Longitudinal analysis of metalloproteinases, tissue inhibitors of metalloproteinases and clinical parameters in gingival crevicular fluid from periodontitis-affected patients

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Background: The aim of this work was to improve the assessment of the periodontal disease status through measurements of extracellular matrix metalloproteinases (MMPs) and their tissular inhibitors (TIMPs) in the gingival crevicular fluid from patients diagnosed with chronic periodontitis.

Methods: Gingival crevicular fluid samples from patients ($n = 13$) were taken from 60 sites initially, and from 51 and 41 sites, respectively, 3 and 6 months after scaling and root planing. Gingival crevicular fluid samples were also taken from healthy subjects ($n = 11$, 24 sites). The presence of MMP-9 and MMP-8 was assessed by zymography and immunowestern blotting, respectively. The actual MMP activity (gelatinase and collagenase) was measured using the fluorogenic substrate assay. TIMP-1 and -2 levels were measured by immunodot blot.

Results: The fluorogenic substrate assay determinations showed higher MMP activity in sites with probing depth ≥ 4 mm, with significant reduction post-treatment. Gelatinase activity followed by zymography consisted mainly of MMP-9. A different pattern of MMP-8 in control and patient sites was found. Controls only showed species of a partially active form (69 kDa), whereas patient sites showed a high frequency of the active form (56 kDa), and in some cases the latent form (85 kDa) was also observed. The active form reduced its frequency in sites with probing depth ≥ 4 mm. TIMP-1 and -2 levels in patients were significantly lower than in controls, and after treatment the recovery of TIMP-1 level similar to control was observed.

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Conclusion: Significant correlations between the severity of the periodontal disease and the actual MMP activity, the active form of MMP-8 and the low level of both TIMP-1 and TIMP-2 were found.

The periodontal disease periodontitis, characterized by tissue destruction, is a chronic inflammation with the formation of a periodontal pocket, destruction of supporting connective tissue, and loss of the alveolar bone (1–3). Chronic peridontitis is usually related to polybacterial infection including, among others, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Tannerella forsythia* (4). The tissue destruction appears to result from a complex interaction between these bacteria and the host's immune and inflammatory system. Proteolytic activities from these bacteria, including collagenase, may participate in collagen degradation (5). Activation and over-expression of host matrix metalloproteinases (MMPs) by periodontal pathogens such as *A. actinomycetemcomitans*, *P. gingivalis* and *Chlamydia pneumoniae* and by inflammatory cytokines has been reported (6–11).

MMPs are a family of proteolytic enzymes involved in the physiological degradation of extracellular matrix proteins and basement membrane (1, 11). It has been shown that an imbalance between them and their host inhibitors (TIMPs) may lead to periodontitis initiating structural protein destruction including collagens (8, 12). There are several subgroups of MMPs: collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11) and membrane-type associated MMPs (11). Evidence of the role that MMPs could play in periodontal disease is supported by the presence of elevated levels, both in tissues and gingival crevicular fluid, of several MMPs, including MMP-1, -2, -3, -7, -8, and -9, which in some cases are in their activated rather in the latent form (2, 3, 13–19). A number of inflammatory mediators can be secreted by these activated cells, which in turn stimulate the production of MMPs from fibroblasts, epithelial cells, and polymorphonuclear leukocytes (3, 10, 17).

Several methods have been used to measure MMP activities: (i) zymography in sodium dodecyl sulfate–polyacrylamide gel electrophoresis with gelatin as substrate that allows determination of MMP-2 and MMP-9 (19); (ii) assays with [^{14}C]collagen or biotinylated-collagen (18, 20); (iii) enzyme-linked immunosorbent assays (ELISA) (14, 18, 21, 22); (iv) time-resolved immunofluorescence assay (23–26). Recently a more simple and sensitive assay using a fluorogenic septapeptide has been reported (27). This method is based on the incorporation of a fluorescent-leaving group to a peptide that mimics both collagenase (MMP-8, MMP-13) and gelatinase (MMP-2, MMP-9) cleavage sites, making possible the measurement of the actual metalloproteinase activity (28, 29). The inclusion of 4-aminophenylmercuric acetate (APMA) to the assay medium allows the quantification of both active and latent forms.

Controversial results on TIMP-1 levels in gingival crevicular fluid and gingival tissue when comparing samples from controls and periodontitis-affected patients have been described. In tissue, lower levels in patients have been reported (18, 21, 30), and also absence of changes (3, 13). In gingival crevicular fluid, both increased (22, 31) or decreased levels in diseased sites have been described (14, 18, 30).

A longitudinal evaluation of gingival crevicular fluid total MMP activity (collagenase/gelatinase) and TIMPs levels could be useful for monitoring the effectiveness of a non-surgical treatment. In the literature, there are a number of reports on MMP-8 levels, both as severity index and effectiveness of treatment (with or without antibiotic treatment) (21, 23–25, 30, 32, 33). No studies have been carried out on the possible changes in the proportion of its latent and active forms after treatment. This data is important to evaluate the degree of proteolytic environment related with the tissue destruction.

In this study we followed the actual proteolytic activity due to the action of collagenolytic and gelatinase activies using the fluorogenic peptide matrix metalloproteinase assay to assess a simple, rapid and sensitive method to measure metalloproteinase activity in gingival crevicular fluid. This data is important for the diagnosis and progression of the disease, and for the responses to the different periodontal therapies. Gelatinase profile was determined by zymography with gelatin as substrate, and both the different species of neutrophil collagenase (MMP-8) with characteristic molecular weights and the levels of TIMP-1 and -2 were followed by immunoanalysis. Finally, we have evaluated whether the amount of MMPs and TIMPs and the MMP-8 species correlate with the clinical parameters obtained before and after non-surgical treatment (scaling and root planing).

Materials and methods

Patient selection

The initial population studied comprised 13 adult patients (11 men and two women) with chronic moderate periodontitis: two of them dropped out of the study. Control patients (11 individuals, six women and five men) exhibited no clinical signs of periodontitis. Control and patient groups were duly informed of the research protocol approved by the Human Ethics Experimentation Committee at the Facultad de Odontología (Santiago, Chile), and all of them agreed to participate in this study.

Patients with chronic periodontitis ranged from moderate to advanced periodontitis, with at least five teeth,

had sites with probing depth ≥ 5 mm and with attachment loss ≥ 3 mm and extensive bone loss in radiography. These patients fulfilled the following criteria: (i) no history of systemic disorders, antibiotics and/or inflammatory drugs, had no periodontitis treatment within the past 6-month period prior to the studies, and (ii) initiation age between 30 and 35 years, slow progression, inflamed gum, bacterial plaque, records of radiographic bone loss. All patients were subjected to a non-surgical treatment, monitoring the maintenance phase of treatment for up to 6 months. The control group was selected from healthy volunteers with no evidence of periodontal disease (absence of clinical attachment loss or increased probing pocket depths). Prior to the study, all subjects received supragingival prophylaxis to remove gross calculus and allow probing depth. The protocol was clearly explained to all patients and controls, and Institutional Reviews Board-approved informed consents were signed.

Clinical measurements and sampling

The clinical parameters measured at the time of the sample collection included plaque index, bleeding on probing, probing depth, and clinical attachment loss. The examination and collection of gingival crevicular fluid were carried out by one well-trained and calibrated examiner (Dr Claudio Melej). On successive days a group of 20 subjects were examined who had the full range of periodontal conditions expected to be assessed during the survey. Examinations of the subjects were repeated until acceptable consistency was achieved.

Gingival crevicular fluid samples were obtained from three locations based on probing depth: sites with < 4 mm, sites ≥ 4 and < 6 mm, and sites ≥ 6 mm of randomly selected teeth in each patient. Six sites were examined for each tooth: mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual. An automated disk probe was used for attachment level and probing depths (Florida Probe Corporation, Gainesville, FL, USA).

Prior to collection of gingival crevicular fluid samples, the area of the mouth to be sampled was isolated with cotton rolls, the supragingival plaque was removed, and filter paper strips (ProFlow, Amityville, NY, USA) were inserted non-traumatically just into the crevice for 30 s. All patients underwent therapy (scaling and root planing) including oral hygiene instructions (24, 30).

Gingival crevicular fluid was removed from the strips by incubation with 50 μl of a solution of 50 mM Tris HCl, 5 mM $CaCl_2$, 200 mM NaCl, 0.01% Triton X-100, pH 7.5 for 30 min at 4°C. After centrifugation ($10,000 \times g$ for 5 min) treatment of the strips was repeated one more time. Both eluted fractions were pooled and stored at -20°C until use. Protein concentration was measured using the Bio-Rad microassay (Bio-Rad Laboratories, Hercules, CA, USA), following the instructions of the manufacturer, with bovine serum albumin as standard.

Gelatin zymography

MMP-2 and MMP-9 were analysed from gingival crevicular fluid by electrophoresis on 7.5% sodium dodecyl sulfate–polyacrylamide gel containing 0.1% gelatin under non-reducing conditions (34). After destaining, gelatinolytic activity was visualized as clear bands against a blue background. The area of proteolysis was estimated by densitometric analysis using the 'Uni-Scan-it Gel-Digitizing System' software (Silk Scientific Corporation, Orem, UT, USA). Quantified data were expressed as a percentage of the area of the bands of interest relative to the total bands. Pre-stained molecular weight standards and a standard mixture of MMP-2/MMP-9 were used to assess the molecular weight of the different gelatinase forms.

Determination of metalloproteinase activity using a fluorometric assay

Samples of gingival crevicular fluid were assayed using 2.5 μM MOC-Ac-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2 (Cat. 3163-v, Peptide Inc., Minoh-Shi, Osaka, Japan), in an assay

buffer containing 0.1 M Tris-HCl pH 7.5, 0.1 M NaCl and 50 mM $CaCl_2$ at 47°C. These conditions of Ca^{2+} and temperature showed to be more adequate for the kinetic analysis. The fluorescence was followed at λ_{ex} 328 nm (with slit 3 nm), and λ_{em} 393 nm (with 15 nm slit) using a Shimatzu RF 5301 PC-S spectrofluorometer (28). The calibration curve was performed with the reference peptide MOCA-Pro-Leu-Gly (Cat. 3164-s, Peptide Inc, Japan) using a range between 0.025 and 0.100 μM. The scale of 1000 arbitrary units (AU) was fixed with the concentration 0.100 μM of the reference peptide. The condition of linearity of the product release with time was initially determined using samples with different protein concentration and assay times.

Western blotting/dot blot analysis

For western blotting of MMP-8, protein separated by 12% sodium dodecyl sulfate–polyacrylamide gel under reducing conditions (35) was electro-transfered to a nitrocellulose membrane (0.45 μm, Bio-Rad) in a semidry transfer cell (Transfer-blot SD semidry transfer cell, Bio-Rad) for 15 min using 25 mM Tris-HCl and 192 mM glycine at pH 8.3 with 20% methanol at 15 V (36). For dot blot of TIMPs (after determination of the specificity of the antibodies by immunowestern blot), portions of 2.5 μl of gingival crevicular fluid were applied to the same nitrocellulose membrane, testing simultaneously samples from controls and patients. Both in western blot and dot blot analysis, non-specific binding sites were blocked for 4 h with a solution of Tris-buffered saline with Tween 20 (TBS-T buffer: 20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20, 6% non-fat milk). The following primary antibodies were used: (i) for MMP-8 detection: monoclonal anti-MMP-8 (Cat. MAB 3316, Chemicon International, Temecula, CA, USA) diluted 1 : 2500 in TBS-T buffer; (ii) for TIMPs measurements: polyclonal anti-TIMP-1 and TIMP-2 (Cat. sc-6832 and sc-6835, respectively, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 250 in TBS-T were

incubated overnight at 4°C. After rinsing with TBS-T buffer without milk, the blots were incubated for 1 h with the adequate secondary antibody conjugated with peroxidase using an anti-mouse antibody (Pierce Biotechnology, Rockford, IL, USA) diluted 1 : 10,000 in TBS-T for the monoclonal or anti-goat antibody (Pierce) or diluted 1 : 1500 in TBS-T for the polyclonal antibody. After rinsing with TBS-T (without milk), the positive reaction was identified using enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Pierce). Control experiments in the absence of the primary antibodies and employing only the two secondary antibodies did not yield any chemiluminescent signal. Pre-stained molecular weight standards from Gibco BRL (Bench Mark pre-stained ladder) were used to assess the molecular weight of the different MMP-8 species.

Dots were scanned and quantified using the same software mentioned in gelatin zymography, expressing the results in arbitrary units (AU, pixels/mg protein).

Statistical analysis

Data from patient and control groups pre-treatment were not normally distributed. Mathematical transformation to logarithm did not change the distribution, therefore the non-parametric test of Mann–Whitney U for two independent samples to determine the significance of the differences between both groups was used. Data were expressed as median values together with 25 and 75 percentiles. The chi-square test was used to compare the changes in the presence of the different MMP-8 forms. Correlations between the data were analysed using Spearman's rho rank correlation test. Analysis of statistical significance in the longitudinal study of the same sites between initial values and 2 and 6 months after scaling and root planing treatment was performed using one-way analysis of variance (ANOVA, post hoc multiple comparison Tukey test). In the case of TIMPs we only had enough samples for studying up to

2 months after treatment, therefore a paired samples *t*-test was employed for the longitudinal analysis. Covariance analysis (ANCOVA) was used to determine statistical differences between the actual MMP and TIMP levels, and sex and age of the two subject groups. SPSS version 8 and Systat 5 statistical softwares were used. Statistical significance was set at a value of $p < 0.05$.

Results

Clinical assessments

Demographic characteristics and measurements before and after scaling and root planing are summarized in Table 1. The periodontitis-affected patients showed significantly higher probing depth, clinical attachment loss, plaque index and bleeding on probing compared to the healthy group (with pockets < 4 mm). From the 60 selected sites of the patients with periodontitis, 24 corresponded to pockets < 4 mm, 25 sites to pockets ranging from 4 to 6 mm, and 22 sites > 6 mm. After treatment all the increased parameters reverted to similar values as the control group, except bleeding on probing values.

Zymographic analysis of gelatinase activity

The gelatinase profile was followed by a functional assay using gelatin-substrate zymography. A stronger band, with molecular weight around 90 kDa as MMP-9 standard, was observed both in patients and controls (Fig. 1). Two additional gelatinolytic bands with higher molecular weight were also visualized, a 130-kDa band (a MMP-9–lipocalin complex) (37), and a 200-kDa band, reported to be recognized by anti-MMP-2 antibodies (38). In some cases a gelatinolytic band of 50 kDa was observed, but no MMP-2 species was visualized. In pathological sites the broad gelatinolytic band between 100 and 80 kDa probably represents both the latent and active forms of MMP-9, whereas in controls only the latent form was observed.

For comparison purposes this analysis was performed using portions of 0.60 µg of protein per lane. Nevertheless, we observed visual differences in the MMP-9 band between sites with different depths, and also between sites before and after scaling and root planing. Data obtained from densitometric analysis did not allow us to

Table 1. Mean patient and control clinical parameters

	Control group (n = 11)	Periodontitis patients pre-treatment (n = 13, sites 60)	Periodontitis patients 2 months post-SRP (n = 10, sites 51)	Periodontitis patients 6 months post-SRP (n = 9, sites 41)
Age (years) mean ± SD	27.3 ± 7.6	43.5 ± 8.9	43.1 ± 9.7	42.7 ± 10.2
Females (%)	54.5	84.6	90.9	88.8
PD (mm) mouth average mean ± SD	2.1 ± 0.9	4.0 ± 1.5*	1.9 ± 0.9†	1.6 ± 0.8†
PD (mm) in sampled sites mean ± SD	1.8 ± 0.5	4.1 ± 1.4*	2.5 ± 1.2*†	2.1 ± 1.1†
CAL (mm) mean ± SD	0	4.2 ± 1.8*	2.7 ± 1.5†	2.4 ± 1.5†
PI mean ± SD	17.6 ± 13.4	56.8 ± 8.7*	34.8 ± 23.5†	16.3 ± 10.7†
BOP (%)	2.7 ± 1.8	52.6 ± 17.3*	20.4 ± 14.4	17.9 ± 17.2

*Significant differences between periodontitis-affected patients and control group with *p*-values < 0.05.
†Significant differences between initial values of periodontitis-affected patients and post-SRP treatment.
BOP, bleeding on probing; CAL, clinical attachment loss; PD, probing depth; PI, plaque index; SRP, scaling and root planing.
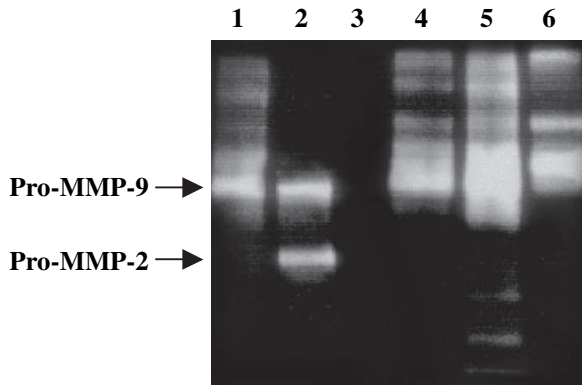
Fig. 1. Zymogram in sodium dodecyl sulfate–polyacrylamide gel electrophoresis under non-reducing conditions using gelatin as substrate. Portions of gingival crevicular fluid samples containing 0.6 µg of protein were placed in each lane. Lane 1, a sample from a control; lane 2, a standard mixture of matrix metalloproteinase (MMP)-2 and -9; lane 3, pre-stained molecular weight standards; lanes 4, 5 and 6, samples from one patient (probing depth 4–6 mm) before, 2 and 6 months after scaling and root planing treatment, respectively.

associate this parameter with the severity of the disease.

Fluorometric assay of collagenase and gelatinase activities

Unlike zymographic analysis, which detects both active and latent forms of gelatinase, a simple method for measuring the active form of metalloproteinases, including gelatinase and collagenase activities, is based on the hydrolysis of a synthetic peptide that after the cleavage increases its fluorescence. The conditions required to work at initial velocity conditions included a protein concentration of 5 µg, 50 mM $CaCl_2$ and 47°C, thus yielding a linear increase in fluorescence during 30 min of incubation. Data were expressed using the reference peptide MOCA-Pro-Leu-Gly, as
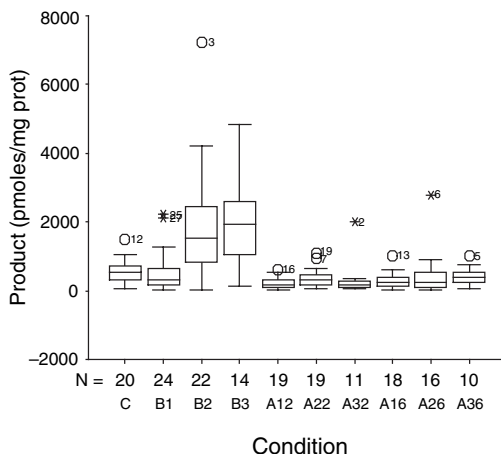
$pmol/(min \times mg$ protein). Figure 2 shows median values together with 25 and 75 percentiles of the MMP activity of the control group and studied sites from the patient group before and after scaling and root planing. Almost all patient studied sites reverted to normal values after treatment. Before treatment, actual MMP activity in patient sites < 4 mm were similar to controls, whereas in patient sites with depth ≥ 4 mm, MMP activity was significantly higher compared to controls ($p < 0.05$).

Longitudinal analysis of 17 sites with probing depth < 4 mm, 14 sites with probing depth 4–6 mm, and eight sites with probing depth > 6 mm followed up to 6 months after scaling and root planing treatment, showed significant differences between initial values and 2 and 6 months post-treatment ($p < 0.05$), whereas no differences between 2 and 6 months post-treatment were found.

Presence of different forms of neutrophil collagenase

The pattern of MMP-8 forms in the gingival crevicular fluid from controls and patients, before and after scaling and root planing, was followed by western blotting. Sites of the control group showed only a species of 69 kDa (Table 2), corresponding to a partially activated form of the neutrophil MMP (9, 15, 17). However, periodontitis-affected patients showed the presence of this form in only 64% of samples, together with a significantly high frequency (in 59% of the samples) of a lower molecular weight species (56 kDa, active form), and in some sites (three out of 39 sites) a higher molecular weight form (85 kDa, latent form) was also observed. Two months after scaling and root planing treatment, 95% of the studied patient sites showed the 69-kDa MMP-8 (21 out of 22 sites) and, with the exception of sites with probing depth 4–6 mm, the 56-kDa isoform was also present in 27% of the cases. The 85-kDa species showed a higher frequency compared to initial data, being detected in 36% of the studied sites. No statistical analysis was done with data 6 months

Fig. 2. Metalloproteinase activity measured using the fluorogenic substrate. Box plot representation based on the median (line across the box): the box limits contain 50% of the values (including 25 and 75 percentiles). The whiskers are lines that extend from the box to the highest and lowest values excluding outliers. C, control; B1, B2 and B3, initial values of sites < 4 mm, 4–6 mm, and > 6 mm; A12 and A16 correspond to sites < 4 mm after 2 and 6 months, respectively; A22 and A26 correspond to sites 4–6 mm after 2 and 6 months, respectively; A32 and A36 correspond to sites > 6 mm after 2 and 6 months, respectively. *N*, number of analysed sites.

Table 2. Distribution of the different matrix metalloproteinase-8 (MMP-8) species in gingival crevicular fluid from controls and patients before and after the scaling and root planing treatment. The first number represents the presence of the species, the second the total number of study sites. Data after scaling and root planing represent the longitudinal study of several of the same initial sites (see text)

	Initial	2 months after SRP	6 months after SRP
Controls			
PD < 4 mm			
69 kDa	15/15		
Patients			
PD < 4 mm			
56 kDa	6/14*	3/5*	2/4*
69 kDa	12/14	5/5	4/4
85 kDa	1/14	2/5	3/4*
PD 4–6 mm			
56 kDa	9/13*	0/10	0/4†
69 kDa	9/13*	10/10	4/4
85 kDa	0/13	3/10	4/4†
PD ≥6 mm			
56 kDa	8/12*	3/7*	0/4
69 kDa	4/12*	6/7†	4/4†
85 kDa	2/12	3/7*	2/4†

*Values significantly different from controls according to a chi-squared test.
†Values significantly different from the initial values of the same patient group.
PD, probing depth; SRP, scaling and root planing.

after treatment, considering the diminution of the patient number.

Analysis of TIMP-1 and TIMP-2 in peridontitis-affected patients

The comparison of the relative level of TIMPs was performed by immunodot blot analysis instead of western blotting for at least three reasons: (i) quantitative analysis by western blotting presents difficulties for the large variability and loss of protein in the transference process to the nitrocellulose membrane; (ii) the impossibility of doing the simultaneous analysis of a large number of samples; (iii) the requirement of low sample volume compared with the ELISA kit, thus allowing to perform a large number of analyses (MMPs and TIMPs) per sample.

We observed significantly reduced values of TIMP-1 in all sites of periodontitis patients that after treatment reached values similar to controls (Fig. 3). Paired samples t-test indicates a significant difference between initial TIMP-1 values and 2 months after scaling and root planing in the 10 sites studied with probing depth < 4 mm, and in the 12 sites with probing depth 4–6 mm, but no differences with the three sites with depth >6 mm. TIMP-2 values in 14 control sites showed an average value of 1726 ± 30 (AU, arbitrary units), with significantly lower values in patient sites. In patients, the average of 24 sites with probing depth < 4 mm presented 264 ± 22 AU, the 21 sites with probing depth 4–6 mm and the 12 sites with probing depth >6 mm showed average values between 145 and 98 AU, respectively. In the few sites where we could determine the level of TIMP-2 ($n = 11$ after 2 months post-treatment and 2 after 6 months) we did not observed a recovery to the control values similar to the TIMP-1 case.

Correlations between clinical parameters and biochemical measurements

Table 3 summarizes correlations done taking together all data (initial, 2 and 6 months after the treatment). Positive statistically significant correlations between actual metalloproteinase and probing depth, clinical attachment loss and bleeding on probing were found, whereas a negative correlation with TIMP-1 levels was found. TIMP-2 level negatively correlated with probing depth and bleeding on probing, whereas the presence of active MMP-8 positively correlated with probing depth and clinical attachment loss.

The possible effects of factors such as sex and age on actual MMP and TIMPs levels were analysed by ANCOVA testing sites with probing depth < 4 mm, which are the only common sites of control and patient groups. No
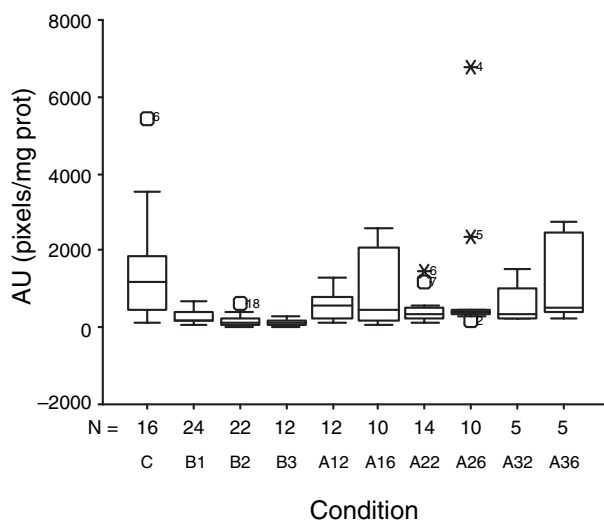

Fig. 3. Box plot representation of the relative tissue inhibitor of metalloproteinase-1 (TIMP-1) levels measured by immunodot blot. C, control; B1, B2, and B3, initial values of sites < 4 mm, 4–6 mm, and >6 mm; A12 and A16 correspond to sites < 4 mm after 2 and 6 months, respectively; A22 and A26 correspond to sites 4–6 mm after 2 and 6 months, respectively; A32 and A36 correspond to sites >6 mm after 2 and 6 months, respectively. N, number of analysed sites.

Table 3. Correlations between clinical parameters and biochemical measurements. All data from patients have been taken, initial, and 2 and 6 months after scaling and root planing treatment. Correlations between the data were analysed using the Spearman's rho rank correlation test

Measurement		PD	CAL	BOP
Actual MMP	Correlation coefficient	0.400*	0.288*	0.196*
activity	Sig (2-tailed)	0.001	0.001	0.015
MMP-8	Correlation coefficient	0.398*	0.295*	0.209
(56 kDa)	Sig (2-tailed)	0.001	0.012	0.078
MMP-8	Correlation coefficient	−0.184	−0.109	−0.365*
(69 kDa)	Sig (2-tailed)	0.122	0.362	0.002
MMP-8	Correlation coefficient	−0.487*	−0.435*	−0.269*
(85 kDa)	Sig (2-tailed)	0.001	0.001	0.023
TIMP-1	Correlation coefficient	−0.343*	−0.266*	−0.269*
	Sig (2-tailed)	0.001	0.007	0.006
TIMP-2	Correlation coefficient	−0.263*	−0.131	−0.422*
	Sig (2-tailed)	0.039	0.309	0.001

*Significant differences according to Spearman's rho test with p-values < 0.05.
BOP, bleeding on probing; CAL, clinical attachment loss; PD, probing depth; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

statistically significant differences were found, thus validating our results, although the sex and age of the subject groups were not balanced.

Discussion

The severity of periodontitis is associated with high levels of the actual metalloproteinase activity (including collagenase and gelatinase activities) (29), together with a high frequency of the MMP-8 active form (molecular weight of 56 kDa). These results indicate that in sites with probing depth ≥4 mm, an elevated proteolytic environment is present. The concerted action of several MMPs, including MMP-9 and MMP-8 among others, causes the destruction of periodontal extracellular matrix molecules (21, 24, 39).

Measurements of these proteolytic activities in gingival crevicular fluid, fluid derived from the circulation after having permeated through the diseased tissue of the periodontal pocket, are important because gingival crevicular fluid is considered a source of biochemical disease markers (24). Additionally, the reduced levels of the endogeneous inhibitors (TIMP-1 and -2) would not be able to counterbalance this abnormal proteolytic activity. According to the literature, TIMP-1 shows greater inhibition against MMP-9, whereas TIMP-2 shows strong inhi-

bition against MMP-8 (21). Therefore, the overexpression of MMP activity and the activation of latent proforms of MMPs (MMP-8 and probably also MMP-9), together with the reduction in TIMPs, would be responsible for the degradation of collagen fibers and other extracellular matrix components in chronic periodontitis. In this tissue degradation, in addition to host-derived matrix metalloproteinases, bacterial proteases may have an important role (1, 4, 12). For example, *P. gingivalis* can activate (transforming the latent pro-enzyme form to the active enzyme form) and stimulate expression of MMP-8 and MMP-9 (6, 7, 11). The multiple species of MMP-8 derived from degranulating neutrophils detected in gingival crevicular fluid can be triggered by periodontopathogenic bacteria such as *A. actinomycetemcomitans* (4, 9, 15). But, these lower molecular weight forms could also represent less glycosylated forms induced by proinflammatory mediators (17).

Similar to previous reports, the detection of gelatinase activity by gelatin zymography indicated that MMP-9 is the main activity present (20, 37, 38, 40). The high sensitivity of the zymographic analysis (where we used 0.6 μg of protein) compared with that of the fluorescent method (5 μg of protein) is noteworthy. However, the utilization of the zymographic assay

for comparison of different samples (controls and patients with different severity) would require the initial search of adequate dilution samples in order to obtain a linear response of gelatinase activity.

Data obtained using the fluorogenic septapeptide substrate of sites with a probing depth of ≥4 mm from periodontitis-affected patients were significantly higher than those of controls and sites with a probing depth of < 4 mm from patients. In addition, these measurements were useful for following site recoveries after scaling and root planing treatment. This proteolytic activity was determined in the absence of APMA, the organometallic compound that activates the latent forms (27, 29), with the purpose of determining the actual hydrolytic environment. A recent publication reports the second-order rate constant of several MMPs for the hydrolysis of the fluorogenic peptide used in this work, showing the higher specificity towards MMP-9, followed by MMP-13, MMP-2 and MMP-8 (29). These data indicate that the use of this synthetic peptide applying a fluorometric assay is a reliable method for the determination of the total metalloproteinase activity.

Several studies report an increase in MMP-8 levels, followed by ELISA (14, 22), immunofluorescence assays (23–25, 33) or other assays (20, 32). The presence of multiple species of MMP-8 has been described only in diseased sites (15, 17), but no further studies on changes in the MMP-8 species profile post-treatment have been reported. The relevance of our results is related with the demonstration of a reduction of the active form, mainly in sites with probing depth 4–6 mm, after scaling and root planing treatment, suggesting that the reduction of MMP-8 hydrolytic activity facilitates tissue recovery.

Our results using dot blot analysis for TIMP-1 and TIMP-2 present in gingival crevicular fluid, showed that both inhibitors were significantly reduced in periodontitis-affected patients compared to healthy subjects, similar to other reports (14, 18, 21, 30). The recovery of TIMP-1 level, 2 months after scaling and root

planing together with oral hygienic instructions, also agrees with data of at least two publications that also followed the recovery after scaling and root planing with doxycycline therapy (21, 30). The increase of TIMP-1 after treatment is proposed to be due to the reduction of MMPs, such as MMP-9, which would bind to TIMP-1 (21). The lack of recovery of TIMP-2, with higher affinity towards MMP-8, is not easily explained considering the literature data of a reduced expression of MMP-8 after scaling and root planing (14, 20, 21, 23–25,30, 32, 33).

Our observations of a positive correlation between either probing depth or clinical attachment loss and actual metalloproteinase activity and the active MMP-8 form, together with a negative correlation with TIMP-1, strengthened the proposed imbalance between the MMPs and their host inhibitors (TIMPs) leading to tissue destruction. Although the measurements of the TIMPs levels or the presence of the active MMP-8 species correlate with the severity of the disease, the evaluation of the actual metalloproteinase activity by the fluorometric assay would be more useful to follow both the severity of the damage and the efficacy of the treatment because of its simplicity, low cost, speed, and reproducible quantitative analysis.

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References

1. Reynolds JJ, Meikle MC. Mechanism of connective tissue matrix destruction in periodontitis. *Periodontol 2000* 1997;**14:** 144–157.
2. Tervahartiala T, Pirilä E, Ceponis A et al. The *in vivo* expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *J Dent Res* 2000;**79:**1969–1977.
3. Séguier S, Gogly B, Bodineau A, Godeau G, Brousse N. Is collagen breakdown during periodontitis linked to inflammatory cells and expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human gingival tissue? *J Periodontol* 2001;**72:**1398–1405.
4. Eley BM, Cox SW. Proteolytic and hydrolytic enzymes from putative periodontal pathogens: characterization, molecular genetics, effects on host defences and tissues and detection in gingival crevice fluid. *Periodontol 2000* 2003;**31:**105–124.
5. Sela MN, Kohavi D, Krausz E, Steinberg D, Rosen G. Enzymatic degradation of collagen-guided tissue regeneration membranes by periodontal bacteria. *Clin Oral Implants Res* 2003;**14:**263–268.
6. DeCarlo AA, Windsor LJ, Boden MK, Harber GJ, Birkedal-Hansen B, Birkedal-Hansen H. Activity and novel processing of matrix metalloproteinases by a thiol-proteinase from the oral anaerobe *Porphyromonas gingivalis. J Dent Res* 1997; **76:**1260–1270.
7. Okamoto T, Akaike T, Suga M et al. Activation of human matrix metalloproteinases by various bacterial proteinases. *J Biol Chem* 1997;**272:**6059–6066.
8. Ryan ME, Golub LM, Modulation of matrix metalloproteinase activities in periodontitis as a treatment strategy. *Periodontol 2000*, 2000;**24:**226–238.
9. Claesson R, Johansson A, Belibasakis G, Hanstrom L, Kalfas S. Release and activation of matrix metalloproteinase-8 from human neutrophils triggered by the leukotoxin of *Actinobacillus actinomycetemcomitans. J Periodont Res* 2002;**37:** 353–359.
10. Chang YC, Yang SF, Lai CC, Lui JY, Hsieh YS. Regulation of matrix metalloproteinase production by cytokines, pharmacological agents and periodontal pathogens in human periodontal ligament fibroblast cultures. *J Periodont Res* 2002; **37:**196–203.
11. Mäntylä P, Stenman M, Paldanius M, Saikku P, Sorsa T, Meurman JH. *Chlamydia pneumoniae* together with collagenase-2 (MMP-8) in periodontal lesions. *Oral Dis* 2004;**10:**32–35.
12. Uitto VJ, Overall CM, McCulloch C. Proteolytic host cell enzymes in gingival crevice fluid. *Periodontol 2000* 2003; **31:**77–104.
13. Ejeil AL, Igondjo-Tchen S, Ghomrasseni S, Pellat B, Godeau G, Gogly B. Expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in healthy and diseased human gingiva. *J Periodontol* 2003;**74:**188–195.
14. Ingman T, Tervahartiala T, Ding Y et al. Matrix metalloproteinases and their inhibitors in gingival crevicular fluid and saliva of periodontitis patients. *J Clin Periodontol* 1996;**23:**127–1132.
15. Romanelli R, Mancini S, Laschinger C, Overall CM, Sodek J, McCulloch CA. Activation of neutrophil collagenase in periodontitis. *Infect Immun* 1999;**67:**2319–2326.
16. Dahan M, Nawrocki B, Elkaïm R et al. Expression of matrix metalloproteinases in healthy and diseased human gingival. *J Clin Periodontol* 2001;**28:**128–136.
17. Kiili M, Cox SW, Chen HW et al. Collagenase-2 (MMP-8) and collagenase-3 (MMP-13) in adult periodontitis: molecular forms and levels in gingival crevicular fluid and immunolocalisation in gingival tissue. *J Clin Periodontol* 2002;**29:**224–232.
18. Soell M, Elkaim R, Tenenbaum H. Catepsin C, matrix metalloproteinases, and their tissue inhibitors in gingiva and gingival crevicular fluid from periodontitis-affected patients. *J Dent Res* 2002; **81:**174–178.
19. Apajalahti S, Sorsa T, Ingman T. Matrix metalloproteinase-2, -8, -9, and -13 in gingival crevicular fluid of short root anomaly patients. *Eur J Orthod* 2003; **25:**365–369.
20. Mancini S, Romanelli R, Laschinger CA, Overall CM, Sodek J, McCuloch CA. Assessment of a novel screening test for neutrophil collagenase activity in the diagnosis of periodontal diseases. *J Periodontol* 1999;**70:**1292–1302.
21. Choi DH, Moon IS, Choi BK et al. Effects of sub-antimicrobial dose doxycycline therapy on crevicular fluid MMP-8 and gingival tissue MMP-9, TIMP-1 and IL-6 levels in chronic periodontitis. *J Periodont Res* 2004;**39:**20–26.
22. Alpagot T, Bell C, Lundergam W, Chambers DW, Rudin R. Longitudinal evaluation if GCF MMP-3 and TIMP-1 levels as prognostic factors for progression of periodontitis. *J Clin Periodontol* 2001; **28:**353–359.
23. Chen HY, Cox SW, Eley BM, Mäntylä P, Rönkä H, Sorsa T. Matrix metalloproteinases-8 levels and elastase activities in gingival crevicular fluid from chronic adult periodontitis patients. *J Clin Periodontol* 2000;**27:**366–369.
24. Kinane DF, Darby IB, Said S et al. Changes in gingival crevicular fluid matrix metalloproteinase-8 levels during periodontal treatment and maintenance. *J Periodont Res* 2003;**38:**400–404.
25. Buduneli N, Vardr S, Atilla G, Sorsa T, Luoto H, Baylas H. Gingival crevicular fluid matrix metalloproteinases-8 levels following adjunctive use of meloxicam and initial phase of periodontal therapy. *J Periodontol* 2002;**73:**103–109.
26. Mäntylä P, Stenman M, Kinane DF et al. Gingival crevicular fluid collagenase 2 (MMP-8) test stick for chair-side monitoring of periodontitis. *J Periodont Res* 2003;**38:**436–439.
27. Bhide VM, Smith L, Overall CM, Birek P, McCulloch CAG. Use of fluorogenic septapeptide matrix metalloproteinase

assay to assess responses to periodontal treatment. *J Periodontol* 2000;**71:**690–700.

28. Fasciglione GF, Marini S, D'Alessio S, Politi V, Coletta M. pH- and temperature-dependence of functional modulation in metalloproteinases. A comparison between neutrophil collagenase and gelatinases A and B. *Biophys J* 2000;**79:**2138–2149.

29. Neumann U, Kubota H, Frei K, Ganu V, Leppert D. Charaterization of Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg. NH$_2$, a fluorogenic substrate with increased specificity constants for collagenases and tumor necrosis factor converting enzyme. *Anal Biochem* 2004;**328:** 166–173.

30. Tüter G, Kurtis B, Serdar M. Effects of phase I periodontal treatment on gingival crevicular fluid levels of matrix metalloproteinases-1 and tissue inhibitor of metalloproteinase-1. *J Periodontol* 2002;**73:** 489–493.

31. Nomura T, Ishii A, Oishi Y, KohmaH, Hara K. Tissue inhibitors of metalloproteinases level and collagenase activity in gingival crevicular fluid: the relevance to periodontal diseases. *Oral Dis* 1998;**4:**231–234.

32. Golub LM, McNamara TF, Ryan ME *et al*. Adjunctive treatment with subantimicrobial doses of doxycycline: effects on gingival fluid collagenase activity and attachment loss in adult periodontitis. *J Clin Periodontol* 2001;**28:**146–156.

33. Emingil G, Atilla G, Sorsa T, Luoto H, Kirilmaz L, Baylas H. The effect of adjunctive low-dose doxycycline therapy on clinical parameters and gingival crevicular fluid matrix metalloproteinase-8 levels in chronic periodontitis. *J Periodontol* 2004;**75:**106–115.

34. Valenzuela MA, Cartier L, Collados L *et al*. Gelatinase activity of matrix metalloproteinases in the cerebrospinal fluid from various patient populations. *Res Commun Mol Pathol Pharmacol* 1999;**104:** 42–52.

35. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227:**680–685.

36. Kettlun AM, Cartier L, García L *et al*. TIMPs and MMPs expression in CSF from patients with TSP/HAM. *Life Sci* 2003;**72:**2863–2876.

37. Westerlund U, Ingman T, Lukinmaa PL *et al*. Human neutrophil gelatinase and associated lipocalin in adult and localized juvenile periodontitis. *J Dent Res* 1996;**75:** 1553–1563.

38. Mäkelä M, Sato T, Uitto VJ, Larjava H. Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status. *J Dent Res* 1994;**73:**1397–1406.

39. Kivelä-Rajamäki M, Maisi P, Srinivas R *et al*. Levels and molecular forms of MMP-7 (matrilysin-1) and MMP-8 (collagenase-2) in diseased human peri-implant sulcular fluid. *J Periodont Res* 2003;**38:**383–390.

40. Teng YT, Sodek J, McCulloch CA. Gingival crevicular fluid gelatinase and its relationship to periodontal disease in human subjects. *J Periodont Res* 1992; **27:**544–552.