J Clin Periodontol 2010; 37: 180–190 doi: 10.1111/j.1600-051X.2009.01512.x

Journal of Clinical Periodontology

Gingival crevicular fluid levels of MMP-8, MMP-9, TIMP-2, and MPO decrease after periodontal therapy

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Marcaccini AM, Meschiari CA, Zuardi LR, de Sousa TS, Taba M, Teofilo JM, Jacob-Ferreira ALB, Tanus-Santos JE, Novaes AB, Gerlach RF. Gingival crevicular fluid levels of MMP-8, MMP-9, TIMP-2, and MPO decrease after periodontal therapy. J Clin Periodontol 2010; 37: 180–190. doi: 10.1111/j.1600-051X.2009.01512.x.

Abstract

Background: This study aimed at comparing the levels of matrix metalloproteinase (MMP)-8, tissue Inhibitor of MMPs (TIMP)-1 and TIMP-2, Myeloperoxidase (MPO), and MMP-9 in the gingival crevicular fluid (GCF) of chronic periodontitis (CP) patients and controls at baseline and 3 months after non-surgical therapy.

Materials and Methods: GCF was collected from one site of 15 control subjects and 27 CP patients. MMP-8, MMP-9, TIMP-1, and TIMP-2 were determined by Enzyme-linked immunoabsorbent assay; different forms of MMP-9, by gelatin zymography; and MPO, colorimetrically.

Results: At baseline, higher levels of MMP-8, TIMP-2, MPO, and the 87 kDa-MMP-9 were found in patients compared with controls ($p < 0.001$), and these molecules decreased after therapy ($p < 0.03$). There were no differences between the groups with respect to the higher molecular forms of MMP-9 (180, 130, 92 kDa) or total MMP-9 at baseline. No differences were observed in TIMP-1 levels. In controls, decreased levels of TIMP-2 and the higher molecular forms of MMP-9 (180, 130, 92 kDa) were found 3 months after therapy compared with baseline ($p < 0.01$).

Conclusions: Higher levels of MMP-8, TIMP-2, MPO, and 87 kDa MMP-9 were found in the GCF of patients compared with controls, and these markers decreased 3 months after periodontal therapy.

Key words: chronic periodontitis; non-surgical therapy; GCF; gingival crevicular fluid; MMP-8; MMP-9; MPO; TIMP-1; TIMP-2

Accepted for publication 25 October 2009

Matrix metalloproteinases (MMPs) are key proteolytic enzymes for periodontal tissue destruction (Uitto et al. 2003). Because MMPs are responsible for the degradation of all extracellular matrix proteins, precise information about their presence, amounts, and activities is thought to be very important for the in-depth knowledge and characterization of different forms of periodontal disease, for determination of active destruction, and for monitoring purposes.

Tissue extracts and culture tissue explants of inflamed human gingiva were shown to contain more collagenase activity than extracts and explants from healthy human gingiva, and collagenase activity in the GCF also increased and correlated with the severity of the periodontal disease (Sorsa et al. 2004). Sorsa et al. (1988) demonstrated that the major collagenase in periodontitis is collagenase-2 (MMP-8), followed by MMP-9 (Sorsa et al. 1995). MMP-8 and MMP-9 were described to be increased in the gingival crevicular fluid (GCF) of sites with active periodontal disease (Lee et al. 1995). MMP-8 and MMP-9 are present in the granules of polymorphonuclear neutrophils (PMNs) (Hartog et al. 2003), but they are also expressed by a variety of other cells present in the normal and diseased periodontium (Sorsa et al. 2004). Interestingly, in 1996, it was shown that while adult (chronic) periodontitis GCF contained high amounts of MMP-8 and MMP-9 (possibly derived from neutrophils) and no TIMP-1, the GCF collected from patients

Conflict of interest and sources of funding statement

The authors declare that they have no conflict of interests.

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with localized juvenile periodontitis exhibited high concentrations of TIMP-1 and MMP-1 (Ingman et al. 1996). Although other MMPs have been described in the gingival tissue (such as MMP-2, MMP-7, MMP-14) (Tervahartiala et al. 2000), the most widely reported MMPs in the GCF are MMP-8, MMP-9, and MMP-13 (Choi et al. 2004, Tuter et al. 2005, Beklen et al. 2006, Kumar et al. 2006, Soder et al. 2006). It has also become clear that from the three collagenases (MMP-1, MMP-8, and MMP-13), MMP-8 accounts for 80% of the total collagenase protein found in the GCF, with much smaller relative amounts of MMP-13 and MMP-1 (in this order) in chronic periodontitis (CP) (Golub et al. 2008).

In neutrophils, six different types of granules can be distinguished (Gullberg et al. 1997). The different granules are synthesized subsequently during different stages of myeloid differentiation and each contains specific proteases as well as other enzymes and proteins (Cowland & Borregaard 1999). Myeloperoxidase (MPO) is found in primary or azurophil granules, while gelatinase B (92 kDa MMP-9) is part of tertiary granules. The secretion of the different neutrophil granules depends on the stimulus (Van den Steen et al. 2002). PMN activation during periodontal inflammation results from the action of prostaglandins, cytokines, and periodontopathogenic bacteria and/or their virulence factors (Birkedal-Hansen 1993). Activated PMNs degranulate the 25 kDa neutrophil gelatinase-associated lipocalin (NGAL) molecule mostly from the secondary PMN granules (Kjeldsen et al. 1994), which is a covalently bound 130 kDa complex containing the 92 kDa MMP-9 form, also found in the tertiary PMN granules (Sengelov et al. 1994). This NGAL–MMP-9 complex is unique for PMNs (Kjeldsen et al. 1994). MPO has been considered a promising marker of periodontal inflammation (Yamalik et al. 2000, Wei et al. 2004, Kaner et al. 2006), but there is limited knowledge about the association between proteases and MPO in CP disease.

Non-surgical periodontal therapy has been demonstrated to reduce MMP-8 levels and activity by approximately 50% (Kinane et al. 2003), while sites that do not respond to treatment have been associated with elevated MMP-8 concentrations (Mantyla et al. 2003). MMP-8 has been suggested as a promising marker of active disease (Sorsa et al. 1999). Results from a chair-side test based on the immunological detection of elevated levels of MMP-8 in the GCF revealed that it was possible to differentiate periodontitis from gingivitis and healthy sites as well as to monitor the treatment of periodontitis (Mantyla et al. 2006). On the other hand, commercial MMP-8 enzyme-linked immunoabsorbent assay (ELISA) results showed that periodontitis-affected sites continue to display high levels of MMP-8 and collagenolytic activity after effective periodontal treatment, and these levels and activity were in the range of those found in gingivitis sites. Furthermore, the authors did not find correlations between the results of clinical treatment and proteolytic activity at baseline or after treatment (Figueredo et al. 2004). These contrasting results point to the need for more in-depth information on MMPs in the GCF. Although the diagnostic tools based on enzyme activity are very appealing and have a considerable potential for application, as pointed by Uitto et al. (2003) ''there is a need for new data to determine if crevicular fluid-based tests will lead to improved disease detection and patient outcomes''.

Despite the increase in the number of studies on MMPs and TIMPs in the GCF over the past years, some limitations have still not been overcome, such as: (1) the use of GCF pools to determine MMPs and TIMPs, instead of a GCF sample from one site; (2) the lack of comparison before and after treatment; and (3) the lack of inclusion of a control group. Therefore, we have attempted to characterize the most widely described MMPs and TIMPs at the protein level in CP, both before and after non-surgical therapy. We have also concentrated our efforts on performing the assays only in samples collected from one site, to avoid site-dependent variability.

This study aimed at comparing the levels of MMP-8, MMP-9, tissue inhibitor of MMPs (TIMP)-1 and TIMP-2, and MPO in the GCF of CP patients and controls at baseline and 3 months after non-surgical therapy. This study also aimed to test the correlations between these markers.

Materials and Methods

Patients

A total of 42 patients were enrolled in this study, and they gave their informed consent to participate in the investigation. This study was approved by the Institutional Review Board, and is part of a larger study, whose results on inflammatory markers in the plasma (Marcaccini et al. 2009a) and MMPs in the plasma (Gerlach et al. 2009, Marcaccini et al. 2009b) are published elsewhere.

All patients met all the enrollment criteria, which included: being aged 35–55 years; having $\geqslant 20$ teeth in the mouth, none of which with untreated periapical lesions; and not having previous subgingival periodontal debridement or periodontal surgery in the preceding 6 months. Additional exclusion criteria were applied: the presence of systemic diseases that could influence the course of the periodontal disease; any chronic inflammatory or immunological conditions, such as cardiovascular disease, diabetes, arthritis, gastrointestinal disorders, skin conditions, bronchitis, or other chronic obstructive airway diseases; smoking (current or former smoker for <10 years); and pregnancy or intention to become pregnant during the study period. Patients regularly taking any of the following types of drugs in the previous 6 months were also excluded: anti-inflammatory, steroids, immunosupressants, statins, lipid-lowering drugs, or anticoagulants. Subjects who had received antimicrobial therapy for the previous 6 months were also excluded (Ide et al. 2003).

After applying the inclusion and exclusion criteria, two groups were selected: a CP group and a control (C) group, according to the periodontal status. A single previously calibrated examiner (A. M. M.) recorded the following clinical variables: bleeding on probing (BOP), pocket depth (PD), and clinical attachment loss (CAL). These variables were assessed in all the teeth, at six sites around each tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual locations). The cemento-enamel junction was accepted as the reference point in CAL measurements. PD and CAL measurements were performed using an automated pocket probe (Florida Probe, Gainesville, FL, USA). BOP (deemed positive if occurring within 15 s after periodontal probing) was recorded dichotomously. Patients were assigned to the CP group when at least two teeth had probing PD $\geqslant 5$ mm, CAL $\geqslant 6$ mm, and radiographic evidence of alveolar bone loss (Machtei et al. 1992). Control subjects exhibited no

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clinical signs of gingival inflammation (no BOP) or presented varying degrees of gingival inflammation, but no radiographic evidence of alveolar bone loss, and PD < 3 mm.

Study design

Both study groups underwent an initial examination consisting of complete radiographic series, clinical history, and assessment of clinical periodontal variables. All patients who met the inclusion criteria (from CP and control groups) received instructions on oral hygiene techniques and supragingival prophylaxis in the next session. The treatment of the CP group was a standard phase of non-surgical, cause-related periodontal therapy consisting of scaling and root planing over a 3- to 4-week period using Gracey curettes (HuFriedy® Manufacturing Inc., Chicago, IL, USA) and ultrasonic instrumentation (Cavitron® Ultrasonics Inc., Long Island City, NY, USA) performed by one periodontologist (A. M. M.), under local anaesthesia and without time limitation.

In the control group ($n = 15$), there were 10 women and 5 men (mean age, 44.88 years and range, 35–50 years). They had 25.22 teeth with healthy or minimally inflamed gingiva (gingivitis). In the CP group (CP) ($n = 27$), 17 women and 10 men were included (mean age, 44.13 years and range, 36–55 years) with 25.46 teeth, and 12.7% of all sites presented pockets deeper than 5 mm. Subjects were followed up at 3 months after completion of the non-surgical periodontal therapy. In each visit, oral hygiene instructions were reinforced, and supragingival prophylaxis was carried out. Clinical periodontal measurements and GCF sampling were repeated 3 months after the therapy was completed.

Collection of GCF

GCF samples were taken from mesiobuccal or disto-buccal aspects of one single-rooted tooth, before therapy (baseline) and after 3 months. The randomly selected site was chosen among those with greater PD and bleeding for CP. In the control group, GCF samples were collected from one site with ⩽2 mm PD. After isolating the tooth with a cotton roll, the supragingival plaque was removed with a curette without touching the marginal gingiva. The crevicular site was then dried gently with an air syringe. The GCF samples were collected using one Periopaper® absorbent paper strip (Pro-Flow Corp., Amityville, NY, USA), which was placed into the sulcus/pocket until mild resistance was felt and left in place for 30 s (Lamster et al. 1985). Samples were always taken from the same sites at the two visits (at baseline and 3 months after non-surgical therapy). Strips contaminated by saliva or blood were excluded. The volume of GCF was determined by means of a previously calibrated electronic device (Periotron 6000, ProFlow) and converted into an actual volume (μl) by reference to the standard curve. All strips with GCF were immediately and individually placed in Eppendorf vials containing 100 μl buffer (50 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM $CaCl_2$, 0.02% Triton X-100) for 30 min. at 4°C (Pozo et al. 2005). The GCF was centrifuged at 13,000 *g* for 10 min. at 4°C, and the supernatants were aliquoted and stored at − 70°C until further analysis (Song et al. 2003). All analyses were performed directly on this 100 μl sample (one sample per patient, from only one site per patient). Each aliquot was thawed only at the time of use.

MPO assay

GCF MPO concentrations were measured by means of an MPO technique. Briefly, the amount of MPO in each sample was measured enzymatically by suspending the material in 2.0 ml 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO, USA) in 50 mM potassium phosphate buffer, pH 5.4, to solubilize MPO (Bradley et al. 1982). After this, the MPO was assayed spectrophotometrically by addition of 1.6 mM tetramethyl benzidine (Sigma Chemical Co.) diluted in δ-dianisidine dihydrochloride (Sigma Chemical Co.) and 0.5 mM hydrogen peroxide in a Costar 96-well plate (Corning Inc., New York, NY, USA). The colorimetric reading was accomplished at a wavelength of 450 nm after addition of 4 M H_2SO_4, and the plates were read using a μQuant™ microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). A standard curve was generated using human PMNs. The PMNs were isolated from human blood by density-gradient centrifugation (Bozeman et al. 1990) and suspended to 1×10^6 cells/ml in PBS.

SDS-polyacrilamide gel electrophoresis (PAGE) gelatin zymography of MMP-9

Gelatin zymography of MMP-9 from the GCF was performed as described previously (Gerlach et al. 2005, 2007, Souza-Tarla et al. 2005). Briefly, GCF samples were subjected to electrophoresis on 12% SDS-PAGE co-polymerized with gelatin (0.1%) as the substrate. Foetal calf serum was used as the molecular weight standard for gelatinases, because it contains both MMP-2 and the different forms of MMP-9 found in serum. After electrophoresis was completed, the gel was incubated two times for half an hour at room temperature in a 2% Triton X-100 solution, and incubated at 37°C for 16 h in Tris-HCl buffer, pH 7.4, containing 10 mmol/l $CaCl_2$. The gels were stained with 0.05% Coomassie Brilliant Blue G-250, and then de-stained with 30% methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of the Coomassie blue-stained gelatin. Enzyme activity was assayed by densitometry using a Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Kodak, Rochester, NY, USA).

GCF ELISA analysis of MMP-8, MMP-9, TIMP-1, and TIMP-2

Concentrations of MMP-8, MMP-9, TIMP-1, and TIMP-2 in the GCF samples were assayed by the sandwich (ELISA) kit (DuoSet R&D Systems Inc., Minneapolis, MN, USA). All assay procedures were carried out according to the manufacturer's instructions. The ELISA plates were then assessed spectrophotometrically at OD 490 nm. The levels of GCF MMP-8, MMP-9, TIMP-1, and TIMP-2 in each sample were determined using the concentration values of standards included in the kit contents. GCF samples were assayed at the following dilutions: 1:20 for MMP-8, 1:100 for MMP-9, 1:10 for TIMP-1, and 1:20 for TIMP-2 according to standardization performed in our laboratory, and always 100 μl of these dilutions were used in each well of the ELISA plates, as recommended by the manufacturer. The results for MMPs and TIMPs were obtained by multiplying the ELISA results by the dilutions. These results were expressed in ng/ml, then being multiplied by the initial sample volume (0.1 ml buffer+GCF volume) to obtain results as ng/sample.

Statistics

The mean patient clinical parameters, GCF volumes, and the total MPO,

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MMP-8, MMP-9, TIMP-1, and TIMP-2 levels were calculated and analysed for distribution. Only BOP and 87 kDa MMP-9 did not pass the normality test, and non-parametric statistics was used to analyse these data. All other data were analysed with parametric statistics.

Baseline comparisons between controls and patients with periodontitis were accomplished using an unpaired *t*-test or the Mann–Whitney test, as necessary. At follow-up (3 months), comparisons within groups were performed using a paired *t*-test or the Wilcoxon test. Differences were considered significant when $p < 0.05$.

The differences in the proportions of 87 and ~170 kDa MMP-9 (expressed as percentage of patients positive for those MMP-9 forms) between the control and the CP groups were compared by means of the χ^2-test, followed by Yates correction, and the significance level accepted was $p < 0.05$.

Correlations between clinical parameters (from the very site of GCF collection) and inflammatory markers were performed, using baseline and 3-month data for both groups in each correlation analysis. In this case, after the Bonferroni correction, correlations were considered statistically significant when $p < 0.0045$; the Pearson correlation test was used for normally distributed variables, whereas the Spearman correlation test was used for correlation when non-normally distributed data were analysed.

Table 1. Clinical parameters and GCF volumes from the sites in which GCF was collected in this study

	Control group ($n = 15$)		CP group ($n = 27$)		p-value*
	baseline	3 months	baseline	3 months	
Bleeding on probing [n (%)]	8 (53.34)	1 (6.67)†	21 (77.78)	4 (14.81)**	>0.05
Pocket depth (mm)	2.24 ± 0.68	1.74 ± 0.37‡	4.50 ± 1.84	2.50 ± 1.04††	<0.0001
Clinical attachment loss (mm)	2.3 ± 0.6	1.9 ± 0.4§	4.8 ± 1.9	3.0 ± 1.5‡‡	<0.0001
GCF volumes (μl)	0.43 ± 0.35	0.42 ± 0.27	0.7 ± 0.41	0.6 ± 0.41	0.03

Pocket depth, clinical attachment loss, and GCF volumes are shown as mean ± SD.
*p-value for comparisons between the control and chronic periodontitis groups at baseline (unpaired *t*-test and Mann–Whitney test).
†$p = 0.01$ for comparison within the control group between baseline and 3 months (Wilcoxon *U*-test).
‡$p = 0.02$ for comparison within the control group between baseline and 3 months (paired *t*-test).
§$p = 0.04$ for comparison within the control group between baseline and 3 months (paired *t*-test).
**$p = 0.0001$ for comparison within the chronic periodontitis group between baseline and 3 months (Wilcoxon *U*-test).
††$p < 0.0001$ for comparison within the chronic periodontitis group between baseline and 3 months (paired *t*-test).
‡‡$p = 0.0001$ for comparison within the chronic periodontitis group between baseline and 3 months (paired *t*-test).
GCF, gingival crevicular fluid; CP, chronic periodontitis.

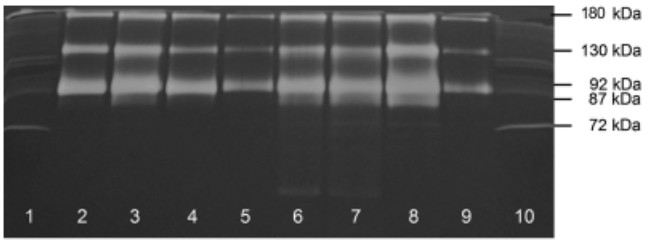

Fig. 1. Representative gelatin zymogram of gingival crevicular fluid samples (4 μl/lane). Lanes 1 and 10: molecular weight standard for gelatinases. Lanes 2, 4, 6, and 8: samples from four chronic periodontitis patients at baseline. Lanes 3, 5, 7, and 9: samples from these same four patients at 3-month follow-up.

Results

Clinical findings

The study groups (controls and patients with CP) included patients of similar age and proportions of males and females per group ($p > 0.05$, not shown). Patients' clinical parameters (PD, CAL, and BOP) for all sites of these patients are presented elsewhere (Marcaccini et al. 2009b). The mean PD, CAL, BOP, and GCF volumes for the sites collected in this study are shown in Table 1. There was no difference in BOP between the control and the CP groups at baseline ($p > 0.05$), probably due to gingivitis in the controls, because after 3 months there were significant decreases in BOP between the two groups ($p = 0.01$ for controls and $p = 0.0001$ for the CP group). Differences between controls and CP groups in terms of PD and CAL reached $p < 0.0001$ before non-surgical therapy. Treatment led to significant decreases in these parameters. There was a difference in the GCF volume between controls and CP groups ($p = 0.03$) at baseline, and no reduction in the volume of fluid collected in 30 s per site was observed 3 months after therapy ($p > 0.05$).

GCF MPO, MMP-8, MMP-9, TIMP-1, and TIMP-2 levels

The amounts of the different molecular forms of MMP-9 were quantified in gelatin zymograms. Figure 1 shows one representative zymogram used for MMP-9 quantification. Gelatin zymography was performed in all patients and control subjects. On the basis of literature studies on the analysis of GCF by means of zymograms, as well as preliminary tests using various volumes of fluid, we defined that 4 μl of the 100 μl GCF sample would be used throughout this study. This volume was adequate for the quantification of all the bands, except for the most abundant form of MMP-9, the 92 kDa pro-MMP-9 form, whose degradation of gelatin lay outside a linear range in most samples. The MMP-2 molecular form was absent from the GCF of most CP patients and controls. Gelatinolytic bands of 180, 130, and 92 kDa were detected in all the samples (Fig. 1). These bands corresponded to the typical banding pattern of neutrophil-derived MMP-9 consisting of pro-MMP-9 (92 kDa), human neutrophil lipocalin–pro-MMP-9 complex (HNL or 130 kDa), and the homodimeric form of MMP-9 (180 kDa). Two additional gelatinolytic bands were detected in some samples: the active MMP-9 form (87 kDa) and a second (lower molecular) form of the homodimeric MMP-9, which was observed as

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an ~ 170 kDa band (possibly a dimer of the active 87 kDa MMP-9). Because these two lower molecular forms of MMP-9 (87- and ~ 170 kDa) were not observed in all patients, we tested whether in some subjects their absence was a consequence of the small volume of GCF applied to the gel (4 μl).

We therefore performed zymograms with increasing volumes of sample from control patients and decreasing volumes of sample from CP patients. One representative gel of this experiment is shown in Fig. 2. Even when increasing volumes of control GCF were applied, the 87 kDa form of MMP-9 was not observed, and conversely, this form was still observed when smaller volumes of CP patients' GCF were applied, suggesting that the volume used was probably not a problem for the detection of this form in the patients of this study. One striking observation is that the ~170 kDa form (Fig. 2, arrow) could be distinguished in CP patient samples even when 0.5 μl of the sample was applied. This band was probably not observed in Fig. 1 because of a shorter run, as it can be observed that the higher molecular bands are not as well resolved as they are in Fig. 2. This ~170 kDa gelatinolytic band is compatible with a dimeric MMP-9 form, because a band of such a molecular mass is reactive with anti-MMP-9 antibodies (results not shown).

Table 2 shows the quantification of the molecular forms of MMP-9 in zymograms. There were no statistically significant differences between the control and the CP groups at baseline with respect to the 180, 130, and 92 kDa MMP-9 forms (although the information on this last form is not suitable for quantification, as detailed above). The 87 kDa MMP-9 (active) was increased in CP patients at baseline ($p = 0.004$), compared with control subjects. In the control group, there were statistically significant decreases in the 180 kDa ($p = 0.007$), 130 kDa ($p = 0.01$), and 92 kDa ($p = 0.007$) MMP-9 molecular forms 3 months after therapy. In the CP group, there were statistically significant decreases in the 180 kDa ($p = 0.002$) and 87 kDa ($p = 0.007$) molecular forms 3 months after therapy. The lack of difference in the 92 kDa MMP-9 form in this group is probably an artefact due to the very high amounts of this form found in CP patients, and we therefore performed quantification of total MMP-9 by ELISA (around 2/3 of the MMP-9 present in the GCF samples corresponds to the 92 kDa form).

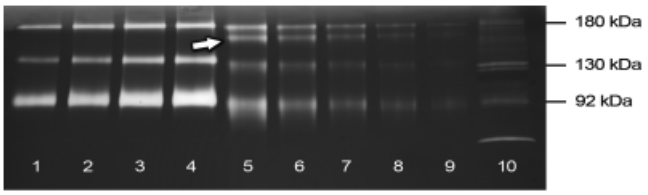

Fig. 2. Representative gelatin zymogram of gingival crevicular fluid (GCF) samples. Lanes 1–4: increasing volumes (4, 8, 12, and 16 μl/lane, respectively) of GCF from one control subject. Lanes 5–9: decreasing volumes (4, 2, 1, 0.5, and 0.25 μl/lane) of GCF from one chronic periodontitis patient. Lane 10: molecular weight standard for gelatinases. The arrow indicates the 150 kDa dimeric MMP-9 form that is present in chronic periodontitis GCF. The 87 kDa active MMP-9 form can be observed in lanes 5 and 6.

Table 2. Concentrations of different molecular forms of MMP-9 in the GCF of the controls and chronic periodontitis patients, at baseline and 3 months after scaling and root planing treatment

MMP-9	MW (kDa)	Control group ($n = 15$)		CP group ($n = 27$)		p-value*
		baseline	3 months	baseline	3 months	
Complex	180	4.7 ± 3.0	3.3 ± 1.9†	5.4 ± 2.8	4.1 ± 2.7**	> 0.05
MMP-9+Lipocaline	130	7.5 ± 5.1	4.6 ± 2.9‡	7.6 ± 4.6	7.3 ± 4.7	> 0.05
MMP-9	92	18.1 ± 9.9	13.0 ± 7.0§	20.0 ± 11.3	17.7 ± 9.5	> 0.05
Active MMP-9	87	0.36 ± 0.6	0.1 ± 0.3	4.0 ± 5.1	1.6 ± 2.3††	0.004

Data are shown as Mean ± SD.

*p-value for comparisons between the control and chronic periodontitis groups at baseline (unpaired t-test and Mann–Whitney test).

†$p = 0.007$ for comparison within the control group between baseline and 3 months (paired t-test).

‡$p = 0.01$ for comparison within the control group between baseline and 3 months (paired t-test).

§$p = 0.007$ for comparison within the control group between baseline and 3 months (paired t-test).

**$p = 0.002$ for comparison within the chronic periodontitis group between baseline and 3 months (paired t-test).

††$p = 0.007$ for comparison within the chronic periodontitis group between baseline and 3 months (Wilcoxon U-test).

MMP-9, matrix metalloproteinase 9; GCF, gingival crevicular fluid; CP, chronic periodontitis.

The percentage of subjects who exhibited the active forms of MMP-9 was determined. While the ~170 kDa MMP-9 form was found in 81.5% of the CP patients at baseline, being found only in 20% of these patients 3 months after treatment ($p < 0.001$), this same MMP-9 form was found in only 35.3% of the control subjects at baseline ($p < 0.01$ for comparison with CP patients before treatment) and in 15.4% of those after treatment ($p = 0.15$). The same trend was observed when we compared the percentage of subjects who showed the active MMP-9 form (87 kDa). At baseline, this form was found in 66.7% of the CP patients and 23.5% of the controls ($p < 0.01$), decreasing to 40% of the CP patients ($p = 0.2$) and to 7.7% of the controls ($p < 0.05$) 3 months after therapy.

Figure 3 displays the results of total MMP-9 quantified by ELISA. No difference was found between the controls and CP patients at baseline, while treatment resulted in significant decreases in total MMP-9 both in the controls ($p < 0.0001$) and in the CP patients ($p = 0.0006$).

As shown in Fig. 4, at baseline, higher levels of MMP-8 were detected in the GCF samples of CP patients compared with the controls ($p < 0.0007$). Three months after non-surgical periodontal therapy, the MMP-8 levels decreased significantly only in the CP group ($p = 0.0009$). Treatment had no significant effect on the MMP-8 levels of the control samples.

Figure 5 shows that higher amounts of MPO were found in the CP group compared with controls ($p = 0.001$). After 3 months of non-surgical periodontal therapy, MPO amounts decreased ($p = 0.03$) in CP patients; however, this difference was not as pronounced as that of the other variables tested in this study.

Results from the TIMP-1 and TIMP-2 levels are shown in Figs 6 and 7, respectively. No differences in the

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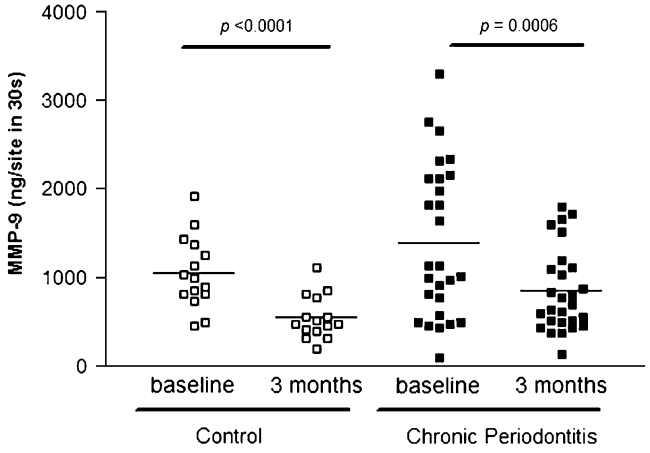

Fig. 3. Gingival crevicular fluid matrix metalloproteinase 9 (MMP-9) levels determined by enzyme-linked immunoabsorbent assay of control ($n = 15$) and chronic periodontitis ($n = 27$) subjects.

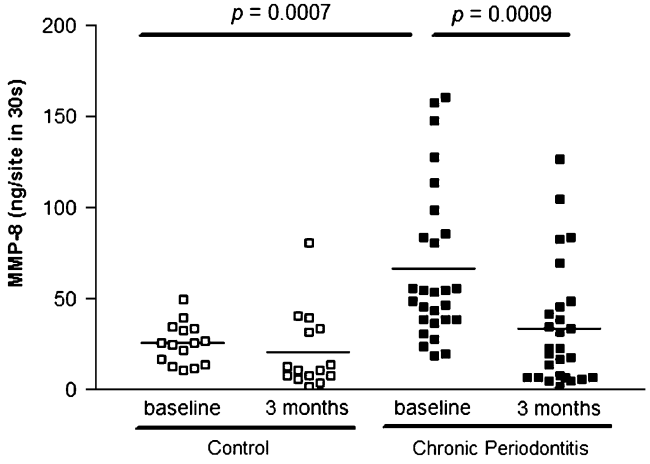

Fig. 4. Gingival crevicular fluid matrix metalloproteinase 8 (MMP-8) levels determined by enzyme-linked immunoabsorbent assay of control ($n = 15$) and chronic periodontitis ($n = 27$) subjects.

TIMP-1 levels were observed between the groups. Significantly increased TIMP-2 levels were observed in the CP group at baseline ($p < 0.0001$ *versus* controls), and these levels decreased 3 months after non-surgical periodontal therapy in the CP patients ($p = 0.0009$).

The MMP-8/TIMP-1 and MMP-9/TIMP-1 ratios were calculated (data not shown). There was a difference between control and CP data at baseline in terms of the MMP-8/TIMP-1 ratio ($p = 0.03$), while no difference was observed between control and CP at baseline with respect to the MMP-9/TIMP-1 ratio. When baseline results from the control group were compared with follow-up results, a significant decrease in the MMP-9/TIMP-1 ratio was observed ($p = 0.001$). In the CP patients, therapy resulted in lower MMP-8/TIMP-1 ($p = 0.001$) and MMP-9/TIMP-1 ($p = 0.008$) ratios. These data further support that increased MMP-8 and MMP-9 levels result in imbalanced MMP/TIMP ratios.

Correlation analysis

Correlation data between the several variables tested in this study are shown in Table 3 for the controls and in Table 4 for the CP patients. Those with a p-value < 0.0045 are considered significant correlations .

In the control patients, a significant correlation was found between the 130 and the 180 kDa MMP-9 forms, and the 130 kDa MMP-9 and the total MMP-9 ($r = 0.67$ and $p < 0.0001$ for both comparisons). Significant correlations were also found between total MMP-9 and MPO ($r = 0.77$, $p < 0.0001$), total MMP-9 and PD ($r = 0.67$, $p = 0.0001$), and MPO with PD ($r = 0.59$, and $p = 0.001$).

A larger number of significant correlations were observed in the CP patients. In these individuals, all the MMP-9 forms were positively correlated (r_s values varied between 0.42 and 0.68, $p \leqslant 0.002$ for all comparisons). TIMP-2 was also positively correlated with the 180, 87 kDa, and total MMP-9 ($r_s = 0.56$, $= 0.44$, and $= 0.60$, respectively). TIMP-2 was positively correlated with MMP-8 ($r = 0.54$; $p < 0.0001$). MPO was positively correlated with all forms of MMP-9 (r_s values varying from 0.42 to 0.60, $p \leqslant 0.002$), and with TIMP-2 ($r = 0.48$; $p = 0.0006$). PD was positively correlated with the 87 kDa and total MMP-9 ($r_s = 0.39$ and $= 0.41$, $p \leqslant 0.004$), and with MMP-8 ($r = 0.39$, $p = 0.0043$). BOP was positively correlated with MMP-8 ($r_s = 0.39$, $p = 0.004$), TIMP-2 ($r_s = 0.45$, $p = 0.001$), and PD ($r_s = 0.48$, $p = 0.0003$). A moderate positive correlation was found between the GCF volume and the 87 kDa MMP-9 form ($r_s = 0.39$, $p = 0.003$), and the TIMP-1 levels ($r = 0.43$, $p = 0.001$).

Discussion

Our results show that there is a clear distinction in the amounts of the markers tested in this study that were all assayed in a GCF sample collected for 30 s in one site. MMP-8, MPO, and TIMP-2 appeared to follow the same pattern, with increased levels found in CP patients *versus* controls at baseline, and a decrease 3 months after therapy in the former patients, with no changes in the control after 3 months. There was no difference in the TIMP-1 levels between the groups at baseline, or within the control or the CP group when samples collected before and after therapy were compared.

Total MMP-9 was increased both in the controls and in the CP patients, and it decreased in both groups after therapy. Therefore, the total MMP-9 results suggest that this MMP is increased both in gingivitis and in periodontitis. However, the total MMP-9 results reflect mostly the presence of the 92 kDa (inactive) MMP-9 form (which constitutes on average 2/3 of the MMP-9 forms resolved on our zymograms). Detailed information on the other MMP-9 forms revealed that differences in the 87 and $\sim$ 170 kDa MMP-9 forms (probably

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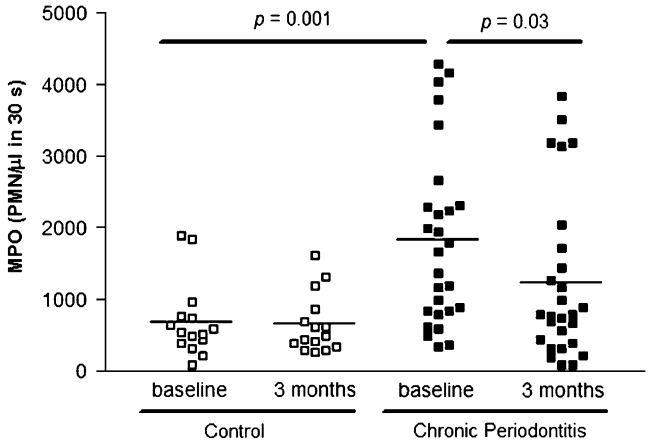

Fig. 5. Gingival crevicular fluid myeloperoxidase (MPO) levels enzymatically determined of control ($n = 15$) and chronic periodontitis ($n = 27$) subjects.

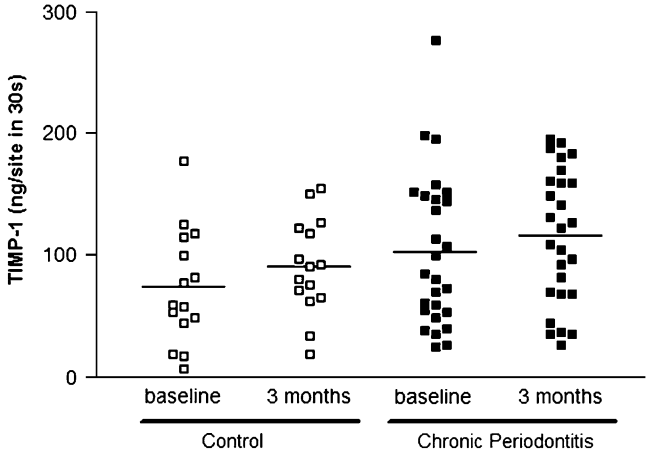

Fig. 6. Gingival crevicular fluid tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) levels determined by enzyme-linked immunoabsorbent assay of control ($n = 15$) and chronic periodontitis ($n = 27$) subjects.

active enzymes) were also found when the groups were compared. The differences in these active forms of MMP-9 are probably very important clinically, because these forms are capable of degrading many tissue components, and, in concert with other proteases, will cause tissue destruction.

According to Olson et al. (2000), pro-MMP-9 is unique among the members of the MMP family in that it forms dimers consisting of covalently tethered monomers via a disulphide bond, and such dimers can also be found in tissues. Biochemical assays on monomeric and dimeric MMP-9 forms showed that both cleave gelatin (used as the substrate) with similar catalytic efficiency; both are activated with MMP-3 (stromelysin-1). However, the dimeric pro-MMP-9 form is activated with a catalytic efficiency 10-fold lower than that of the monomeric pro-MMP-9, and the dimeric form is substantially more stable than the monomer form (Olson et al. 2000). This distinct activation efficiency suggests that in vivo, these two forms may behave differently, with the monomeric form being readily activated, while the slow activating dimer might provide an additional level of control during proteolysis by MMP-9 species.

While the $\sim$ 170 kDa MMP-9 form was present in 81.5% of the CP patients at baseline, it was only present in 20% of these same patients after therapy. Therefore, this particular form of MMP-9, together with the 87 kDa form, may represent a good marker of tissue degradation. Although many studies have determined MMP-9 in GCF samples, few have attempted to describe the precise amounts of the different forms of MMP-9. To this end, two techniques need to be used (zymograms and ELISA, or only zymograms using different sample volumes). It will be interesting to see further studies with a larger number of patients comparing sites with different degrees of PD, to observe whether biochemical differences in GCF MMP-9 forms correlate with CP activity. In Fig. 2, it can be observed that while the control patient samples generated sharp bands, the samples of the CP patients (irrespective of the volume of sample applied) appeared as cloudy bands, and such bands many times indicate a higher degree of glycosylation. MMP-9 is a heavily glycosylated MMP (Rudd et al. 1999), and its characteristic cloudy appearance in zymograms is due to this post-transcriptional modification. This distinction indicates that the source of MMP-9 may be different in controls and in patients, because different cells glycosylate proteins differently.

This observation is in line with the evidence from our correlation data. In controls, a stronger correlation ($r = 0.77$, $p < 0.0001$) was found between total MMP-9 and MPO [which is an enzyme found in PMNs (Borregaard & Cowland 1997)] compared with the correlation between these two markers in the CP patients ($r_s = 0.47$, $p = 0.0006$). This suggests that most of the MMP-9 found in the GCF of controls stems from PMNs, while part of the MMP-9 found in the CP patients comes from another source. Other authors have already described that MMP-9 is expressed by tissue macrophages (Mainardi et al. 1984) and fibroblasts (Salo et al. 1985, 1991). The correlation between total MMP-9 and the 130 kDa MMP-9 [conjugated to lipocaline, a typical form of MMP-9 found in PMNs (Hibbs et al. 1985, Betsuyaku et al. 1999)], further supports this statement, because this correlation is stronger in the controls ($r = 0.67$, $p < 0.0001$) than in the CP patients ($r_s = 0.52$, $p < 0.0001$).

Correlation data from controls also show that PD is correlated with total MMP-9 ($r = 0.67$, $p = 0.0001$) and MPO ($r = 0.59$, $p = 0.001$), revealing that increased MMP-9 and MPO are found in patients with gingivitis (which are those with increased PD). PMNs are known to secrete different pro-forms (zymogens) of lytic enzymes stored in granules including MMPs (Borregaard

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& Cowland 1997). This fact is demonstrated in our study by the correlation data from CP patients between 130 kDa MMP-9 and MPO ($r = 0.42$, $p = 0.002$). The lack of correlation between MPO and MMP-8 in the controls is probably due to the low levels of MMP-8 and MPO found in these patients (baseline and 3 months). In these control subjects, only MMP-9 varied between baseline and follow-up, and this variation range is important to find correlations. Increased MMP-8 and MPO levels were found in the CP patients, and even so no correlation of MMP-8 with MPO was found ($r = 0.11$, $p = 0.44$). These data suggest that most MMP-8 comes from other cells, not PMNs.

Azurophilic granules of PMNs contain the enzyme MPO, which is an important component of GCF (Aras et al. 2007). This enzyme is suggested to be involved in the pathogenesis of periodontal diseases (Kowolik & Grant 1983, Smith et al. 1986, Cao & Smith 1989). Increased activity of the MPO in periodontal disease sites and decreased activity after treatment is suggested to support the role for MPO in destructive periodontal diseases (Yamalik et al. 2000, Wei et al. 2004).

Kinane et al. (2003) showed that MMP-8 is increased in periodontal diseased pockets and decreases after periodontal therapy. These and other authors have also suggested that MMP-8 is a good marker for detection of periodontal sites with active periodontal disease (Romanelli et al. 1999, Kiili et al. 2002, Kinane et al. 2003). Our correlation data from the CP patients also show that PD is moderately correlated with the 87 kDa MMP-9, total MMP-9, and MMP-8, while BOP was moderately correlated with MMP-8 and TIMP-2. Therefore, MMP-8 indeed appears to be moderately correlated with the clinical disease parameters, although a larger number of subjects would be needed for definitive data to be obtained.

Interestingly, a recent study has shown that MMP-8 levels in the GCF of shallow crevices are associated with the extent of periodontal disease, and the authors suggested that MMP-8 might be a valid prognostic marker for periodontal disease (Passoja et al. 2008).

We think that the moderate correlations found ($r < 0.5$) between MMP-8 in the GCF and MPO, MMP-9, TIMP-2, and even disease parameters such as PD and BOP may indicate that the MMP-8 levels depend on the local environment.

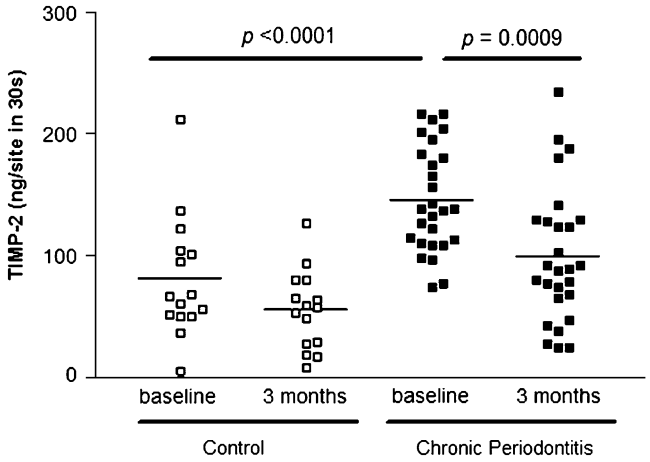

Fig. 7. Gingival crevicular fluid tissue inhibitor of matrix metalloproteinase 2 (TIMP-2) levels determined by enzyme-linked immunoabsorbent assay of control ($n = 15$) and chronic periodontitis ($n = 27$) subjects.

Table 3. Correlations between different markers found in GCF of control subjects

	MMP-9			MMP-8	MPO	TIMP-1	TIMP-2	PD	BOP	Volume (μl/30 s)
	130 kDa	87 kDa	total							
180 kDa MMP-9	**$r = 0.67$**	$r_s = 0.003$	$r = 0.35$	$r = 0.24$	$r = 0.28$	$r = 0.09$	$r = 0.27$	$r = 0.09$	$r_s = 0.09$	$r = 0.39$
(arbitrary units)	**$p < 0.0001$**	$p = 0.98$	$p = 0.07$	$p = 0.19$	$p = 0.14$	$p = 0.62$	$p = 0.18$	$p = 0.61$	$p = 0.61$	$p = 0.03$
130 kDa MMP-9	–	$r_s = -0.03$	**$r = 0.67$**	$r = 0.15$	$r = 0.34$	$r = 0.18$	$r = 0.27$	$r = 0.26$	$r_s = 0.05$	$r = 0.42$
(arbitrary units)	–	$p = 0.84$	**$p < 0.0001$**	$p = 0.41$	$p = 0.07$	$p = 0.36$	$p = 0.17$	$p = 0.16$	$p = 0.81$	$p = 0.01$
87 kDa MMP-9	–	–	$r_s = -0.09$	$r_s = 0.01$	$r_s = -0.19$	$r_s = 0.19$	$r_s = -0.07$	$r_s = -0.26$	$r_s = -0.08$	$r_s = -0.13$
(arbitrary units)	–	–	$p = 0.64$	$p = 0.95$	$p = 0.32$	$p = 0.32$	$p = 0.72$	$p = 0.16$	$p = 0.66$	$p = 0.50$
Total MMP-9	–	–	–	$r = -0.06$	**$r = 0.77$**	$r = 0.25$	$r = -0.008$	**$r = 0.67$**	$r_s = 0.17$	$r = 0.29$
(ng/sample)	–	–	–	$p = 0.76$	**$p < 0.0001$**	$p = 0.21$	$p = 0.97$	**$p = 0.0001$**	$p = 0.38$	$p = 0.13$
MMP-8	–	–	–	–	$r = -0.09$	$r = -0.33$	$r = 0.17$	$r = -0.09$	$r_s = 0.013$	$r = -0.015$
(ng/sample)	–	–	–	–	$p = 0.64$	$p = 0.08$	$p = 0.39$	$p = 0.62$	$p = 0.95$	$p = 0.93$
MPO	–	–	–	–	–	$r = 0.41$	$r = 0.16$	**$r = 0.59$**	$r_s = 0.14$	$r = 0.35$
(PMN/μl)	–	–	–	–	–	$p = 0.03$	$p = 0.43$	**$p = 0.001$**	$p = 0.49$	$p = 0.07$
TIMP-1	–	–	–	–	–	–	$r = 0.166$	$r = 0.24$	$r_s = -0.09$	$r = 0.36$
(ng/sample)	–	–	–	–	–	–	$p = 0.42$	$p = 0.22$	$p = 0.65$	$p = 0.06$
TIMP-2	–	–	–	–	–	–	–	$r = 0.16$	$r_s = 0.10$	$r = 0.10$
(ng/sample)	–	–	–	–	–	–	–	$p = 0.41$	$p = 0.32$	$p = 0.61$
PD (mm)	–	–	–	–	–	–	–	–	$r_s = 0.47$	$r = 0.28$
	–	–	–	–	–	–	–	–	$p = 0.007$	$p = 0.13$
BOP (%)	–	–	–	–	–	–	–	–	–	$r = 0.16$
	–	–	–	–	–	–	–	–	–	$p = 0.38$

Statistically significant correlations are marked in bold. CGF, crevicular gingival fluid; MMP, matrix metalloproteinase; MPO, myeloperoxidase; TIMP, tissue inhibitor of matrix metalloproteinase; PD, pocket depth; BOP, bleeding on probing; Correlation coefficients: r, Pearson's r and r_s, Spearman's r.

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Table 4. Correlations between different marke r_s found in GCF of chronic periodontitis patients

	MMP-9			MMP-8	MPO	TIMP-1	TIMP-2	PD	BOP	Volume (μl/30 s)
	130 kDa	87 kDa	total							
180 kDa MMP-9 (arbitrary units)	$r_s = \mathbf{0.65}$ $\mathbf{p < 0.0001}$	$r_s = \mathbf{0.64}$ $\mathbf{p < 0.0001}$	$r_s = \mathbf{0.68}$ $\mathbf{p < 0.0001}$	$r_s = 0.20$ $p = 0.16$	$r_s = \mathbf{0.60}$ $\mathbf{p < 0.0001}$	$r_s = 0.09$ $p = 0.51$	$r_s = \mathbf{0.56}$ $\mathbf{p < 0.0001}$	$r_s = 0.27$ $p = 0.05$	$r_s = 0.20$ $p = 0.15$	$r_s = 0.21$ $p = 0.13$
130 kDa MMP-9 (arbitrary units)	– –	$r_s = \mathbf{0.41}$ $\mathbf{p = 0.002}$	$r_s = \mathbf{0.52}$ $\mathbf{p < 0.0001}$	$r = 0.20$ $p = 0.15$	$\mathbf{r = 0.42}$ $\mathbf{p = 0.002}$	$r = 0.21$ $p = 0.13$	$r = 0.36$ $p = 0.01$	$r = 0.11$ $p = 0.45$	$r_s = -0.001$ $p = 0.99$	$r = 0.03$ $p = 0.82$
87 kDa MMP-9 (arbitrary units)	– – –	– –	$r_s = \mathbf{0.42}$ $\mathbf{p = 0.002}$	$r_s = 0.11$ $p = 0.43$	$r_s = \mathbf{0.49}$ $\mathbf{0.0002}$	$r_s = 0.30$ $p = 0.03$	$r_s = \mathbf{0.44}$ $\mathbf{p = 0.001}$	$r_s = \mathbf{0.39}$ $\mathbf{p = 0.004}$	$r_s = 0.22$ $p = 0.11$	$r_s = \mathbf{0.39}$ $\mathbf{p = 0.003}$
Total MMP-9 (ng/sample)	– – –	– –	– –	$r_s = 0.19$ $p = 0.18$	$r_s = \mathbf{0.47}$ $\mathbf{p = 0.0006}$	$r_s = 0.03$ $p = 0.83$	$r_s = \mathbf{0.60}$ $\mathbf{p < 0.0001}$	$r_s = \mathbf{0.41}$ $\mathbf{p = 0.002}$	$r_s = 0.26$ $p = 0.06$	$r_s = 0.07$ $p = 0.60$
MMP-8 (ng/sample)	– –	– –	– –	– –	$r = 0.11$ $p = 0.44$	$r = 0.13$ $p = 0.35$	$\mathbf{r = 0.54}$ $\mathbf{p < 0.0001}$	$\mathbf{r = 0.39}$ $\mathbf{p = 0.004}$	$r_s = \mathbf{0.39}$ $\mathbf{p = 0.004}$	$r = 0.34$ $p = 0.01$
MPO (PMN/μl)	– –	– –	– –	– –	– –	$r = 0.30$ $p = 0.03$	$\mathbf{r = 0.48}$ $\mathbf{p = 0.0006}$	$r = 0.08$ $p = 0.56$	$r_s = 0.29$ $p = 0.04$	$r = 0.28$ $p = 0.04$
TIMP-1 (ng/sample)	– –	– –	– –	– –	– –	– –	$r = 0.24$ $p = 0.09$	$r = 0.001$ $p = 0.99$	$r_s = -0.12$ $p = 0.38$	$\mathbf{r = 0.43}$ $\mathbf{p = 0.001}$
TIMP-2 (ng/sample)	– –	– –	– –	– –	– –	– –	– –	$r = 0.33$ $p = 0.02$	$r_s = \mathbf{0.45}$ $\mathbf{p = 0.001}$	$r = 0.34$ $p = 0.01$
PD (mm)	– –	– –	– –	– –	– –	– –	– –	– –	$r_s = \mathbf{0.48}$ $\mathbf{p = 0.0003}$	$r = 0.26$ $p = 0.05$
BOP (%)	– –	– –	– –	– –	– –	– –	– –	– –	– –	$r = 0.23$ $p = 0.09$

Statistically significant correlations are marked in bold. CGF, crevicular gingival fluid; MMP, matrix metalloproteinase; MPO, myeloperoxidase; TIMP, tissue inhibitor of matrix metalloproteinase; PD, pocket depth; BOP, bleeding on probing; Correlation coefficients: r, Pearson's r and r_s, Spearman's r.

We found that MMP-8 and MMP-9 are increased in the plasma of the CP patients at baseline (but not in the controls at baseline), and these two MMPs decrease 3 months after non-surgical periodontal therapy (Marcaccini et al. 2009b). These results are in agreement with many studies showing that periodontal disease causes increases in systemic inflammatory markers such as C-Reactive Protein (Paraskevas et al. 2008) and IL-6 (D'Aiuto et al. 2005, Marcaccini et al. 2009a), which decrease after effective periodontal therapy.

The use of a single GCF sample/person is one limitation of the study that makes comparisons with earlier studies difficult (many studies used the mean of three or more GCF samples/person). In apparent contrast with the current literature (Kinane et al. 2003, Gurkan et al. 2005, Correa et al. 2008), no statistically significant difference was found when the GCF volume was compared in CP sites before and after periodontal therapy. This may be due to the difference in periodontal disease, because the mean PD in the diseased sites collected in this study at baseline was 4.5 mm (± 1.8), while in other studies the mean (SD) was 6.1 mm (± 1.4) (Kinane et al. 2003) or 7.3 mm (± 0.2) (Gurkan et al. 2005).

Measurement of GCF enzymes might be valuable tools to aid in the diagnosis and as additional information for clinical follow-up.

In conclusion, in this study, we have shown higher levels of MMP-8, TIMP-2, MPO, and the active 87 kDa-MMP-9 form in the GCF of CP patients compared with controls, and these markers decreased 3 months after periodontal therapy.

Acknowledgements

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

This study was financially supported by The State of Sao Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de Sao Paulo, FAPESP, Sao Paulo, SP; grants 05/60527-6 and 05/60526-0), and by the (Brazilian) National Council of Technological and Scientific Development (Conselho National de Ciência e Tecnologia, CNPq, Brasilia, DF).

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Clinical Relevance

Scientific rationale for the study: GCF levels of MMPs, TIMPs, and their ratios have been proposed as a promising way of diagnosing active periodontal disease and monitoring the periodontal treatment site specifically. However, such a test is still not in use.

Principal findings: Higher levels of MMP-8, active MMP-9, MPO, and TIMP-2 were found in the GCF of CP patients compared with controls, and these markers decreased in the CP patients 3 months after therapy, suggesting that these molecules can be valuable for site monitoring.

Practical implications: Improvements in the biochemical assays of MMPs and TIMPs will aid the development of site-specific tests.

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