6 production of macrophage-like cells

when infected with *T. forsythia* and *P. gingivalis.*[45] Gingipains enhance phagocytosis of *T. forsythia*, but diminish their killing by macrophages.[46] Further research should focus on the potentially synergistic role of KLIKK proteases and gingipains in pathogenesis of periodontal and peri-implantitis diseases.

Miropin, a protease inhibitor belonging to the serpin superfamily was highly expressed. The miropin mRNA level in tested samples was higher than that of *sod,* the house-keeping gene used as the reference. Bacterial serpins are mainly found in commensals. *Eubacterium sireaum*, an inhabitant of the human gut, synthesizes serpins which inhibit neutrophil elastase, protease 3 and certain gut proteases and in this way may contribute to homoeostasis in the gut.[47] Also miropin is the very potent inhibitor of serine endopeptidases, such as cathepsin G and neutrophil elastase.[13] *T. forsythia* obviously acquired this serpin gene by horizontal gene transfer from eukaryotes.[14] Miropin is attached to the bacterial cell surface and also located in the periplasm exerting protection against *T. forsythia* own proteases as well as host and other bacteria enzymes, which may degrade the surface S-leyer.[13] At the teeth, expression of miropin was not only strongly associated with the *T. forsythia* proteases, but also correlated with gingipains. This suggests that gingipains might be involved in regulation of miropin or *vice versa*. It can be speculated that the pathogenetic role of miropin is exerted by maintenance of inflammation at a low chronic level.

Correlation assessment made separately or by the principal components analysis showed differences between the teeth and implants. It is of interest to note, that significant associations between biomarkers' levels, and expression of gingipains and *T. forsythia* proteases and miropin was found only at teeth. Specifically, expression of the KLIKK proteases, as well as the protease inhibitor *miropin* by *T. forsythia* was correlated with gingipains. Furthermore, *karilysin* positively correlated with pro-inflammatory biomarkers and negatively with the anti-inflammatory cytokine IL-10. At implants there was no significant association between *miropin* and gingpains or between *miropin* and the *T. forsythia* proteases. Also no correlation was found between bacterial proteins and the analyzed biomarkers. This may suggest a substantial difference in regulation of bacterial virulence factor expression and host responses to bacteria at implants and teeth, which may partly be due to the existing anatomical differences between implants and teeth. For example, Sharpey's fibres form a complex network around the teeth together with a vascular plexus, whereas dental implants are surrounded by collagen fibres run in parallel and there are a few blood vessels[48] thus the established peri-implant soft tissue resembles a scar tissue.[49] Further, it has been shown that implant sites respond with a stronger inflammatory reaction than teeth sites when exposed to biofilm.[50] Another factor contributing to differences may be

related to variations in biofilm formation on the tooth and implant surface. Recently an *in vitro* study found that miropin expression was lower at titanium implants than at dentine disks.[32] However, the miropin expression by *T. forsythia* adhered either to the dentin or titanium surface exceeded up to 500-fold the expression of proteases (miropsin-1, miropsin-2, mirolase.[32] It is clear that more research is needed on *T. forsythia,* not only as the periodontal pathogen, but also a potentially pathogenic member of the peri-implant biofilm consortium.

In summary, the focus of this study was on *in vivo* expression (mRNA levels) of the proteases and the protease inhibitor by periodontal pathogens. Our results showed much higher expression of gingipains of *P. gingivalis* and *miropin* of *T. forsythia* than the KLIKK proteases of the latter bacterium. Taking into account a broad inhibitory spectrum of miropin, this data suggest that this protein may play a regulatory role in a multispecies dysbiotic biofilm forming on teeth and implant surfaces and may contribute to the initiation and/or progression of both periodontal and peri-implant diseases.

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Table 1

Primer pairs used in real-time PCR for detection of bacterial proteases (*rgpA*, *rgpB*, *kgp*, *miropsin-1*, *miropsin-2*, *mirolase*) and of *T. forsythia* protease inhibitor (*miropin*) as well as the house keeping genes (*sod*)

Species	Gene	Primer	Reference
P. gingivalis	*rgpA*	fwd: 5′-TAT CCT TCG TGA TGT GCG TG-3′ rev: 5′-GCT GTA ACG GGA GAA GCA AT-3′	Frohlich *et al*. [51]
	rgpB	fwd: 5′-CAT TCT CCT CTC TGT TGG GA-3′ rev: 5′-CGT AGG GGA TTT GAT CAG GA-3′	Frohlich *et al*.[51]
	kgp	fwd: 5′-TCA AGC AGT TCG ATG CAA GC -3′ rev: 5′-ACT TGG GTC AGT TCT TGT CC-3′	Frohlich *et al*.[51]
	sod	fwd: 5′-AAT TCC ACC ACG GTA AGC AC-3′ 5′- TTC TCG ATG GAC AGT TTG CC-3′	Frohlich *et al*.[51]
T. forsythia	*miropsin-1*	fwd: 5’-CGT GCG TGA AGA AGC CAT TA-3′ rev: 5’-AAC CCG GAT GTT CAT ACC CC-3′	according to Ksiazek *et al*.[40]
	miropsin-2	fwd: 5’-TCC TGA CCG ACC TGA TCA AA-3’ rev: 5’-TCG GCA TTG GAA ATT TCG GA-3′	according to Ksiazek *et al*.[40]
	karilysin	fwd: 5’- TTA CAG TTG CGG CAC ATG AG-3’ rev: 5’- TGT TAA TGG TTG CTC GCA CT -3′	accession: Tf 0367; BFO2683
	mirolysin	fwd: 5’- CGA ACA TCG ACT TCC ACA GA-3’ rev: 5’- TGT TTT AGG GAA CGA AGG ACA -3′	accession: Tf0341; BFO2661
	mirolase	fwd: 5’-TGC CGC AAA TCA TAA TGG TA rev: 5’-GTC CAT CCC TTC CTT GAG TG-3′	according to Ksiazek *et al*.[40]

	forsylisin-1	fwd: 5’- GAT GAT GGG TTT ACA ATT GAC G-3’ rev: 5’- TCT AGT AAT TTG TTC TCC AAT TTG C -3′	accession:Tf2162; BFO1168
	miropin	fwd: 5‘-ATG CCT TTG CCT TCG ATC TG-3′ rev: 5‘-CTT CCC GTA GTG AAT GGC TG-3′	according to Ksiazek *et al*.[13]
	sod	fwd: 5’-GCA CGT CTG TTC TGG TAA TCC-3′ rev: 5’-CCT GCA ATT CAA GCC TCA GA-3′	accession: JUET01000058.1

Table 2

Presence of selected species in subgingival and peri-implant biofilm

Species	**Healthy**		**gingivitis / mucositis**		**periodontitis / peri-implantitis**	
	tooth (n=10)	**implant (n=10)**	**tooth (n=12)**	**implant (n=12)**	**tooth (n=10)**	**implant (n=10)**
A. actinomycetemcomitans	1 (10.0)	2 (20.0)	3 (25.0)	3 (25.0)	3 (30.0)	3 (30.0)
P. gingivalis	3 (30.0)	2 (20.0)	4 (33.4)	2 (16.7)	5 (50.0)	6 (60.0)
T. forsythia	2 (20.0)	4 (40.0)	5 (41.7)	6 (50.0)	8 (80.0)	10 (100)
T. denticola	1 (10.0)	3 (30.0)	5 (41.7)	6 (50.0)	8 (80.0)	10 (100)

Table 3

DNA (dichotomized results) and mRNA expression of gingipains in samples with less (dichotomized results) and equal or more than 10^5 (dichotomized results and quantitative data related to *sod*) *Porphyromonas gingivalis*

	positive <100,000 (n=11)		**positive ≥100,000 (n=11)**				
	DNA	**mRNA**	**DNA**	**mRNA**	**related to *sod***		
	n (%)	**n (%)**	**n (%)**	**n (%)**	**median**	**minimum**	**maximum**
rgpA	4 (36.4)	1 (9.1)	11 (100)	11 (100)	0.246	<0.001	24.859
rgpB	6 (54.6)	1 (9.1)	11 (100)	11 (100)	0.011	<0.001	15.122
kgp	8 (72.7)	2 (18.2)	11 (100)	11 (100)	27.611	<0.001	566.65
sod	10 (90.9)	7 (63.6)	11 (100)	11 (100)	1	1	1

Table 4

DNA (dichotomized results) and mRNA expression of *Tannerella forsythia* proteases and miropin in samples with less (dichotomized results) and equal or more than (dichotomized results and quantitative data related to *sod*) 10^5 *T. forsythia*

	positive <100,000 (n=6)		**positive ≥100,000 (n=29)**				
	DNA	**mRNA**	**DNA**	**mRNA**	**related to *sod***		
	n (%)	**n (%)**	**n (%)**	**n (%)**	**median**	**minimum**	**maximum**
miropsin-1	5 (83.3)	1 (16.7)	27 (93.1)	17 (58.6)	<0.001	0.000	0.615
miropsin-2	3 (50.0)	0 (0)	29 (100)	14 (48.3)	0.001	0.000	3.051
karilysin	4 (66.7)	1 (16.7)	21 (72.4)	11 (37.9)	0.000	0.000	0.792
mirolysin	4 (66.7)	0 (0)	29 (100)	11 (37.9)	0.000	0.000	0.008
mirolase	4 (66.7)	0 (0)	29 (100)	15 (51.7)	0.006	0.000	0.380
forsylisin-1	0 (0)	0 (0)	25 (86.2)	6 (20.7)	0.000	0.000	0.012
miropin	6 (100)	6 (100)	29 (100)	29 (100)	2.340	<0.001	17.609
sod	6 (100)	3 (50)	29 (100)	29 (100)	1	1	1

Table 5

mRNA expression (dichotomized results) of *Tannerella forsythia* proteases and miropin related to clinical data

	Tooth			**implant**		
	healthy	**gingivitis**	**periodontitis**	**healthy**	**mucositis**	**peri-implantitis**
	n (%)	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Total	2	4	8	2	4	9
miropsin-1	1 (50.0)	2 (50.0)	7 (87.5)	0 (0)	2 (50.0)	5 (55.6)
miropsin-2	0 (0)	2 (50.0)	7 (87.5)	0 (0)	0 (0)	5 (55.6)
karilysin	0 (0)	0 (0)	5 (62.5)	0 (0)	2 (50.0)	5 (55.6)
mirolysin	1 (50.0)	1 (25.0)	3 (37.5)	0 (0)	1 (25.0)	5 (55.6)
mirolase	0 (0)	2 (50.0)	6 (75.0)	0 (0)	1 (25.0)	6 (66.7)
forsylisin-1	0 (0)	0 (0)	3 (37.5)	0 (0)	1 (25.0)	2 (22.2)
miropin	2 (100)	4 (100)	8 (100)	2 (100)	4 (100)	9 (100)

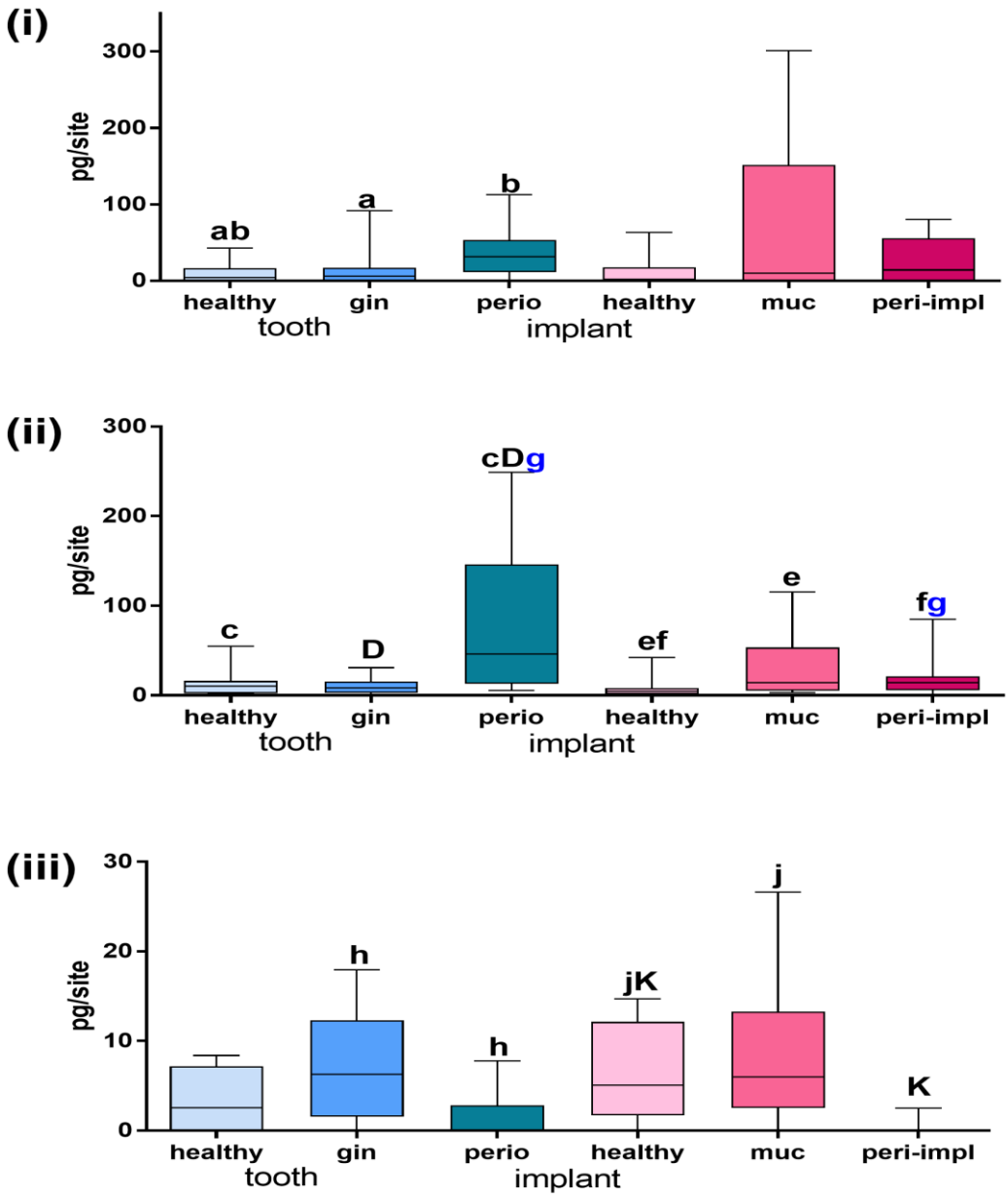
(only samples with *T. forsythia* ≥10^5)

Table 6

Principal components analysis (loadings of the main components) at teeth and implants

Variable	Principal component teeth		Principal component implants	
	Component 1	**Component 2**	**Component 1**	**Component 2**
rgpA	0.616	0	0	0.939
rgpB	0	0	0	0.937
kgp	0	0	0	0.885
miropsin-1	0.743	0	0	0
miropsin-2	0	0	0.873	0
karilysin	0	0.645	0	0
mirolysin	0	0	0.708	0
mirolase	0.906	0	0.910	0
forsylisin-1	0.877	0	0.855	0
miropin	0.741	0	0	0
IL-8	0	0.549	0	0
IL-1β	0	0.867	0	0
IL-10	0	-0.600	0	0
% of variance	25.23	21.00	25.57	23.09

Components with a variance ≥ 20% are presented. Loadings of the principal components < |0.5| are set to 0 for greater clarity.



Comparison between two groups
at teeth: **a**, **b**, **c**, **h** ($p<0.05$); **D** ($p<0.01$)
at implants: **e**, **f**, **j** ($p<0.05$); **K** ($p<0.01$)
at teeth and implants: **g** ($p<0.05$)

Figure 1

Levels of the chemokine interleukin (IL)-8 (i), and of the cytokines IL-1β (ii) and IL-10 (iii) at teeth being periodontally healthy (healthy), with gingivitis (gin) and with periodontitis (perio) sites as well as at implants being healthy, with mucositis (muc) and with peri-implantitis (peri-impl)

Statistical analysis compared cytokines levels at teeth and at implants as well as between the healthy sites and sites with inflammation (gingivitis, mucositis) and between sites with alveolar bone loss (periodontitis, peri-implantitis).