

ORIGINAL ARTICLE

Detection of gingival crevicular fluid MMP-8 levels with different laboratory and chair-side methods

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OBJECTIVE: The aim of the study was to compare four methods for gingival crevicular fluid (GCF) matrix metalloproteinase (MMP)-8 detection.

METHODS: Matrix metalloproteinase-8 levels from 20 GCF samples from two periodontally healthy subjects, 18 samples from two patients with gingivitis and 45 samples from six patients with moderate to severe periodontitis, altogether 83 samples, were analysed using (1) a time-resolved immunofluorometric assay (IFMA), (2) an MMP-8 specific chair-side dip-stick test, (3) a dentoAnalyzer device and (4) the Amersham ELISA kit. Western immunoblot using same monoclonal anti-MMP-8 as in IFMA and dentoAnalyzer was used to identify molecular forms of MMP-8 in GCFs.

RESULTS: Correlation between IFMA and dentoAnalyzer results calculated with Spearman's correlation coefficient was 0.95 ($P = 0.01$). The chair-side dip-stick test results were well in line with these assays. Periodontitis sites with unstable characteristics were differentiated with these methods. The Amersham ELISA results were not in line with the findings by other methods.

CONCLUSIONS: Immunofluorometric assay and dentoAnalyzer can detect MMP-8 from GCF samples and these methods are comparable. Using Western immunoblot, it was confirmed that IFMA and dentoAnalyzer can detect activated 55 kDa MMP-8 species especially in periodontitis-affected GCF. dentoAnalyzer is among the first quantitative MMP-8 chair-side testing devices in periodontal and peri-implant diagnostics and research.

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Keywords: chair-side test; GCF MMP-8; periodontitis; biomarker

Introduction

Periodontal diagnosis and treatment decisions are mostly based on clinical and radiological findings combined with the background information about patient's general health and behaviour (Armitage, 2004). This same information is also the basis by which the intervals of the maintenance visits are decided. Objective means to guide our treatment decisions either at tooth site level, which is informative about the risk of the progression of periodontitis, or on patient level, which tells about the general risk of periodontitis, are not available. A feasible biochemical test for oral fluids in a chair-side setting for the specification of the level of the host response would help clinicians to direct the treatment measures at an optimal level. A biochemical test could also help identify those subjects who are at risk of periodontitis or of further progression of periodontitis; it would give information about the prognosis of the treatment and would also work as an indicator of successful periodontitis treatment (McCulloch, 1994; Christodoulides *et al*, 2007; Herr *et al*, 2007; Kinney *et al*, 2007; Rai *et al*, 2008).

Matrix metalloproteinase (MMP)-8 or collagenase-2 is one of the central biomarkers in the connective tissue breakdown of periodontitis (Lee *et al*, 1995; Mancini *et al*, 1999; Sorsa *et al*, 1988, 2004, 2006) and it has been found to be a potential candidate as a diagnostic aid (Mäntylä *et al*, 2003, 2006). Treatment of periodontitis with scaling and root planning (SRP) decreases the gingival crevicular fluid (GCF) MMP-8 levels, while periodontal pockets at risk of irreversible tissue destruction indicate repeatedly elevated MMP-8 levels (Mäntylä *et al*, 2006). Consequently, a chair side test for the analysis of MMP-8 level in GCF or oral rinse would be informative and give guidance in the patient care.

The aim of this study was to compare four different methods for GCF sample MMP-8 detection in site specific periodontal diagnostics: a time-resolved immunofluorometric assay (IFMA; Hanemaaijer *et al*, 1997), an MMP-8 specific chair-side dip-stick test previously introduced by Sorsa *et al* (1999, 2004, 2006)

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and Mäntylä *et al* (2003, 2006), a device for rapid MMP-8 detection applying a sandwich based immunoassay system using ABICAP filters (dentoAnalyzer by dentognostics GmbH, Jena, Germany; Munjal *et al*, 2007), and periodontitis patients' samples were evaluated also by a commercially available ELISA kit by Amersham. We further identified the molecular forms of MMP-8 in the studied GCF samples using Western immunoblotting.

Materials and methods

Twenty GCF samples from two periodontally healthy subjects, 18 samples from two patients with gingivitis and 45 samples from six patients with moderate to severe periodontitis (altogether 83 GCF samples) were analysed. All samples for this study were selected from samples which have been part of a larger scale study in which the patient inclusion criteria have been explained (Mäntylä *et al*, 2003, 2006). The study protocol was approved by the ethical committee of the Institute of Dentistry, University of Helsinki and Helsinki University Central Hospital, and the experiments were undertaken with the understanding and written consent of each subject and according to ethical principles, including the World Medical Association Declaration of Helsinki.

From six representative periodontitis patients included in this study, one site from each was selected. In each site, the collection of GCF and measurement of clinical parameters were performed (1) before any treatment measures, (2) 1 month after hygiene phase periodontal treatment (SRP and oral hygiene instructions) and (3) bimonthly during the 10 to 12-month maintenance phase. Thus the selected sites had each been sampled 7–8 times between baseline and the end of maintenance, making total number of 45 periodontitis GCF samples. Gingivitis patients' and periodontally healthy subjects' sites were sampled only once, and no treatment or oral hygiene instructions preceded the sampling. The procedure of the GCF sampling with paper strips and further processing of the samples have been described by Mäntylä *et al* (2003). Samples were stored in -60°C .

All samples were analysed with an IFMA (Hanemaaijer *et al*, 1997) in the research laboratory, and by two feasible methods for chair-side testing: the MMP-8 specific immunochromatographic chair-side dip-stick test (Mäntylä *et al*, 2003, 2006) where the result is provided in 5 min and graded by eye as positive or negative (the principle of the test is represented by Sorsa *et al*, 1999; and clinical testing protocol by Mäntylä *et al*, 2003) and the dentoAnalyzer device which applies a sandwich based immunoassay system and provides the quantitative numeric chair-side result in about 15 min (Munjal *et al*, 2007). Both chair-side testing methods and IFMA use same monoclonal antibodies (Hanemaaijer *et al*, 1997). Periodontitis samples were also analysed with the ELISA kit by Amersham.

The dentoAnalyzer is designed as a portable user-friendly bench-top instrument. It automatically con-

ducts the whole assay process, that is, steps like liquid handling as well as readout based on a software program and a robust algorithm. [For a more detailed description of the dentoAnalyzer, its key components and the art of quantification of MMP-8 (as well as other biomarkers) see Munjal *et al*, 2007]. The key component is a cartridge consisting (i) of a liquid-handling module containing all relevant reagents for immunological reactions like clinical sample, conjugate, wash buffers, and substrate and (ii) a reaction chamber containing six filters including positive and negative controls, where the immunological reactions take place (Munjal *et al*, 2007). Two antibodies directed against specific epitopes of the antigens are used in a sandwich based immunoassay technology known as antibody immuno column for analytical process (ABICAB) which is based on an immunoaffinity filter design using flow through solid phase filters with extremely high binding potential (Hartmann *et al*, 1993; Stove *et al*, 1995; Cavuslu *et al*, 2003; Meyer *et al*, 2007).

The IFMA method has been described by Hanemaaijer *et al* (1997) and the principle of the MMP-8 specific chair-side dip-stick test by Sorsa *et al* (1999, 2004). The analysis by Amersham ELISA kit was performed according to manufacturer's instructions. Western immunoblot was carried out as described previously using anti-MMP-8 antibody (Hanemaaijer *et al*, 1997). Recombinant human MMP-8 was purchased from Chemicon Inc., Temecula, CA, USA.

Moreover, the treatment response of the periodontitis patients' sites seen as a change of pocket probing depth (PPD) and attachment loss (AL) and indicated by the dip-stick test, IFMA, dentoAnalyzer and Amersham ELISA levels was analysed. The MMP-8 levels of the GCF samples in question have previously been found to correlate with the periodontal status of the tested sites in a larger scale study (Mäntylä *et al*, 2003, 2006).

For the statistical analyses non-parametric tests (for paired samples Wilcoxon signed ranks test and for multiple independent samples Kruskal Wallis test) and correlation coefficients (Spearman's) were used. The level of significance was set at 0.05.

Results

Periodontitis sites

Table 1 shows the baseline and after treatment (SRP) pocket probing depths (PPD), clinical attachment levels (CAL) and GCF MMP-8 levels of periodontitis sites analysed using IFMA, dentoAnalyzer and Amersham ELISA. Decrease of PPD from baseline values after treatment (SRP) was not statistically significant ($P = 0.14$) but CAL change reached significance ($P = 0.027$). The baseline MMP-8 IFMA level decreased significantly ($P = 0.028$), as well as the dentoAnalyzer result ($P = 0.043$) after SRP. Based on the data shown in Table 1, treatment by SRP reduced the mean MMP-8 IFMA value by 63.1%, while the dentoAnalyzer detected a reduction of mean MMP-8 value by 71.4%. The Amersham ELISA recorded only a 34% reduction of baseline mean MMP-8 level. Change

Table 1 Effect of treatment (SRP and oral hygiene instructions) registered in periodontitis patients' PPD, CAL, and GCF MMP-8 levels analysed by IFMA and dentoAnalyzer level one month after the treatment was ready. Number of patients (6), one site per each patient was sampled; mean \pm s.d. (median; range)

	Baseline	After treatment	P-value*
PPD (mm)	6.75 \pm 0.9 (7; 5.5–8)	5.1 \pm 2.1 (4; 3.5–9)	ns
CAL (mm)	5.4 \pm 2.5 (4.5; 3–9)	3.8 \pm 2.1 (3; 2–7)	0.027
IFMA (ng ml ⁻¹)	5 899 \pm 4 823 (3 615; 1 687–13 421)	2 176 \pm 2 286 (1 134; 457–6 423)	0.028
dentoAnalyzer (ng ml ⁻¹)	213 \pm 119 (256; 17–311)	61 \pm 123 (11; 5–311)	0.043
Amersham ELISA (ng ml ⁻¹)	15.9 \pm 13.4 (1.8–36.6)	10.5 \pm 4.6 (10.2–17.6)	ns

SRP, scaling and root planning; PPD, pocket probing depth; CAL, clinical attachment level; GCF, gingival crevicular fluid; MMP, matrix metalloproteinase; IFMA, immunofluorometric assay; ns, non-significant.

*Wilcoxon signed ranks test, level of significance 0.05.

of GCF MMP-8 levels after treatment analysed by Amersham ELISA were not statistically significant.

When each of the six sites from six representative periodontitis patients were examined separately, three of them could be interpreted as stable: the treatment response after SRP detected in CAL and PPD values as well as in the decrease of MMP-8 level remained to the end of the maintenance phase (Figure 1a, b). The only exception was the site of one of these patients in which 8 month CAL was exceptionally high. This was obviously an error in measurement with a manual probe, which is reflected in the curve representing mean CAL values shown in Figure 1a. The sites of three other patients could be interpreted as unstable: the change of PPD and CAL to positive direction after treatment (SRP) was lost in the beginning of the maintenance (Figure 1c). In the latter cases also MMP-8 levels had a trend to increase (Figure 1d). In unstable sites the chair-

side dip-stick test result was positive in 59% of samples when all time points of sampling were taken into consideration from baseline to the end of the maintenance, while in stable sites the corresponding percentage was 35.

To compare IFMA and dentoAnalyzer results more conveniently, IFMA results need to be divided by factor 70 (indicated by IFMA/70). The cut off level for positive chair-side dip-stick test result is set at IFMA level of 1 000 ng ml⁻¹, thus IFMA/70 level 14 ng ml⁻¹ represents this level. The results obtained from 45 periodontitis samples using IFMA, dentoAnalyzer and the dip-stick test were compared with each other to find out the comparability of studied methods. For this purpose, the samples were grouped according to MMP-8 levels detected using IFMA: <1 000 ng ml⁻¹ (*n* = 17), 1 000–4 000 ng ml⁻¹ (*n* = 16), and >4 000 ng ml⁻¹ (*n* = 12). The dentoAnalyzer quantifies samples with

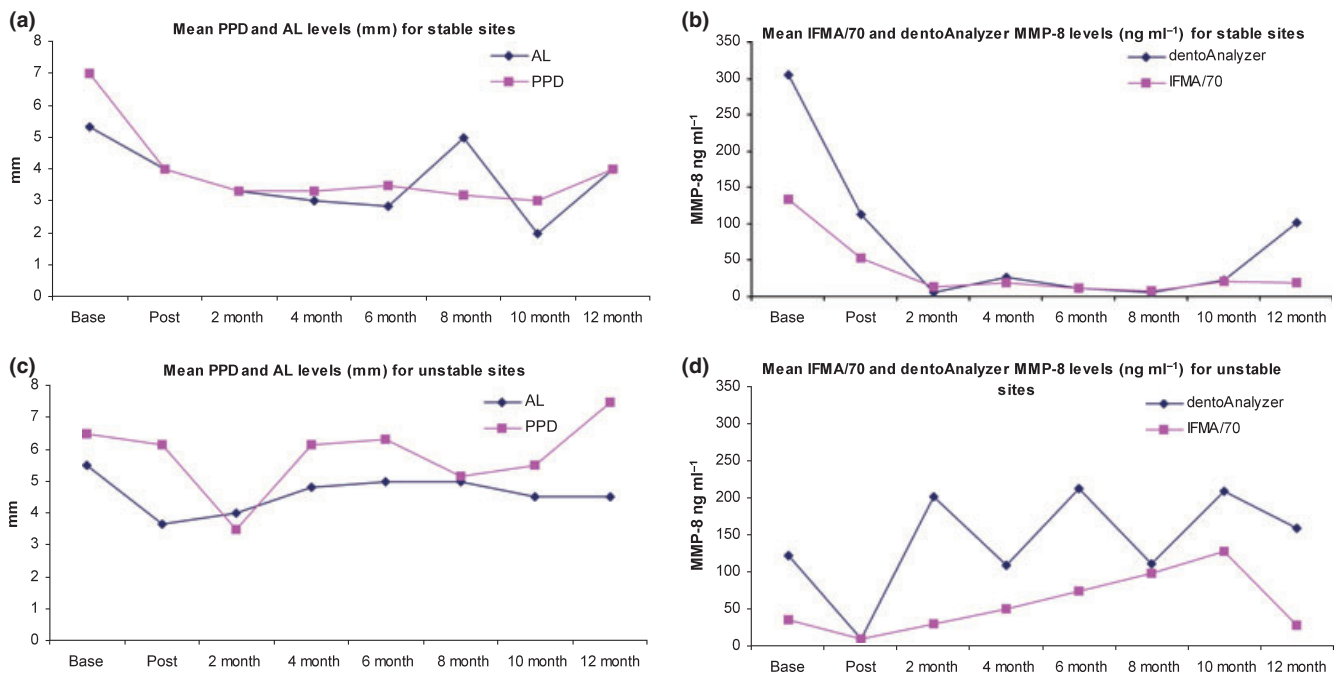


Figure 1 Mean values of (a) PPD and attachment loss and (b) IFMA/70 and dentoAnalyzer gingival crevicular fluid matrix metalloproteinase-8 levels for three periodontitis patients with stable sites, and (c, d) respectively for three periodontitis patients with unstable sites

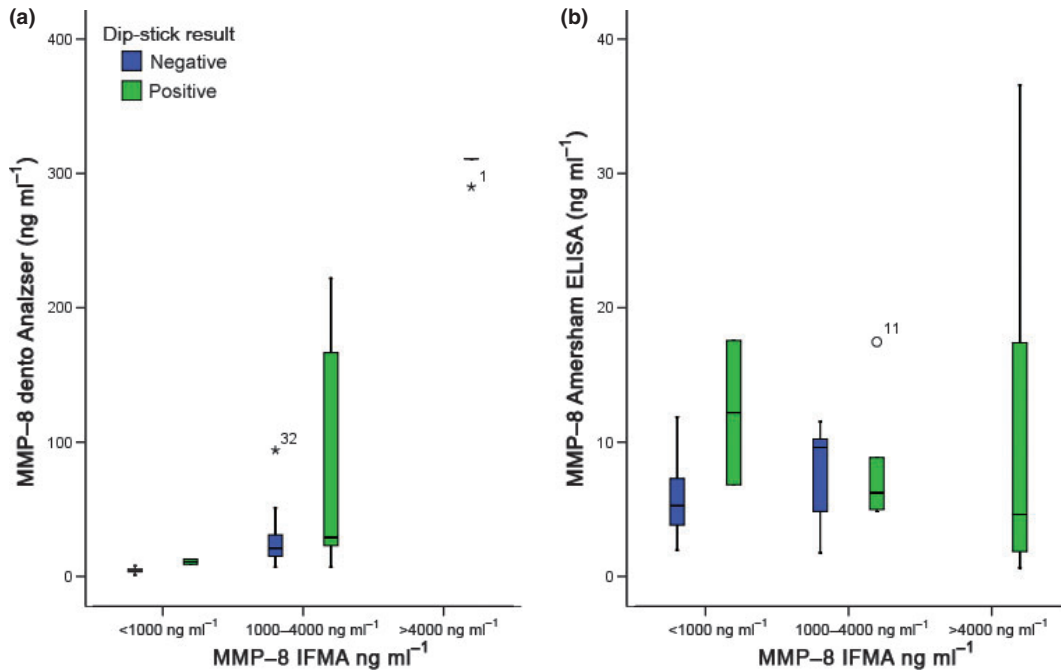


Figure 2 Boxplots (medians and interquartile percentiles) showing the distributions of dentoAnalyzer (a) and Amersham ELISA (b) matrix metalloproteinase (MMP)-8 levels in periodontitis gingival crevicular fluid samples in three categories of MMP-8 immunofluorometric assay levels labelled with the chair-side dip-stick results

IFMA level exceeding about 4 000 ng ml⁻¹ with a standard result of 311 ng ml⁻¹ (Figure 2). Therefore this concentration was chosen as a second cut off for the comparisons. The calibration of the dentoAnalyzer, which is meant to serve as a chair-side tool, is optimized to mirror the clinical relevant interval of MMP-8 concentrations from healthy and gingivitis sites up to periodontitis affected sites. However, the biochemical set up as well as the corresponding software were not developed to discriminate extremely high from very high MMP-8 values. In Figure 2, the distributions of the dentoAnalyzer and Amersham ELISA levels of samples in all IFMA level groups are shown in boxplots which also are labelled with the chair-side dip-stick results (negative vs positive). The Amersham ELISA results' correlation with other studied methods was weak, and the response to treatment (SRP) could not be seen as a significant decrease in the concentra-

tion measured by this method while IFMA and dentoAnalyzer results were in line with each other (Table 1). Table 2 shows the results of periodontitis samples obtained with all tested methods and the *P*-values for the differences between the three IFMA level groups of samples.

In Figure 3 dip-stick test results of 45 periodontitis GCF samples are correlated with the IFMA levels. Correlation between IFMA and dentoAnalyzer results of periodontitis GCF showed a Spearman's correlation coefficient 0.95 (significant at the <0.01 level). Correlation between IFMA and dentoAnalyzer results is shown in Figure 4. Western immunoblot analysis revealed 55 kDa MMP-8 immunoreactive species in periodontitis GCF; 65 kDa immunoreactivities were detected in gingivitis GCF but hardly any in healthy control GCF (Figure 5). Only the periodontitis GCF exhibited activated forms of MMP-8 while high molecular

Table 2 Levels of MMP-8 in GCF from 45 periodontitis samples analysed by IFMA (and converted to IFMA/70), dentoAnalyzer and Amersham ELISA [mean ± s.d. (median; range)], and percentages of positive chair-side dip-stick test results

	< 1 000 ng ml ⁻¹ n = 17	1 000–4 000 ng ml ⁻¹ n = 16	> 4 000 ng ml ⁻¹ n = 12	<i>P</i> -value*
IFMA (ng ml ⁻¹)	487 ± 243 (457; 35–877)	2 076 ± 672 (1 973; 1 197–3 612)	9 245 ± 4 563 (8 258; 4 262–18 127)	< 0.001
IFMA/70 (ng ml ⁻¹)	7 ± 3.5 (6.5; 0.5–12.5)	29.7 ± 9.6 (28.2; 17.1–51.6)	132 ± 65 (118; 61–260)	< 0.001
dentoAnalyzer (ng ml ⁻¹)	5.1 ± 3 (5; 1–13)	57.3 ± 69 (28; 7–222)	309 ± 6 (311; 290–311)	< 0.001
Amersham ELISA (ng ml ⁻¹)	6.7 ± 4.4 (6; 2–17.6)	8 ± 4.1 7.6 (7.6; 1.8–17.5)	9.7 ± 11.4 (4.6; 0.6–36.6)	ns
% of positive dip-stick test results	11.8	43.8	100	< 0.001

GCF, gingival crevicular fluid; MMP, matrix metalloproteinase; IFMA, immunofluorometric assay; ns, non-significant.
*Kruskal Wallis Test, level of significance 0.05.

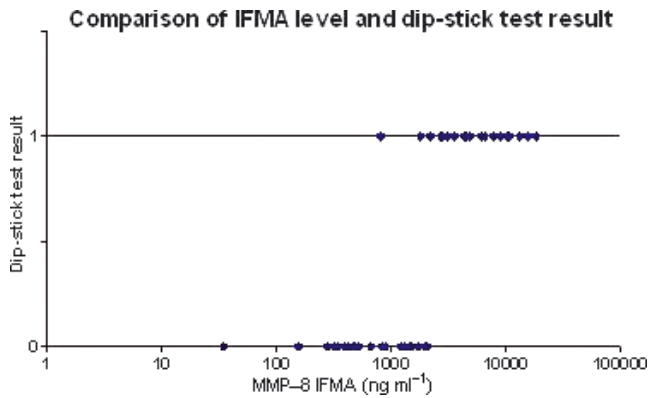


Figure 3 Correlation of matrix metalloproteinase (MMP)-8 chair-side dip-stick test results with the MMP-8 immunofluorometric assay levels (ng ml^{-1}) in 45 periodontitis gingival crevicular fluid samples. Grading for the dip-stick results: 0 = -, 1 = +

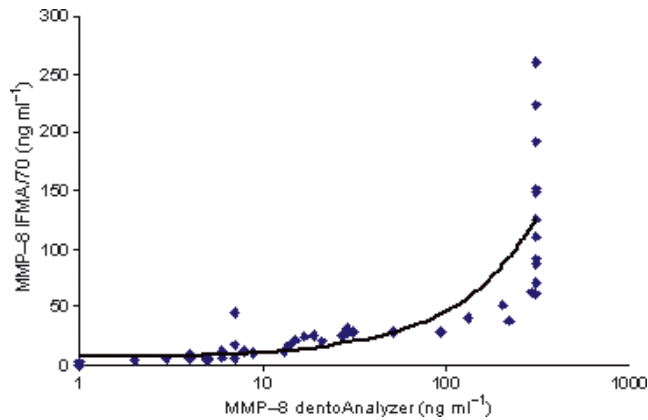


Figure 4 Correlation between matrix metalloproteinase (MMP)-8 IFMA/70 and dentoAnalyzer (ng ml^{-1}) results of 45 periodontitis samples. dentoAnalyzer gives a standard result of 311 ng ml^{-1} for samples with IFMA/70 level exceeding about 57 ng ml^{-1}

size ($> 100 \text{ kDa}$) complex and fragmented (30–40 kDa) MMP-8 species could be observed also in periodontitis GCF (Figure 5).

Periodontally healthy and gingivitis sites

Matrix metalloproteinase-8 concentration measured using dentoAnalyzer in GCF samples ($n = 20$) from periodontally healthy subject sites were $< 1 \text{ ng ml}^{-1}$ in 70% ($n = 14$) of sites and in other six sites $1.2\text{--}3.9 \text{ ng ml}^{-1}$ (for all periodontally healthy sites mean \pm s.d. $1.6 \pm 1 \text{ ng ml}^{-1}$). In gingivitis sites ($n = 18$) the MMP-8 concentration was $< 1 \text{ ng ml}^{-1}$ in 72% ($n = 13$) of sites and in other five sites $1.2\text{--}9.5 \text{ ng ml}^{-1}$ (for all gingivitis sites mean \pm s.d. $2 \pm 2.45 \text{ ng ml}^{-1}$).

Discussion

In this study, we investigated levels of GCF MMP-8 with two different chair-side and two laboratory methods. IFMA, dentoAnalyzer and MMP-8 specific chair-side dip-stick test results were well in line.

The samples which were used in the analyses were a representative subset of samples previously analysed in a longitudinal study (Mäntylä *et al*, 2006). In that particular study 16 periodontitis patients were maintained for 12 months after hygiene phase treatment. During the maintenance, bimonthly collected GCF samples were analysed for MMP-8 levels using IFMA and tested using an MMP-8 specific chair-side dip-stick test. We found that MMP-8 concentrations in sites with unstable character and progression of periodontitis were repeatedly elevated. This with the earlier finding that MMP-8 levels in untreated periodontitis patients' GCFs is high and decreases significantly after hygiene phase treatment have been an incentive to develop a chair-side diagnostic test to analyse MMP-8 levels. A recent study by Golub *et al* (2008) indicated that a collagenase inhibitor subantimicrobial-dose doxycycline (SDD) reduced collagenase activity in GCF representing mainly MMP-8, periodontal collagen breakdown and alveolar bone resorption relative to placebo in postmenopausal women during a 2-year follow up. Moreover, a type-1 collagen breakdown product/bone resorption marker (a carboxyterminal telopeptide cross-link fragment of type I collagen, ICTP) showed a similar pattern of change during SDD treatment (Golub *et al*, 2008). These findings further emphasize the central role of MMP-8

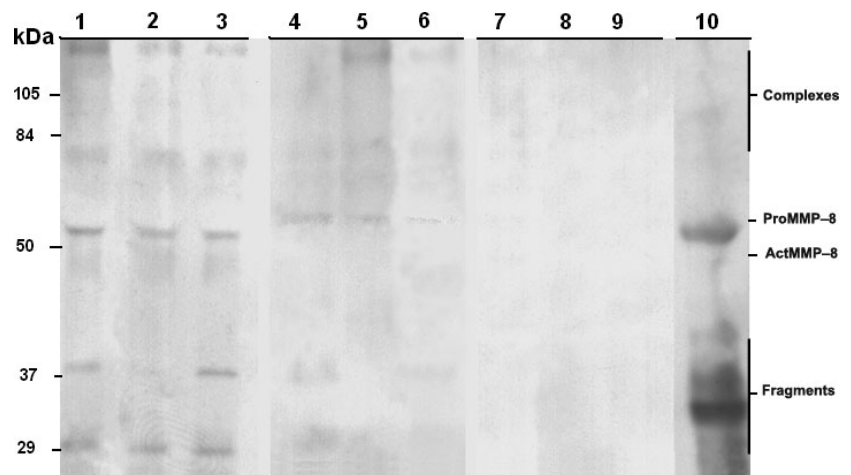


Figure 5 Western immunoblot analysis of matrix metalloproteinase (MMP)-8 immunoreactivities in gingival crevicular fluid (GCF) samples: lanes 1–3, periodontitis GCF; lanes 4–6, gingivitis GCF; lanes 7–9, healthy control GCF; lane 10, recombinant human MMP-8. Mobilities of the molecular weight markers are indicated on the left

in periodontitis' pathogenesis and progression and possible value in diagnostics.

Though several studies have shown the central role of MMP-8 in periodontitis, it has not been possible to show that it is predictive of disease progression, i.e. that the increased MMP-8 concentration in GCF would precede the occurrence of AL. This is a problem which arises from the nature of periodontitis as well as from the accuracy of diagnosing a site as progressing. Disease progression is regarded to be mostly episodic, occurs only infrequently and is slow in most chronic periodontitis patients. During a study period, it is likely that only a small number of sites with AL can be confirmed (Chambers *et al*, 1991; Payne *et al*, 2007; Mäntylä *et al*, 2006). In addition, only a small group of periodontitis patients manifest multiple progressing sites (Persson *et al*, 1995). In our previous study, we could not make a conclusion about the predictive value of MMP-8 testing (Mäntylä *et al*, 2006). However, we concluded that repeatedly elevated GCF MMP-8 levels indicate the sites at risk of periodontal AL and that testing of MMP-8 site specifically from GCF is a valuable diagnostic aid which supplements the traditional methods.

In this study, both IFMA and dentoAnalyzer device detected the GCF samples' MMP-8 levels with equal reliability. The chair-side dip stick test results were in line with results with these two other methods but the capability of the dip-stick test to differentiate the sample levels were rougher. The dip-stick test detected especially the sites with high MMP-8 levels. The cut-off level for the positive chair-side dip-stick test result has been set at 1 000 ng ml⁻¹. This level can differentiate periodontally healthy and gingivitis sites from periodontitis affected sites (Mäntylä *et al*, 2003), but it is obviously too low to indicate the sites at risk of progression of periodontitis (Mäntylä *et al*, 2006). The majority of false positive and false negative test results are obtained from samples with the MMP-8 level near the cut-off level 1 000 ng ml⁻¹. Main reason for this is that the test result is graded by eye, which means that environmental conditions, such as light, have an effect on the reading of the result. To read the dip-stick test result is rough and not very suitable for sensitive small volume samples as GCF collected by paper strips. Though the sample volume is minimal, the concentration of the tested biomarker can differ enormously, and this variation indicated by a line in the test stick is difficult to be analysed by eye. For this reason a chair-side test giving quantitative numeric information is more reliable and can give more precise information.

Moreover, periodontally healthy patients' and gingivitis patients' sites were previously tested with MMP-8 chair-side dip-stick test and the GCF MMP-8 levels of the samples were analysed using IFMA by Mäntylä *et al* (2003). In this study by Mäntylä *et al* (2003), the median concentration and interquartile percentile in GCF from periodontally healthy sites ($n = 59$) measured using IFMA was 100 $\mu\text{g l}^{-1}$ (40, 170 $\mu\text{g l}^{-1}$) and from gingivitis sites ($n = 58$) 470 $\mu\text{g l}^{-1}$ (130, 920 $\mu\text{g l}^{-1}$). Out of these samples, only one site gave positive chair-side dip-stick result. Thus the dentoAnalyzer results of this study

are congruent with the IFMA findings in the earlier study and also with the IFMA findings in the smaller group of periodontally healthy and gingivitis sites' samples analysed in this study. The GCF levels of periodontally healthy sites and gingivitis sites remained in both studies under the cut-off level of the positive dip-stick test result (1 000 ng ml⁻¹; by IFMA/70 14 ng ml⁻¹).

The differences between dentoAnalyzer, IFMA, dip-stick and Amersham ELISA MMP-8 analysis of GCF levels may be, at least in part, explained by the obviously different specificities and sensitivities between antibodies used in these assays. dentoAnalyzer, IFMA and dip-stick assays use same antibody (Hanemaaijer *et al*, 1997). Regarding serum and plasma MMP-8 determinations using both IFMA and Amersham ELISA, significantly higher serum MMP-8 values were recorded in relative to plasma, and the differences were most notable with high serum MMP-8 concentrations as measured using IFMA (Emingil *et al*, 2008; Tuomainen *et al*, 2008). The antibody used in dentoAnalyzer, IFMA and dip-stick exert high sensitivity to target both PMN- and fibroblast-type MMP-8 isotypes (Hanemaaijer *et al*, 1997) and especially their active forms as demonstrated in this study using Western immunoblotting. Active forms of MMP-8 in GCF are associated with progressive periodontitis, whereas latent enzyme is predominant in gingivitis GCF (Lee *et al*, 1995; Romanelli *et al*, 1999; Kiili *et al*, 2002). Pathologically elevated levels of active forms of MMP-8 are characteristic of active periodontitis lesions/pockets (Lee *et al*, 1995; Romanelli *et al*, 1999; Kiili *et al*, 2002).

Regarding chair-side MMP-8 point of care assays, dentoAnalyzer and also the dip-stick, the cut-off level for a periodontitis site at risk of disease progression should be studied in a larger set of samples according to Hernandez *et al*, [2006, 2007; such studies are now in progress and will be reported separately (Hernandez, Sorsa, Gamonal in preparation)]. However, in this study, the range of the MMP-8 concentration during the maintenance phase analysed using dentoAnalyzer was in unstable sites 110–212 ng ml⁻¹, mean level 167 ng ml⁻¹, and in stable sites 5–102 ng ml⁻¹, mean level 28 ng ml⁻¹. This gives some guidance for the interpretation of the results in clinic.

dentoAnalyzer is among the first quantitative MMP-8 chair-side testing devices in periodontal and peri-implant diagnostics and research and applicable in a chair-side use. The device can also be used to analyse biomarkers from other oral samples, like saliva and oral rinse (Sorsa *et al*, 2004; Munjal *et al*, 2007) as well as to diagnose other biomarkers provided the monoclonal antibody is changed accordingly.

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GmbH has been the scientific developer of the dentoAnalyzer. However, dentognostics GmbH does not manufacture, sell or distribute this device.

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