

ORIGINAL ARTICLE

Oral rinse MMP-8 point-of-care immuno test identifies patients with strong periodontal inflammatory burden

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OBJECTIVE: To determine whether oral rinse matrix metalloproteinase (MMP)-8 levels, measured by three different methods, tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) levels and elastase activity differentiate subjects with different periodontal condition; and second, to find out if MMP-8 levels were comparable among the methods used.

METHODS: MMP-8 levels were analysed with an immunofluorometric method (IFMA), dentoELISA and commercial ELISA. Also TIMP-1 levels and elastase activity were measured. For statistical analysis 214 study subjects were categorized into four groups, specified by the presence and number of moderate (4–5 mm) and deep (≥ 6 mm) periodontal pockets, and bleeding on probing percentage.

RESULTS: MMP-8 levels especially measured by dentoELISA and adjusted to the number of teeth per subject differentiated the study group with strong periodontal inflammatory burden from groups with lower levels. This was also verified with receiver operating characteristic (ROC) analysis. Elastase activity associated with higher IFMA and dentoELISA MMP-8 levels. IFMA MMP-8/TIMP and dentoELISA MMP-8/TIMP-1 tended to be higher with the increasing level of periodontal inflammatory burden. TIMP-1 levels decreased with increasing age.

CONCLUSIONS: Oral rinse MMP-8 together with TIMP-1 analysis may have potential in complementary periodontal diagnostics. dentoELISA can be applied in quantitative oral rinse chair side biomarker diagnostics.

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Keywords: oral rinse; biomarker; chair-side test; periodontal diagnostics; MMP-8

Introduction

Periodontitis is a very common inflammatory disease in humans. Though bacteria are the initiating factor, periodontitis is not a classical infectious disease. Majority of the causative organisms are also present in the healthy mouth (Socransky and Haffajee, 2005), and the host response modified by environmental and behavioural factors plays a key role in disease development, progression and in the maintenance of the treatment result. Despite the etiology or type of periodontitis, the products of host response and tissue destruction are similar. Markers of inflammatory process from periodontal tissues migrate into the periodontal pockets within a serum originating fluid, gingival crevicular fluid (GCF) (Uitto *et al.*, 2003) and further from gingival crevices/pockets into oral cavity where GCF associates with saliva. The more inflamed the periodontal tissues and the deeper the periodontal pockets are, the more GCF is excreted and the more it contains inflammatory markers (Uitto *et al.*, 2003). This also means that the inflammatory burden of periodontium may be reflected in saliva (Sorsa *et al.*, 1990; Uitto *et al.*, 1990).

Unlike in medicine, biochemical testing in dentistry has not yet been taken into everyday clinical practice despite the advantages in supplementation of traditional diagnostics. GCF has been used for site-specific diagnostic studies of periodontal disease status, and diagnostic tests for host-derived markers have been developed (Uitto *et al.*, 2003; Sorsa *et al.*, 2004). Inflammatory markers from GCF analysed individually or in combination may be valuable in the identification of individual sites or teeth at risk of periodontitis progression. Saliva can also be regarded as a diagnostic fluid but unlike GCF, it mirrors more generally the oral status and is more unspecific. Whole saliva contains sloughed epithelial cells from oral mucous membranes, nasopharyngeal discharge, food debris, bacteria and their products and, in dentate subjects, its content is also affected by GCF flow from gingival pockets. An oral rinse sample can be regarded as a fluid containing mainly the GCF

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from all periodontal pockets (Sorsa *et al*, 1990; Uitto *et al*, 1990, 2003). Thus, analysis of oral rinse may be useful in the diagnosis of general periodontal disease status, early identification of subjects at risk of periodontitis (Uitto *et al*, 2003), evaluation of periodontal treatment results or the need of hygiene visits during maintenance and in the follow up of the effect of modifying medication over host response (e.g. low-dose doxycycline, LDD) (Reinhardt *et al*, 2010).

Matrix metalloproteinases (MMPs) and their inhibitors and polymorphonuclear leukocyte (PMN) elastase could be candidates as diagnostic markers in oral rinse specimens (Uitto *et al*, 2003; Sorsa *et al*, 2004). Among MMPs, especially GCF MMP-8 levels have been found to be increased in periodontitis (Mäntylä *et al*, 2003, 2006). The deeper the periodontal pockets are, the higher are the levels of MMP-8 at individual sites, and the levels decrease by periodontal hygiene phase treatment. GCF MMP-8 levels remain low in treated and stable periodontitis while in subjects with tendency to progression of periodontitis or poor treatment response (refractory periodontitis) GCF indicates repeatedly high MMP-8 levels. Though smoking may decrease GCF MMP-8 levels, there may also be smoking subjects who repeatedly have especially high GCF MMP-8 levels and poor treatment response (Mäntylä *et al*, 2006).

Salivary MMP-8 levels have been suggested serving as a biomarker of periodontitis and applicable as a point-of-care test because MMP-8 levels in saliva have been found to correlate with periodontal disease severity (Miller *et al*, 2006; Ramseier *et al*, 2009). Herr *et al* (2007) have introduced a microfluidic assay for point-of-care rapid quantification of biomarkers from human saliva which in validation with MMP-8 identified the periodontitis subjects.

Also salivary elastase-like activity has been concluded to reflect the severity of periodontal destruction because elevated activity levels have been found in untreated periodontitis subjects' saliva, which decrease after periodontitis hygiene phase treatment (Nieminen *et al*, 1993). The results were similar in the oral water-rinse samples: a significant correlation was noticed between oral rinse elastase activity and the average of community index of periodontal treatment needs (CPITN) (Uitto *et al*, 1996).

In our previous study (Munjal *et al*, 2007; Sorsa *et al*, 2010) we analysed GCF MMP-8 levels by different methods and found that methods which based on the same monoclonal antibody against MMP-8 [immunofluorometric method (IFMA), MMP-8 specific chair-side dip-stick test and dentoAnalyzer appliance] gave comparable results. In this current paper we have used IFMA, dentoELISA as well as commercially available Amersham ELISA assay to determine MMP-8 levels in oral rinse samples from subjects with different periodontal health status expressed as different levels of periodontal inflammatory burden. Additionally, tissue inhibitor of matrix metalloprotease-1 (TIMP-1) levels and detection of elastase-like activity were screened in oral rinse samples by Amersham ELISA and a test utilizing a chromogenic substrate (Nieminen *et al*, 1993;

Uitto *et al*, 1996), respectively. The aim was to find out if these markers analysed with the applied methods individually or in combination, have diagnostic potential in differentiation of subjects with different periodontal inflammatory burden level. In addition we wished to find out whether the results of MMP-8 analysis obtained by used methods were comparable.

Materials and methods

Patients

In this cross-sectional study 214 adult patients with age range 44–78 years (mean \pm s.d. 57.2 ± 7.9) were randomly selected from dental public health clinics in cities of Helsinki and Vantaa. Patients in need of antibiotic prophylaxis or suffering of a contagious disease were excluded. The protocol was clearly explained to all study subjects and institutional review board-approved informed consents were signed. 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.

All study subjects were examined clinically: bleeding on probing (BOP) was registered dichotomously on four tooth surfaces and pocket probing depths (PPD) by Florida-probe device (Florida Probe Corporation, Gainesville, Florida, USA) on six tooth surfaces. PPD was recorded if ≥ 4 mm. Examinations were done by two calibrated general dentists. Background characteristics were recorded by using questionnaires. Oral rinse samples were taken from all patients.

Patients were categorized into four groups according to their periodontal inflammatory burden, determined by combining the Periodontal Inflammatory Burden Index (PIBI) described by Lindy *et al* (2008) and BOP%. PIBI is calculated by adding the number of periodontal sites indicating moderate periodontitis (number of periodontal pockets with PPD 4–5 mm = Nmod) to the weighted number of periodontal sites indicating advanced periodontitis (number of periodontal pockets with PPD ≥ 6 mm multiplied by two = Nadv).

Periodontal inflammatory markers are conducted into oral rinse within the GCF. Flow of GCF is increased in inflamed tissues and is also proportional to the area of inflamed epithelial surface of the periodontal sulcus/pocket (Armitage, 1995; Weidlich *et al*, 2001). BOP% indicates inflammation in the periodontal tissues and the PIBI reflects periodontal inflammatory injury (Lindy *et al*, 2008). Based on this we reasoned to integrate PIBI and BOP% into one index (PIBI \times BOP%) with the purpose to indicate the GCF flow. We hypothesized that the higher the flow of GCF, the higher is the level of inflammatory markers in oral rinses, and that it reflects the periodontal health status.

According to the categorization methods described above we formed four groups of study subjects: (1) 36 subjects were regarded as periodontally healthy

(BOP <10% of teeth surfaces and no deepened periodontal pockets) (2) 21 subjects with BOP ≥ 10% of teeth surfaces but no deepened periodontal pockets, mild periodontal inflammatory burden, regarded clinically as gingivitis. Patients diagnosed to have deepened periodontal pockets (PPD ≥ 4 mm) were categorized into two groups according the PIBI × BOP%: 104 subjects had PIBI × BOP% 1–100 (group 3; moderate periodontal inflammatory burden) and 53 patients had PIBI × BOP% ≥ 100 (group 4; strong periodontal inflammatory burden). Threshold of 100 was chosen to discriminate moderate inflammatory burden (clinically regarded as minor or early periodontitis) from strong inflammatory burden (clinically more advanced disease).

Oral rinse samples

Oral rinse samples were taken as follows: 1 ml of tap water was placed into the subject’s oral cavity with a disposable plastic pipette. Subjects rinsed their mouth with tap water for 1 min and the rinse was collected into collection tubes (Nieminen *et al*, 1993; Uitto *et al*, 1996). On the same day an aliquot of the specimen was taken for elastase activity testing and the rest of the sample was frozen on the same day for further MMP-8 and TIMP-1 analyses (Uitto *et al*, 1996).

MMP-8, TIMP-1 and elastase determinations

All samples were analysed for MMP-8 levels by three different immunological assays: dentoELISA (Munjal *et al*, 2007; Sorsa *et al*, 2010), a time-resolved immunofluorometric assay (Hanemaaijer *et al*, 1997; Mäntylä *et al*, 2003, 2006) and a commercial ELISA kit by Amersham (Munjal *et al*, 2007; Sorsa *et al*, 2010). TIMP-1 levels were measured with Amersham ELISA (Tuomainen *et al*, 2007; Rautelin *et al*, 2009). Amersham ELISA analyses of MMP-8 and TIMP-1 levels were done according to manufacturer’s instructions. MMP-8 and TIMP-1 results (ng ml⁻¹) were expressed as levels per subject and per total number of teeth/subject.

Elastase activity test was done based on the method described by Nieminen *et al* (1993) and Uitto *et al* (1996). The chromogenic substrate succinyl-alanyl-valine-*p*-nitroanilide (Sigma Co., St Louis, MO, USA) was dissolved in a solution with the following final concentrations: 1 mM substrate, 0.1% dimethyl sulphoxide, 0.2 M NaCl and 0.05 M Tris-HCl buffer, pH 7.5. The enzyme activity is expressed as optical density after

subtracting the 0 h value. Optical densities were measured at nominal/categorical scale by comparing the colour of test plates to a coloured slide designed for eye comparison of optical densities. Optical densities were analysed by two observers to minimize chance of misinterpretation. Optical densities were categorized according to previously published results, which proved good correlation between elastase activity and the number of deep periodontal pockets (Uitto *et al*, 1996). 0.2 OD was used as a cut off level for a significant positive elastase activity and levels below the threshold of 0.2 OD were regarded as negative (Uitto *et al*, 1996).

Data analysis

Non-parametric Kruskal–Wallis or Mann–Whitney *U*-test for independent samples was used for testing statistical significances of differences between study groups and bivariate non-linear Spearman correlation, for testing of correlations between results obtained by different MMP-8 detection methods. An association of nominal variables in crosstables was tested by Cramér V test. The diagnostic accuracy of MMP-8 measuring methods used was evaluated using receiver operating characteristic (ROC) curve analysis. *P*-values < 0.05 were considered to be statistically significant. Statistical analyses and tests were done by SPSS-software (version 17.0).

Results

Clinical parameters and demographic data

Subject groups, clinical parameters and demographic characteristics are shown in Table 1. Association between consensus risk factors (Genco, 1996) for chronic periodontitis; gender, tobacco smoking and diabetes, and different periodontal indices were tested (Table 2). In general terms, male gender and tobacco smoking were associated with higher values of all periodontal indices used in the study: PIBI, BOP%, and PIBI × BOP% (*P* < 0.001), except for the association between male gender and BOP%, that was insignificant (*P* = 0.323). However, diabetes associated with decreased BOP% (*P* = 0.016) in this study population (Table 2). Diabetic subjects were not examined as a separate group, and they could belong to any of the study groups 1–4.

Table 1 Periodontal indices and demographic data of patient groups. Medians (IQR) indicated for each parameter

	Group 1 Periodontally healthy n = 36	Group 2 Mild inflammatory burden n = 21	Group 3 Moderate inflammatory burden n = 104	Group 4 Strong inflammatory burden n = 53	P-value
Teeth (<i>n</i>)	25 (22–27)	26 (22–28)	26 (23–28)	25 (18–27)	0.094
Age (years)	57 (52–64)	55 (50–57)	55 (52–61)	56 (51–63)	0.507
BOP (%)	4.2 (1.8–5.9)	13.7 (12.0–16.0)	6.9 (4.8–13.9)	19.7 (14.5–28.2)	< 0.001
PIBI	0	0	2.5 (2–5)	15 (8–26)	< 0.001
PIBI × BOP (%)	0	0	21.0 (10.9–46.1)	263.9 (166.6–487.2)	< 0.001

Bold value indicates the level of significance 0.05.

Association between MMP-8 and periodontal inflammatory burden

MMP-8 levels increased along with periodontal inflammatory burden (Table 3). Enzyme levels measured by dentoELISA were significantly different among groups ($P = 0.018$) and differences were more pronounced when MMP-8 levels were adjusted to the number of teeth for both, dentoELISA (Figure 1) and IFMA ($P = 0.007$ and $P = 0.032$, respectively).

Correlations between MMP-8 detection methods

Correlation between MMP-8 levels measured with IFMA and dentoELISA were tested with Spearman correlation test which gave a correlation coefficient 0.691 (statistically significant at $P < 0.01$ level). No correlation was found between MMP-8 analysis by dentoELISA or IFMA and Amersham ELISA. MMP-8 levels measured by Amersham ELISA test did not correlate with the values of periodontal parameters.

Statistical outliers

Based on statistical outliers of MMP-8 levels adjusted to the number of teeth (Figure 1), we determined the threshold for peak levels of MMP-8/number of teeth measured by both dentoELISA and IFMA. The thresholds were determined at 15 ng ml⁻¹ level for dentoELISA

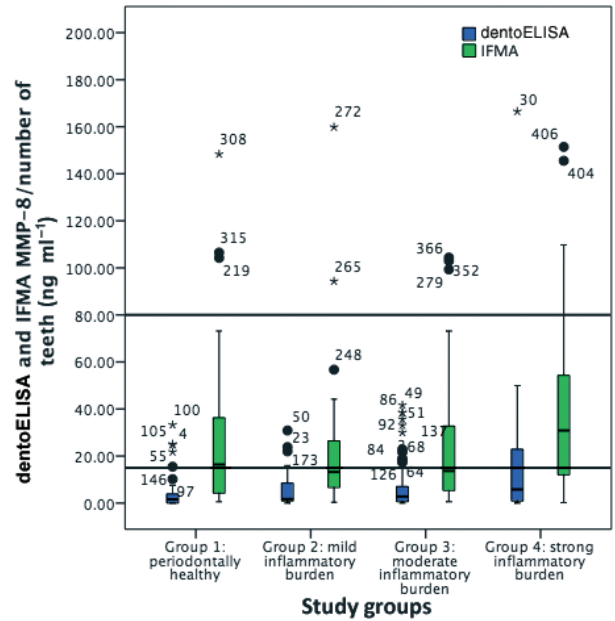


Figure 1 Boxplots representing the distributions of MMP-8 levels detected by dentoELISA and IFMA adjusted to the number of teeth in study groups ($P = 0.007$). Threshold line for peak dentoELISA and IFMA MMP-8 levels is drawn at 15 and 80 ng ml⁻¹ levels, respectively

		BOP%	PIBI	PIBI × BOP%
Gender	Males, $n = 68$	11.6 (6.0–17.3)	5 (1–14)	40 (9–192)
	Females, $n = 146$	9.0 (4.9–15.9)	2 (0–6)	15 (0–70)
<i>P</i> -value		0.323	0.001	0.002
Smoking (Cigarettes daily)	0 (no smoking)	8.3 (4.5–15.3)	2 (0–5)	14 (0–55)
	1–10	11.8 (7.0–19.3)	4 (1–10)	40 (5–203)
	11–20	14.5 (6.9–20.0)	9 (5–21)	112 (59–321)
	>20	13.7 (9.7–47.6)	63 (32–102)	927 (317–5375)
<i>P</i> -value		0.03	< 0.001	< 0.001
Diabetes	No	10.5 (5.3–17.3)	2 (1–8)	23 (0–108)
	Yes	6.1 (4.2–10.1)	2 (0–4)	10 (0–25)
<i>P</i> -value		0.016	0.170	0.055

Bold value indicates the level of significance 0.05.

Table 2 Association between periodontal indices and consensus risk factors. Medians (IQR) indicated for each parameter

Table 3 MMP-8 and TIMP-1 levels among patient groups. Medians (IQR) indicated for each parameter

	Group 1 Periodontally healthy $n = 36$	Group 2 Mild inflammatory burden $n = 21$	Group 3 Moderate inflammatory burden $n = 104$	Group 4 Strong inflammatory burden $n = 53$	<i>P</i> -values
MMP-8 dE	39 (0–100)	44 (23–276)	68 (15–158)	131 (22–462)	0.018
MMP-8 dE/number of teeth	1.6 (0–3.9)	1.7 (0.8–9.9)	2.8 (0.6–7.0)	5.8 (0.9–22)	0.007
MMP-8 IFMA	398 (103–797)	376 (149–591)	341 (135–839)	628 (245–1131)	0.112
MMP-8 IFMA/number of teeth	16.4 (4.2–36.3)	13.2 (5.2–28.7)	13.7 (5.3–32.7)	30.9 (12–54.3)	0.032
MMP-8 AE	35.7 (14.4–82.9)	32.5 (19.8–69.3)	39.2 (17.9–80)	28.8 (15.1–62)	0.576
MMP-8 AE/number of teeth	1.55 (0.77–3.47)	1.31 (0.8–2.48)	1.49 (0.75–3.38)	1.31 (0.68–2.83)	0.921
dE MMP-8/TIMP-1	0.09 (0–0.37)	0.19 (0.03–0.97)	0.2 (0.03–0.59)	0.32 (0.04–2.61)	0.033
IFMA MMP-8/TIMP-1	1.2 (0.3–2.2)	0.9 (0.2–3.0)	1.1 (0.4–2.5)	1.6 (0.2–6.7)	0.298
AE MMP-8/TIMP-1	0.09 (0.04–0.21)	0.1 (0.04–0.21)	0.11 (0.05–0.21)	0.11 (0.06–0.23)	0.935
TIMP-1	172 (111–218)	170 (73–227)	147 (95.6–229)	143 (76.9–258)	0.770

dE, dentoELISA; AE, Amersham ELISA. Bold value indicate the level of significance 0.05.

Table 4 Percentages of subjects (%) representing peak levels of MMP-8 measured by dentoELISA (dE) and IFMA adjusted to numbers of teeth

	Peak threshold	Group 1 Periodontally healthy n = 36	Group 2 Mild inflammatory burden n = 21	Group 3 Moderate inflammatory burden n = 104	Group 4 Strong inflammatory burden n = 53	P-value*
IFMA MMP-8/number of teeth	> 80 ng ml ⁻¹	8.3% (n = 3)	9.5% (n = 2)	3.8% (n = 4)	15.1% (n = 8)	0.104
dE MMP-8/number of teeth	> 15 ng ml ⁻¹	13.9% (n = 5)	19.0% (n = 4)	13.5% (n = 14)	34.0% (n = 18)	0.016

*Contingency coefficient and Cramér V test was used for the statistical analyses. Bold value indicate the level of significance 0.05.

results corresponding 80 ng ml⁻¹ levels for IFMA. Percentages of MMP-8/number of teeth peak levels in each study group are shown in Table 4. The higher the severity of periodontal disease (stronger periodontal inflammatory burden) was, the higher the percentage of peak values of MMP-8/number of teeth, except for group 3 (Table 4). The difference was statistically significant when MMP-8 levels were measured by dentoELISA ($P = 0.016$; Table 4). The Amersham ELISA MMP-8 levels did not express statistical outliers.

MMP-8/TIMP-1 ratio and TIMP-1 levels

MMP-8/TIMP-1 molar ratios increased together with periodontal inflammatory burden when measured by dentoELISA ($P = 0.033$, Table 3).

TIMP-1 levels decreased progressively with inflammatory burden and with age group, but only the latter differences were significant ($P < 0.001$, Figure 2).

Elastase activity

A statistically significant difference ($P < 0.001$) in MMP-8/number of teeth levels between groups with negative and positive elastase activity test results was detected when MMP-8 was measured by dentoELISA or IFMA but not when measured by Amersham ELISA. The difference of dentoELISA MMP-8/number of teeth

median values between elastase activity groups was also tested separately in each patient group and confirmed to be statistically significant (for groups with mild, moderate and strong inflammatory burden, i.e. groups 2, 3 and 4 $P < 0.001$; for periodontally healthy, i.e. group 1, $P = 0.029$; Figure 3). dento ELISA MMP-8/number of teeth levels increased in line with study groups (inflammatory burden), except for group 3 with moderate inflammatory burden, as mentioned earlier in section ‘Results’ and seen in Figure 1. However, the increase was especially pronounced when the study groups were further categorized according to the elastase activity (positive elastase activities: $P = 0.002$; negative elastase activity, $P = 0.322$ ns; Figure 3).

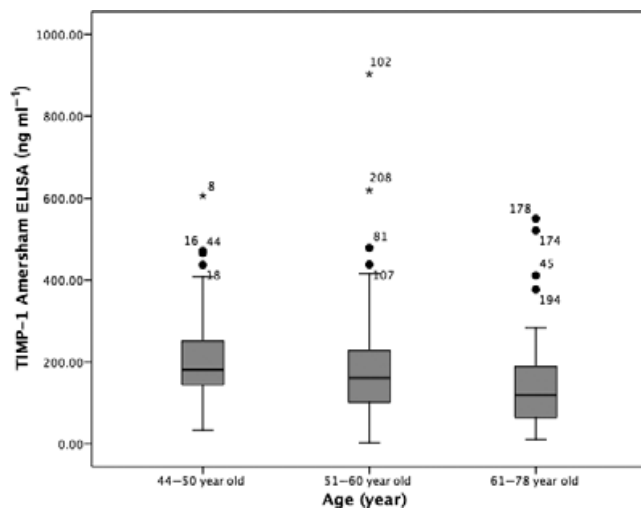


Figure 2 TIMP-1 values grouped according to age of studied subjects ($P < 0.001$)

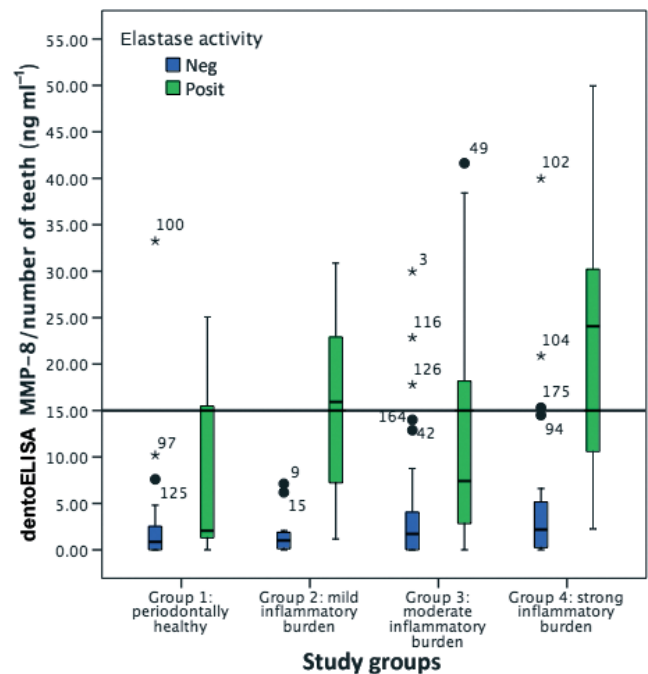


Figure 3 Association between the detection of elastase activity and dentoELISA MMP-8/number of teeth levels in study groups. Green represents the category of positive elastase activity and blue negative elastase activity, respectively. dentoELISA MMP-8 levels v/s elastase activity in subject groups 1-4 with healthy periodontium, and with mild, moderate and strong periodontal inflammatory burden, respectively, $P < 0.05$. dentoELISA MMP-8 levels v/s all study groups 1-4, in subjects with positive elastase activity, $P = 0.002$. There were no statistically significant differences between study groups with negative elastase activities

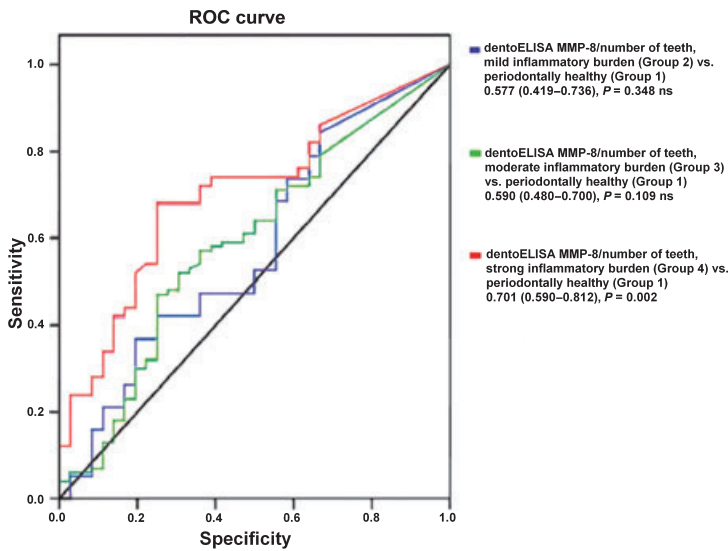


Figure 4 Receiver operating characteristic (ROC) curve analysis for the evaluation of diagnostic sensitivity and specificity of dentoELISA MMP-8/number of teeth levels in different levels of periodontal inflammatory burden (study groups 1–4 with healthy periodontium, mild, moderate and strong periodontal inflammatory burden, respectively). The area under the ROC curve, 95% confidence interval, and P -values are given next to the figure

ROC-curve analysis

A receiver operating characteristic (ROC) analysis was run to evaluate the diagnostic sensitivity and specificity of dentoELISA MMP-8/number of teeth. The ability of dentoELISA MMP-8/number of teeth to distinguish between periodontally healthy subjects and subjects with different levels of periodontal inflammatory burden (groups 2, 3 and 4 with mild, moderate and strong inflammatory burden) was tested separately. The distinction was significant between periodontally healthy (group 1) and subjects with strong periodontal inflammatory burden (group 4) (Figure 4). The area under the curve was 0.701 ($P = 0.002$; 95% confidence interval 0.59–0.81).

Discussion

In this study the subjects were not selected based on a defined periodontal disease status. Instead, the randomly selected study subjects were categorised based on the level of periodontal inflammatory burden expressed as numbers and depths of deepened periodontal pockets and percentage of bleeding on probing.

MMP-8 levels in oral rinse samples were higher in subjects with the strongest periodontal inflammatory burden than in subjects with less inflammatory changes when oral rinse levels were adjusted to number of teeth, especially when measured with dentoELISA. This finding was confirmed with ROC analysis. Similarly, MMP-8/TIMP-1 ratios increased in subjects with strong periodontal inflammatory burden when MMP-8 was measured by dentoELISA. MMP-8 increments were especially pronounced in subjects showing detectable elastase activity.

The measurement of host originating oral biomarker levels in periodontal diagnostics has faced the problem that it has been extremely difficult to define the 'normal' levels of various biomarkers. Biomarkers which are expressed at high levels in periodontal inflammation can be detected at lower levels also in periodontally healthy oral cavity, obviously as an indication of the physiologic

turnover of oral tissues (McCulloch, 1994; Uitto *et al*, 2003). Besides, the individual expression of biomarkers in healthy state as well as in disease may vary because of genetic variation (McCulloch, 1994; Uitto *et al*, 2003).

MMP-8 can be regarded as a central enzyme in periodontitis development and progression (Sorsa *et al*, 2004, 2006). It may be useful in site specific diagnostics to find out if individual sites or teeth are at risk of progression of periodontitis when analysed from GCF. Mäntylä *et al* (2006) found that repeatedly high MMP-8 levels in GCF tested with a MMP-8 specific chair side dip-stick test identified sites/teeth which were at risk of progression of periodontal attachment loss. Site specific MMP-8 testing also identified the subjects with poor response to conventional periodontal treatment, that is scaling and root planing. These subjects had multiple sites with an unstable character, and these sites were repeatedly test positive during the 12-month maintenance phase of periodontal treatment. Repeated testing may thus give more reliable diagnostic information than single testing in site specific biomarker diagnostics.

Smoking has been found in several studies to decrease MMP-8 levels in GCF because of the effects on local blood circulation and lowered signs of inflammation (Söder, 1999; Söder *et al*, 2002). However, Mäntylä *et al* (2006) detected the highest MMP-8 GCF levels in smoking subjects with poor response to conventional periodontal treatment (scaling and root planing, SRP). Thus it cannot be regarded categorically that all smoking periodontitis patients have lower levels of MMP-8 in GCF and further in oral rinse sample.

Periodontitis is regarded to be cyclic by nature, and part of the dentition may be in active and progressing phase of periodontitis while other teeth may be quiescent or even in the phase of recovery (Carranza and Camargo, 2006). Cyclic nature of periodontitis may be even more challenging for oral rinse than for GCF based diagnostics. In an oral rinse sample the GCF is pooled from all periodontal pockets, and the collected oral rinse sample represents GCFs from whole periodontium. For this reason the definition of the cut off level for the biomarker

which would be indicative for the deterioration of periodontal health is demanding and an oral rinse sample biomarker analysis can be used only for a rough assessment of the periodontal inflammatory level. The activity of periodontitis can only be established by monitoring patients longitudinally, and the progression can only be assessed site specifically. In our current study we did not address the question of disease activity or progression. Instead, we wanted to find out, if the surface area for subgingival biofilm accumulation expressed as periodontal pocketing and the simultaneous bleeding on probing, which points out the possibility of an ongoing inflammation, are reflected in an oral rinse sample.

In this current study some subjects with clinically healthy periodontium (no deepened periodontal pockets and bleeding on probing <10%) as well as some subjects with mild to moderate inflammatory burden with limited numbers of deepened periodontal pockets indicating gingivitis or mild periodontitis, expressed high levels of oral rinse MMP-8 which was detected by IFMA and dentoELISA. High oral rinse MMP-8 levels in subjects with no clinical periodontal disease and in subjects with mild to moderate periodontal inflammatory burden were statistical outliers or peak values and were expressed only by some individuals in these groups. The cut off level for 'positive' MMP-8 finding in an oral rinse sample measured by dentoELISA and adjusted to the number of teeth which indicates a person at risk, may be the level above which the statistical outliers or peak values in groups of subjects with mild to moderate periodontal inflammatory burden are situated in box and whiskers plots, that is 15 ng ml^{-1} . These peak values in periodontally healthy subjects' samples may indicate a proinflammatory condition in subjects which may have an aggressive host response and can be susceptible to develop periodontitis, that is subjects with a high 'normal' MMP-8 level. In subjects with mild to strong inflammatory burden, oral fluid MMP-8 at least partly originates from inflamed marginal gingival tissues but levels above the risk level (15 ng ml^{-1}) may also indicate the subjects who are at risk to progress from gingivitis to periodontitis, or at risk of progression of periodontitis.

MMP-8 levels measured by IFMA and dentoELISA, but not with Amersham ELISA, were comparable. The obvious reason for the high correlation between IFMA and dentoELISA MMP-8 levels is the same monoclonal antibody shared by both methods (Hanemaaijer *et al*, 1997; Mäntylä *et al*, 2003, 2006; Sorsa *et al*, 2010), but based on its association with the severity of periodontal inflammation, this antibody may also be applicable and promising in oral fluid periodontal diagnostics. The antibody used in dentoELISA and IFMA exerts high sensitivity to target active forms of MMP-8 (Hanemaaijer *et al*, 1997; Sorsa *et al*, 2010), whereas the Amersham ELISA might test proform and thus target total MMP-8. IFMA is a method which can be used only in research laboratories and for this reason it is not ideal for rapid chair-side use in a dentist's office (Sorsa *et al*, 2010).

It has been suggested that the simultaneous measurement of several oral fluid biomarkers could be beneficial

in periodontal diagnostics. In this current study oral rinse MMP-8 levels and elastase activity, both enzymes which originate especially from PMN cells, associated with the level of periodontal inflammatory burden. However, it is not necessarily useful to measure many biomarkers with the same cellular origin. In this study we also analysed TIMP-1 levels, which is a tissue inhibitor of MMPs, in oral rinse samples. MMP-8/TIMP-1 ratios had a trend to be higher in the group of subjects with strong inflammatory burden, and the ratio was significantly higher when MMP-8 levels were analysed by dentoELISA.

A novel finding was that oral rinse TIMP-1 levels decrease with increasing age which can be regarded as an indication of age related changes in tissue homeostasis. Age as such has not been regarded as a risk factor for periodontitis. In ageing skin, expression of TIMP-1 is decreased with fibroblast senescence (Hornebeck, 2003). Ageing may have a similar effect in oral mucous membranes and periodontal tissues. Relevant age-related changes related to TIMP-1 decrease can also be supported by the findings of Benatti *et al* (2008) indicating that ageing modulates important biological properties of periodontal ligament cells and favours extracellular matrix remodelling.

Diagnostic tests are always somewhat unreliable and they should only be supplementary when the likelihood of a disease is estimated (Pauletto *et al*, 2000; Uitto *et al*, 2003; Sorsa *et al*, 2006). Very seldom a single biochemical test is diagnostically fully reliable but it may be helpful as an adjunct to clinical examination (Mäntylä *et al*, 2003, 2006). No positive test finding in clinic should lead to treatment unless clinical findings are in line, and vice versa, the negative finding should not lead to the neglect of health promoting measures if clinical findings indicate inflammation. Based on our findings, the oral rinse sample analysis of MMP-8 could be clinically useful in rough screening to identify individuals who are at risk to develop periodontitis or in analysing the individual level of host response. Simultaneous analysis of MMP-8 and TIMP-1 could be beneficial. Oral rinse sample analysis may be useful in defining the optimal period between periodontal maintenance visits after active periodontal treatment. During the active treatment phase it would be possible to monitor the decrease of oral rinse MMP-8 levels, and oral rinse MMP-8 levels at the end of the active treatment phase would show the individual level to keep the host response in control. In this regard, the testing could give valuable information about the control of inflammation which today is based on the clinical findings, that is shallower or eliminated periodontal pockets and less bleeding on probing. At best the biomarker level could be monitored by a home test to indicate when the cut off of for possibly unsafe biomarker level is reached. Also the effect of MMP-8 inhibiting low-dose doxycycline (LDD) medication could be monitored by analysing the oral rinse MMP-8 levels to find out when a possible brake in medication would be possible or when the medication should be taken again (Golub *et al*, 2008; Reinhardt *et al*, 2010).

In conclusion, MMP-8 testing of oral rinse samples may be beneficial in periodontal diagnostics, but should be applied at this phase of research with caution. Testing may be adjunctive to other clinical screening methods if it is used correctly. Today it cannot be ruled out that there may be false negative findings because of inadequate knowledge about the safe cut off levels, and the levels may vary between different individuals depending on individual biomarker profiles. Simultaneous analysis of TIMP-1 may be beneficial.

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Author contribution

All authors have substantially contributed the study by designing it, by collecting and analysing the samples, by analysing the data, and/or by drafting and writing the manuscript.

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