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Periodontitis and peri-implantitis tissue levels of *Treponema denticola*-CTLP and its MMP-8 activating ability

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ABSTRACT

Background and Aims: Chymotrypsin-like-proteinase of *Treponema denticola* (Td-CTLP) can stimulate the protein expression and activation of matrix metalloproteinase (MMP)-8 (or collagenase-2), a potent tissue destructive enzyme from gingival cells in vitro. The aims of this study were 1) to demonstrate the proMMP-8 (or latent MMP-8) activation by Td-CTLP *in vitro* and 2) to detect Td-CTLP and MMP-8 protein levels in the tissue samples of peri-implantitis and periodontitis patients.
Materials and Methods: proMMP-8 activation by Td-CTLP was analyzed by immunoblots. Tissue specimens were collected from 38 systemically healthy and non-smoking patients; 14 of whom had moderate to severe peri-odontitis, 10 of whom were suffering from peri-implantitis, and finally 14 of whom showed no sign of peri-odontal inflammation nor radiological bone decay (control group). The immune-expression levels of MMP-8 and Td-CTLP in the epithelium and the connective tissue were analyzed immunohistochemically. A pixel color-intensity analyze was performed with ImageJ software (version 1.46c; Rasband WS, National Institutes of Health, Bethesda, MD, USA) to obtain a comparable numeral score for each patient's epithelium and connective tissue MMP-8 and Td-CTLP enzyme level.
Results: Td-CTLP activated proMMP-8 in vitro by converting the $70-75$ kDa proMMP-8 to 65 kDa active MMP-8. Also, lower molecular size $25-50$ kDa parts of MMP-8 were formed. There was no statistically significant dif-ference between the study groups in terms of their MMP-8 and Td-CTLP levels in the epithelium or in the connective tissue.
Conclusion: Regarding the limits of this study, it can thus be said that the Td-CTLP enzyme can activate the host proMMP-8 enzyme. Tissue protein levels of MMP-8 and Td-CTLP do not seem to be changed in peri-implantitis and in periodontitis.

1. Introduction

Periodontitis is the chronic degenerative infectious-inflammatory disease of tooth supporting tissues. Peri-implantitis, in turn, is the equivalent inflammatory disease affecting the surrounding tissues of tooth implant. Virulence factors of pathogenic periodontal bacteria are the main etiological agents of these two diseases. There are, however, various factors, including smoking and diabetes, that can alone or together increase the risk of developing peri-implant and periodontal

diseases or complicate the existing condition (Könönen et al., 2019).

Treponema denticola is gram-negative, obligate anaerobic spirochete bacterium that has high proteolytic activities. Its elevated numbers in saliva or in dental plaque were related to the severity and extend of periodontitis (Kumawat et al., 2016; Sela, 2001; Simonson et al., 1988). Well-known virulence factors of *T. denticola* include the ability of host-cell invasion (Inagaki et al., 2016), chymotrypsin-like-proteinase (Td-CTLP) enzyme activity (Marttila et al., 2014), and cystalysin secretion, an enzyme contributing to hemolysis (Spyrakis et al., 2014).

In vitro studies revealed that *T. denticola* can induce matrix metalloproteinase (MMP)-8 and -9 secretion and degranulation of inflammatory cells (Ding et al., 1996). Indeed, in periodontitis-affected tissues, presence of *T. denticola* is associated with elevated levels of MMP-8 and -9 (Yakob et al., 2013). aMMP-8 activity in oral fluids has been widely studied as biomarker of periodontitis (Sorsa et al., 2020) and peri-implantitis (Ghassib et al., 2019). Whilst the studies on the contribution of *T. denticola* and its CTLP enzyme activity on periodontal and peri-implant disease pathogenesis, to our knowledge, presence and extension of Td-CTLP has not been investigated in peri-implantitis tissues before.

Recent immunohistochemical studies revealed that disrupted macrophage (Galarraga-Vinueza et al., 2020; Fretwurst et al., 2020) and T-cell (Luo et al., 2013; Gualini and Berglundh, 2003) polarization, and complex proinflammatory cytokine and host protease activation cascades (Ghassib et al., 2019; Kensara et al., 2021; Wang et al., 2016) play critical roles in pathogenesis of peri-implantitis. Microbial profile in peri-implantitis biofilms (obligate anaerobe Gram-negative bacteria, asaccharolytic anaerobic gram-positive rods and other Gram-positive species) was also found to be different than of biofilms associated with healthy implants (Kensara et al., 2021). Even though high *T. denticola* numbers were associated with peri-implantitis in various studies (Gürlek et al., 2017; Costa et al., 2019; Wang et al., 2016), tissue Td-CTLP and MMP-8 enzyme immunoexpression levels in relation to peri-implantitis were not studied so far.

In the present study, the hypothesis was that the elevated *T. denticola*-CTLP enzyme activities in periodontal and peri-implant tissues lead to an increase in MMP-8 levels. Thus, in the present study, the aims were 1) to demonstrate the MMP-8 activation by Td-CTLP *in vitro* and 2) to detect Td-CTLP and MMP-8 immunoexpression levels in tissue samples of periodontitis, peri-implantitis and periodontally healthy individuals.

2. Materials and methods

2.1. Human proMMP-8 activation by Td-CTLP

Purified human polymorfonuclear neutrophil (PMN) proMMP-8 and were prepared as described by Hanemaaijer et al. (1997) and (Sorsa et al., 1992). Then proMMP-8 (20 µg) was incubated with Td-CTLP for 20, 40, and 60 min. Aminophenylmercuric acetate (1 mM, APMA, Sigma, St Luis, MO, USA) was used as positive control. The activations were monitored by western-immunoblotting.

2.2. Western immunoblotting

The molecular forms of MMP-8 were detected by a modified enhanced chemiluminescence (ECL) Western blotting kit according to protocol recommended by the manufacturer (GE Healthcare, Amersham, UK) as described earlier (Gürsoy et al., 2018). After electrophoresis with 11 % sodium dodecyl sulphate (SDS)-polyacrylamide gels, the proteins were electrotransferred onto nitrocellulose membranes (Whatman GmbH, Dassel, Germany). After blocking of non-specific binding the membranes were incubated with polyclonal anti-MMP-8 primary antibody (Hanemaaijer et al., 1997) overnight, and then with horseradish peroxidase-linked secondary antibody (GE Healthcare) for 1 h. The proteins were visualized according the ECL Western blotting kit –system and scanned using GS-700 Imaging Densitometer Scanner (Bio-Rad, Hercules, CA, USA) and Bio-Rad Quantity One program.

2.3. Study population

This study consists of 10 patients suffering from moderate to severe peri-implantitis (7 males and 3 females, ages ranging from 36 to 59 years), of 14 patients suffering from moderate to severe periodontitis (7 males and 7 females, ages ranging from 26 to 54 years), and of 14 periodontally inflammation free patients with no radiological signs of bone loss (7 males and 7 females, ages ranging from 20 to 55 years). All participants were systemically healthy. The exclusion criteria were the following: history of smoking, ongoing orthodontic therapy, carrying oral mucosal diseases or active caries lesions, pregnancy, lactation, and the use of prescribed or non-prescribed medication or antibiotics at the time of participation. The Ethics Committee of the Department of Dentistry of the University of Istanbul approved the study plan according to the Helsinki Declaration (2017/41) (Kasnak et al., 2018).

Each patient in the peri-implantitis and periodontitis groups were qualified to have an either moderate or severe form of the disease. For the selection of patients, an examiner conducted calibrated periodontal examinations including plaque index (PI), clinical attachment level (CAL), bleeding on probing (BOP), the measurement of probing pocket depths (PPD) at 4 sites of each tooth or implant with the use of a periodontal probe (UNC-15, Hu-Friendly, Chicago, IL, USA), and radiographic evidence of alveolar bone loss (Kasnak et al., 2018).

All patients had their orthopantomograph taken with an extra oral radiographic system (KODAK 9000 3D, Carestream Dental LLC, Atlanta, GA, USA). The obtained images were then analyzed using a computer software (Dental Imaging software CS 3D, Carestream Dental LLC, Atlanta, GA, USA) especially produced for the analyze and storage of radiographs. Reference points used to measure the amount of alveolar bone loss until the marginal bone level, were the cemento-enamel junction (CEJ) for the periodontitis and healthy group, and the shoulder of the implants for the peri-implantitis group. Measures in millimeters were taken from two sites, the mesial and the distal side of the tooth.

All patients in the present study had a minimum of 15 teeth. In order to qualify for the peri-implantitis group, one had to have CAL $\geq$ 5 mm, PPD $\geq$ 5 mm on both the mesial and distal sites of the implant, BOP had to occur also no matter whether there was related suppuration or exudate leakage. In addition, $\geq$ 3 mm radiographic bone loss had to be found (Sanz et al., 2012). Accordingly, to qualify for the periodontitis group one had to attain the following criteria: three of more teeth with CAL $\geq$ 5 mm, PPD $\geq$ 5 mm with related BOP in minimum two different quadrants. In addition, the radiographically evaluated loss of alveolar bone had to be minimum 25 % of the root length. Patients not having any sites with PPD $>$ 3 mm and CAL $>$ 2 mm were recruited in the healthy control group (Papapanou et al., 2018).

2.4. Tissue samples

All gingival tissue samples were gathered at the Faculty of Dentistry of the University of Istanbul, Turkey. Peri-implantitis tissue samples were collected from the deepest pocket of bone level implants, which were in function for at least seven years, with a restoration of a single cemented crown at the posterior area. All peri-implant tissue sampling sites had clinical signs of active disease (bleeding on probing). Periodontitis tissue samples were harvested from interdental (mesial or distal) surfaces of intact premolar or molar teeth during the routine periodontal flap surgery. Inclusion criteria of sampling sites were having clinical signs of active disease (bleeding on probing), having a PPD of 5–8 mm, and having no prosthodontic restorations (fillings, crowns, or bridges). Also, the samples of periodontally healthy patients were gathered at the occasion of their crown-lengthening surgery of the intact posterior teeth (Kasnak et al., 2018). One tissue sample from each individual was selected, and the oral epithelium site of each sample was then stained (CDI's® tissue marking dye, 0724−2, Cancer Diagnostics Inc., Dunham, NC, USA) in order to accurately distinguish between the oral and sulcular epithelium while analyzing them under the light microscope. A less then 24 -h long dip in 4% formalin was then performed before fixing the samples in paraffin blocks. The following IHC-evaluations were then performed at the University of Helsinki.

2.5. Immunohistochemical staining

The immunohistochemical stainings of Td-CTLP and MMP-8 were described previously by Listyarifah et al. (2018). Briefly, 4- to 5-μm sections were cut of paraffin blocks. The sections were next deparaffinized automatically and antigens were retrieved in citrate buffer (pH 6) –solution in a microwave oven. The blocking of endogenous peroxidase was performed with 3% H_2O_2 -solution for 30 min. Non-specific staining was inhibited with 2% bovine serum albumin in 10 mM phosphate buffer saline (PBS), (pH 7.4) with 1:50 normal goat serum. Afterwards, tissue sections were incubated with primary rabbit polyclonal antibodies against Td-CTLP and MMP-8 as described previously described Listyarifah et al. (2018) and Mäkinen et al. (2012), respectively. After overnight incubation at 6 °C with primary antibodies the biotinylated secondary antibodies were used as provided in the Vectastin® kit (Vector Laboratories, Burlingame, Ca, USA), at 37 °C for 30 min. Finally, the slides were visualized by using 3,3′-diaminobenzidinetetrahydrochloride (DAB) as chromogen substrate and counterstained with Meyer's hematoxylin -solution. All slides were washed with 0.04 % Tween 20 PBS -solution between each step. The negative controls of stainings were performed with non-immune species-species Rabbit IgG (Vector Laboratories).

2.6. Image analysis and quantification

The tissue samples were first blindly visualized under a light microscope (Leica DMLB, Leica, Wetzlar, Germany) under 5X magnification in order to visually evaluate the sample and choose a 10X magnification frame well representing both the epithelium and connective tissue MMP-8–staining. It was then photographed and saved. If a single frame was judged not to represent well enough at once both the epithelium's and the connective tissue's MMP-8–staining due to the complexity of the histological structure or the uneven staining, two separate high-resolution images – one of the epithelium and another of the connective tissue – were taken. This was then repeated with the Td-CTLP–stained samples in a manner that each sample's frame was taken from the very same spot. Each image's epithelium and connective tissue was then separately analyzed with ImageJ software (version 1.46c; Rasband WS, National Institutes of Health, Bethesda, MD, USA). The obtained pixel color intensity scores hold a positive correlation with the amount of IHC-staining, and in consequent, with the relative amount of MMP-8– and Td-CTLP–enzymes in the tissue's epithelia and connective tissue, and thus opened the possibility to an objective statistical comparison between study groups.

2.7. Statistical analysis

The comparison of the staining levels of MMP-8 and Td-CTLP within and between the sample groups were carried out using one-way ANOVA and followed by post hoc test LSD (Least Significant Differences) test. A p-score < 0.05 was considered as significant. The statistical tests were performed with the aid of a computer software (SPSS v.26, IBM, Chicago, IL, USA).

3. Results

Human 75 kDa proMMP-8 was converted time-dependently to its 65 kDa active species and lower molecular size $25-50$ kDa species by Td-CTLP and by the positive control APMA at all test-time points (Fig. 1).

As showed in Table 1, significant differences were found in PPD and BOP% levels between peri-implantitis and control groups and also between periodontitis and the control groups. Gender and age showed no significant difference between the three groups.

Figs. 2 and 3 demonstrate the immunoexpression levels of MMP-8 and Td-CTLP across the three study groups. In the majority of the samples, both MMP-8 and Td-CTLP were visible both in the epithelium
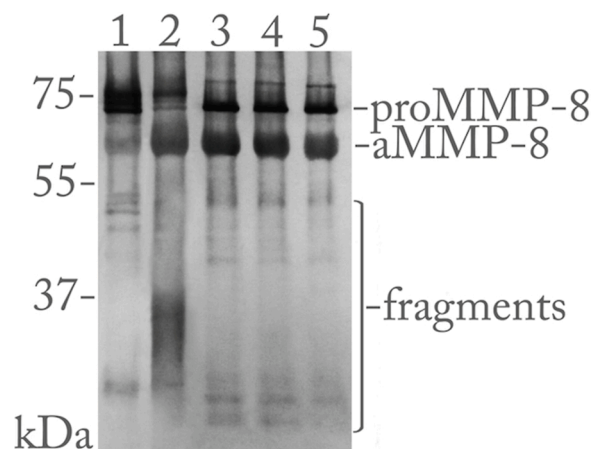

Fig. 1. The activation of human 75 kDa proMMP-8 by *Tremonema denticola* chymothrypsin-like protease (*Td*-CTLP) *in vitro*. In the lane 1: 75 kDa proMMP-8 alone; lane 2: MMP-8 was inbucated with organomercurial compound 1 mM 4-aminophelylmercuric acetate (APMA) for one hour at 37 °C; lanes 3-5: MMP-8 was incubated with *Td*-CTLP for 20, 40, and 60 min at 37 °C. The activation and conversion of 75 kDa proMMP-8 and fragmentations to lower molecular size 65 kDa active and 25-50 kDa MMP-8 species are indicated on left. The mobilities of molecular weight markers are indicated on right.

and in the connective tissue but was more pronounced in the epithelium. The staining distribution of MMP-8 and Td-CTLP varied greatly between samples in all groups; in some samples, both MMP-8 was stronger in the basal layer of the epithelium and in some in the superficial layer of the epithelium. Meanwhile, some were relatively equally stained all through the epithelial tissue. In contrast, the connective tissue was stained more equally, only minor isles of staining existed in some samples. Hence, in connective tissue no difference in staining pattern was identifiable between groups. Through visual inspection, the healthy patients' [Figs. 2 and 3 C, F] tissue samples staining intensities were seeming equal to the two other patient groups.

As illustrated in Fig. 4, no statistically significant differences in the immune-expression levels of MMP-8 or Td-CTLP were observed between study groups in the connective tissue or in the epithelium.

4. Discussion

The bacterial protease Td-CTLP stimulates the expression of MMP-8 by mammalian cells. In the present study we demonstrated that Td-CTLP can time-dependently convert the inactive form of MMP-8, proMMP-8, to its active form, aMMP-8 and lower molecular size species. We also demonstrated that the Td-CTLP is detectable in the epithelium and the connective tissue; however, the staining intensities of neither Td-CTLP nor MMP-8 differ significantly between periodontitis, peri-implantitis or periodontally healthy groups.

To our knowledge, this is the first time MMP-8 and Td-CTLP protein levels have been studied immunohistochemically among peri-implantitis patients. It is however important to notice that with the aid of the used research settings in immunohistochemistry, we cannot determine whether the immunoreactive Td-CTLP enzyme is active or inactive. Therefore, we cannot rule out the option that there is actually a significant difference in the levels of the active enzymes between groups. Further studies using tissue extracts or gingival crevicular fluid to determine the active enzyme levels will give additional information. The periapical radiograph is used in periodontology and more reliable to assess the treatment outcomes such as filling of the bone defects. However, in our study, we preferred orthopantomography to detect the presence of bone ($\geq$ 5 mm) due to its fast and accurate imaging features (Persson et al., 2003; Machado et al., 2020). Inflamed or chronically diseased sulcular and oral epithelium may demonstrate similar

Table 1
Demographic data of the study participants and the clinical parameters of the sampling sites.

|	Control (n = 14)	Peri-implantitis (n = 10)	Periodontitis (n = 14)	Peri-implantitis vs. Periodontitis (p-value)	Peri-implantitis vs. Control (p-value)	Periodontitis vs. Control (p-value)
Age (years)	34.3 ± 9.6	45.5 ± 8.0	40.9 ± 8.4	NS	0.003	0.050
Gender (male %)	50.0	70.0	50.0	NS	NS	NS
PPD (mm)	2.75 ± 0.58	7.67 ± 1.21	5.68 ± 0.93	<0.001	<0.001	<0.001
CAL (mm)	2.93 ± 0.68	8.75 ± 0.88	6.96 ± 0.99	<0.001	<0.001	<0.001
PI%	1.29 ± 0.99	2.30 ± 1.06	2.36 ± 0.84	NS	0.015	0.006
BOP%	0.64 ± 0.50	2.70 ± 0.48	2.64 ± 0.50	NS	<0.001	<0.001
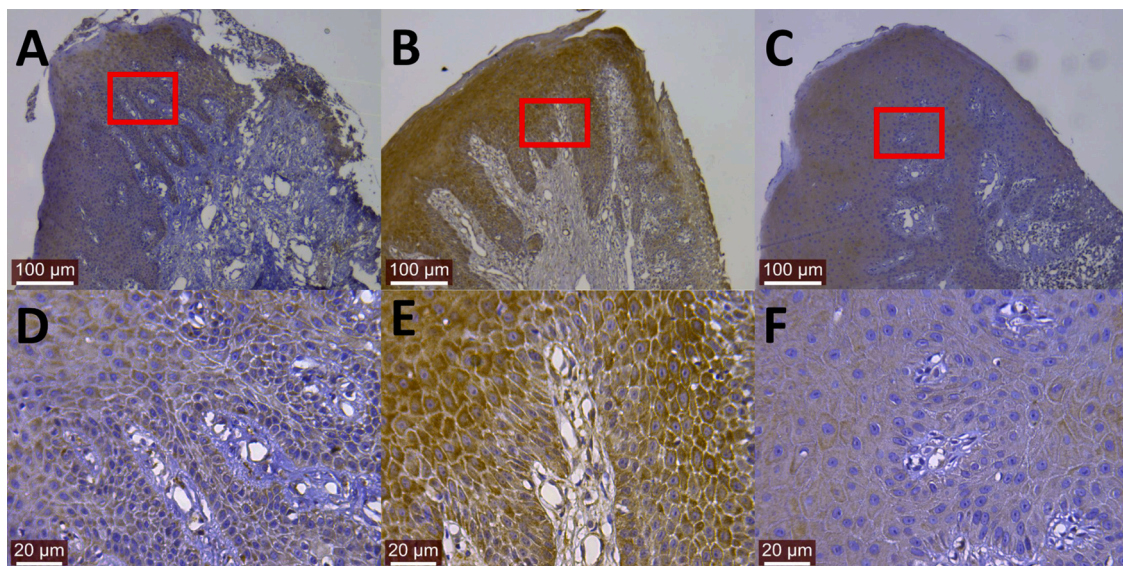
Fig. 2. Td-CTLP stainings with 10x (A, B, and C) and 40x (D, E, and F) magnifications. [A, D] Td-CTLP, peri-implantitis; [B, E] Td-CTLP, periodontitis; [C, F] Td-CTLP, healthy (Marked rectangles on A, B, and C indicates the regions demonstrated on D, E, and F).
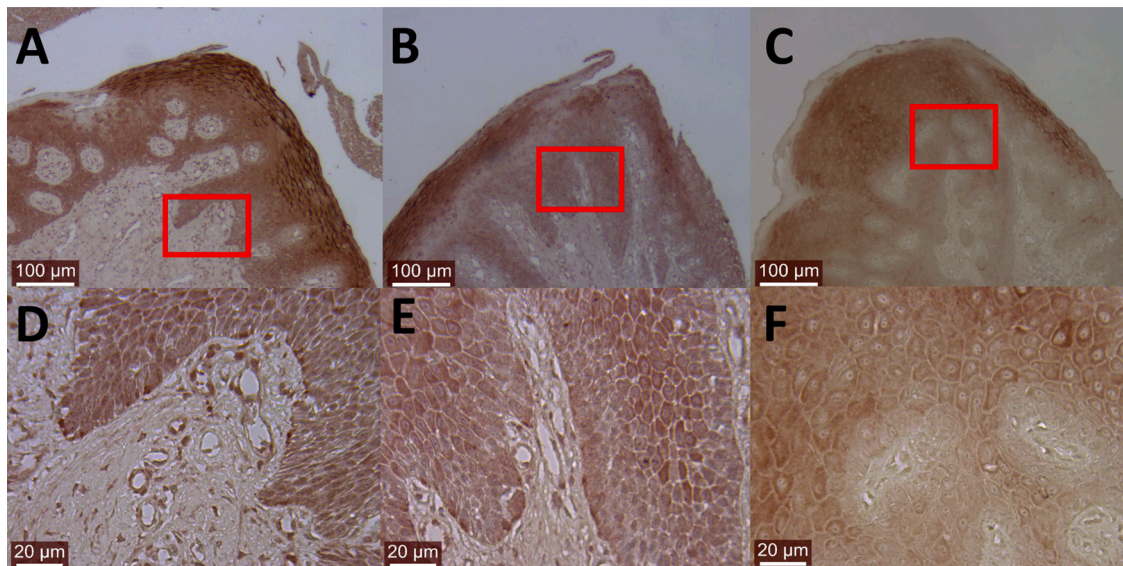

Fig. 3. MMP-8 stainings with 10x (A, B, and C) and 40x (D, E, and F) magnifications. [A, D] MMP-8, peri-implantitis; [B, E] MMP-8, periodontitis; [C, F] MMP-8, healthy (Marked rectangles on A, B, and C indicates the regions demonstrated on D, E, and F).

morphological structures (formation of epithelial ridges, cytokeratin types, and keratinized layers), which do not allow researchers to distinguish easily by microscopy (Pritlove-Carson et al., 1997; Jiang et al., 2014). We therefore stained the oral epithelium with a tissue marking dye immediately after the surgical sampling. While this technique carries its own limitations, this method also allowed us to define the region of interest before microscopic evaluation. Manuel scoring has been used in immunohistochemistry for long time. Yet, this method is
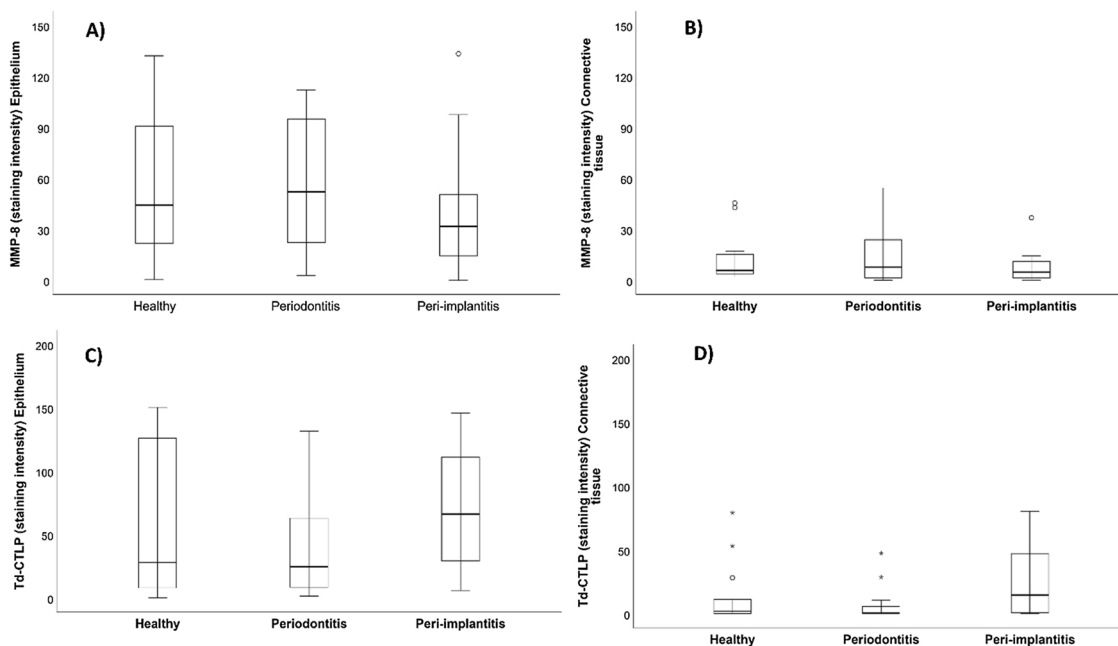
Fig. 4. The staining intensities of MMP-8 (A and B) and Td-CTLP (C and D) between groups in the epithelial and connective tissue.

subjective, has limited that limits reproducibility, and is prone to significant intra- and interobserver variability. (Feuchtinger et al., 2015). On the other hand, using a digital image analyze method to obtain a continuous spectrum of average staining intensity produce reliable and sensitive outcomes and detect biological differences within the tissues with greater accuracy (Braun et al., 2013).

Our findings also demonstrate that Td-CTLP can convert proMMP-8 to aMMP-8 and that the activation readily happens in only 20 min. After that, up until the 60 min timeline, the amount of aMMP-8 stayed stable and did not show signs of augmentation. Our results are in line with previous studies that are showing the ability of Td-CTLP to convert proMMP-8 to its active forms. For instance, Nieminen et al. (2018) used tumor-associated-trypsin-2 as a control activating enzyme and demonstrated that the proMMP-8 to aMMP-8-conversion indeed happens in vitro in 20 min. In the present study, APMA was used as a control activator for proMMP-8. Gürsoy et al. (2018) also used APMA as a control activator for MMP-8 in a similar setting but used different activation times, the shortest being 30 min. Clinically, our in vitro results confirm the interactions between bacterial Td-CTLP and the host cell enzyme proMMP-8 leading to the latter one's activation. Since activated MMP-8 plays a crucial role in the etiology of peri-implantitis, this further confirms the relation between *Treponema denticola* and periodontitis. The fact that the activation of proMMP-8 by Td-CTLP took only 20 min, indicates how rapid this conversion is *in vitro*, and offers keys for future research for adequate further investigation. Yet, Td-CTLP is not the only enzyme that can proteolytically activate proMMP-8, other MMPs, such as MMP-3, -7, and -10 can activate proMMP-8.

According to our results, tissue protein levels of Td-CTLP and MMP-8 do not differ between periodontitis, peri-implantitis, and periodontally healthy control groups. Similar to our results, Karatas et al. (2020), who also conducted an immunohistochemical study, could not find a significant difference in total MMP-8 levels between groups of periodontitis and healthy patients. As the tissue levels of Td-CTLP and MMP-8 in peri-implantitis patients were first time evaluated, no clear comparison could be made with the previous literature. In addition, to our knowledge no comparisons of immunohistochemical Td-CTLP and MMP-8 levels between periodontitis and peri-implantitis groups have been made in the past. Clinically, since no difference in the levels of Td-CTLP and MMP-8 could be found between the 3 study groups, it can be presumed that merely measuring the level of MMP-8

immunohistochemically, without being able to distinct its active forms, is not an adequate measure in the diagnostics of periodontitis or peri-implantitis. Indeed, present results indicate the shortcomings of the use of total MMP-8 and Td-CTLP as biomarkers of periodontitis, as inactive or total forms of these enzymes does not have ability to distinguish disease from health. Finally, both enzymes seem to be a part of healthy gingival environment.

In conclusion, we produced evidence that Td-CTLP can indirectly contribute to the pathogenesis of periodontal diseases as an activator of host MMP-8. Similar levels of Td-CTLP and total MMP-8 in peri-implantitis, periodontitis, and periodontally healthy tissues indicate that total (active and inactive) form of these enzymes cannot distinguish the diseased tissues from the healthy ones.

Declaration of Competing Interest

Timo Sorsa is the inventor of US-patent 10 488 415 B2 and a Japanese patent 2016-554676. Other authors report no conflicts of interest related to this study.

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