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Myeloid-related protein (MRP8/14) expression in gingival crevice fluid in periodontal health and disease and after treatment

E. Andersen[1], I. M. Dessaix[1], T. Perneger[2], A. Mombelli[1]

[1]School of Dental Medicine, University of Geneva, Geneva, Switzerland and [2]Division of Clinical Epidemiology, Geneva University Hospital, Geneva, Switzerland

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Background and Objective: Myeloid-related protein (MRP8/14) and its subunits are biomarkers of inflammation. The present study evaluated whether gingival crevice fluid levels of these markers discriminate periodontitis from healthy sites in patients with chronic periodontitis or diseased from healthy subjects, and whether these biomarkers detect longitudinal changes after therapy.

Material and Methods: Levels of MRP8/14, MRP14 and total protein were quantified in 19 periodontitis patients before non-surgical periodontal therapy, after 3 and 6 mo of treatment, and were measured once in 11 periodontally healthy subjects. In total, diseased subjects contributed 59 sites with probing depths >4 mm (PP) and 21 sites <4 mm (PH); healthy subjects contributed 91 sites (HH).

Results: Overall, in diseased subjects, MRP8/14, MRP14 and total protein were not significantly different between PP and PH sites. However, at baseline, MRP8/14 and total protein had significantly higher values at sites in periodontally diseased than in healthy subjects. Clinical improvement was associated with a significant decrease of MRP8/14 and MRP14 from baseline to month 6 in PP sites. Interestingly, a similar decrease was observed in PH sites for all three markers. At 6 mo, however, levels of MRP8/14 and protein in PP and PH sites of patients were still significantly higher than in healthy subjects.

Conclusion: Gingival crevice fluid levels of MRP8/14 did not differentiate between clinically diseased and healthy sites in patients with chronic periodontitis. However, this marker was elevated in periodontally diseased compared with healthy subjects, and its values decreased following therapy. MRP8/14 may be used to monitor the response to treatment.

Elene Andersen, School of Dental Medicine, University of Geneva, 19, rue Barthélemy-Menn, 1205 Geneva, Switzerland
Tel: +41 22 379 40 30
Fax: +41 22 379 40 32
e-mail: elene.andersen@unige.ch

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Parameters used for periodontal diagnosis in clinical practice essentially measure tissue damage (loss of attachment and alveolar bone) that occurred in the past. Ideally, diagnostic procedures should produce information regarding the present activity, rather than the history of a disease. Various inflammatory mediators can be detected in periodontal tissues affected by gingivitis and periodontitis. Some of these mediators may be directly

implicated in destructive processes, making them candidate markers for progressive disease (1,2). Gingival crevice fluid is a possible source of biomarkers that can be collected non-invasively close to where the disease occurs. However, since it is characteristic of periodontal disease that not all parts of a dentition are affected with equal severity, levels of biomarkers in gingival crevice fluid may vary from site to site, depending on local clinical and biological conditions of the surrounding tissues (3–5), which may decrease their potential diagnostic value at the level of the subject.

In an inflammatory environment, activated polymorphonuclear leukocytes, monocytes and macrophages, as well as certain epithelial cells, release myeloid-related protein (MRP8/14; 6–9), also called S100A8/A9 or calprotectin (10). Studies suggest that MRP8/14 and subunits MRP8 and MRP14 can discriminate types and phases of inflammatory diseases (11–15). Calprotectin is a validated faecal marker of inflammation used in the diagnosis, classification, prevention and treatment of inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease (16). In serum and plasma, high concentrations have been found in patients with cystic fibrosis (17,18), rheumatoid arthritis (19–21), psoriasis (22) and Crohn's disease (11), as well as in microbial infections (23,24). Our group identified MRP8 and MRP14 in the gingival crevice fluid of periodontitis patients using two-dimensional polyacrylamide gel electrophoresis, N-terminal sequencing and mass spectrometry (25). We have since studied the expression of MRP8/14 and its subunits in gingival crevice fluid in various clinical situations, including the early phase of experimental gingivitis (3). In an interventional study, we showed a significant decrease of MRP8/14,10 d after non-surgical periodontal therapy, when supplemented with systemic antibiotics (26). Several other groups have also indicated a relationship between levels of MRP8/14 in gingival crevice fluid and clinical periodontal parameters (27–30).

To further evaluate the diagnostic value of MRP8/14, in the context of chronic periodontitis, the purpose of the present study was to assess whether gingival crevice fluid levels of MRP8/14, MRP14 or total protein can be used to discriminate periodontitis sites (PP) from healthy sites (PH) in periodontally diseased subjects or periodontally diseased subjects from healthy subjects, and to study longitudinal changes of PP and PH sites in periodontitis patients after non-surgical therapy.

Material and methods

Subject and site selection

Nineteen adults with chronic periodontitis, with at least two teeth having a probing pocket depth >4 mm, clinical attachment loss of at least 2 mm and radiographic evidence of bone loss, aged 32–66 years, and 11 subjects with a healthy periodontium with no probing pocket depth >4 mm and no radiographic evidence of bone loss, aged 20–33 years, were included in the study. All subjects were free of systemic disease. Six of the periodontitis subjects and none of the healthy subjects were smokers. Pregnant women or subjects having received antibiotics, immunosuppressive drugs or periodontal treatment within the last 6 mo were not included.

Each of the 19 periodontitis patients contributed between two and four sites with probing pocket depth >4 mm (PP sites). In addition, 12 of these patients also contributed between one and three sites with probing pocket depth <4 mm (PH sites). Probing pocket depth values of 4 mm were eliminated to avoid overlap between clinically diseased and healthy sites. Teeth with suppuration and their adjacent neighbours were not considered. If more than four sites fulfilled the criteria for PP sites, the deepest sites were chosen from non-adjacent teeth. The PH sites were selected to obtain as even a distribution as possible. Each of the 11 subjects without periodontitis contributed between six and nine sites with probing pocket depth <4 mm (HH sites). In total, 59 PP, 21 PH and 91 HH sites were included in the study. In the periodontitis patients, the same sites were sampled at baseline, 3 and 6 mo.

The project was approved by the Ethical Committee of the School of Dental Medicine, University of Geneva, and written informed consent was obtained prior to enrolment of each participant.

Clinical procedures

At baseline, prior to any hygiene instruction or treatment, gingival crevice fluid samples were collected, and the periodontal status was assessed. All patients were then given detailed instructions for proper supragingival plaque control, and the entire dentition was scaled supragingivally. All pockets deeper than 3 mm were thoroughly root planed, once an oral hygiene level with more than 80% tooth surfaces plaque free was reached. The mechanical instrumentation was continued until the operator felt that the surface was hard and smooth. After scaling and root planing, pockets were rinsed with a 0.2% aqueous solution of chlorhexidine. These treatments required between two and four sessions within 1 mo.

Patients were recalled after 3 and 6 mo. At these appointments, gingival crevice fluid was sampled and the periodontal parameters were assessed. If necessary, oral hygiene instructions were reviewed, supragingival calculus was removed and teeth were polished. However, no instrumentation was performed 1 mm below the gingival margin.

Clinical parameters

Plaque scores were recorded dichotomously as presence or absence of visible plaque. Probing pocket depth was measured from the gingival margin to the bottom of the pocket, using a standard probe. Bleeding on probing was recorded dichotomously. Bleeding on probing and plaque scores were expressed as a percentage of positive sites for each site category. The same examiner (I.M.D.) recorded all parameters throughout the study.

Gingival crevice fluid sampling and analysis

Supragingival plaque was carefully removed, if necessary, and the study

sites were isolated from saliva with cotton rolls and gently dried using an aspiration tip. After 2 min, the gingival crevice fluid was collected by means of Durapore membrane strips (2 × 6 mm, 0.22 μm pore size; Millipore, Bedford, MA, USA). The strips were placed at the entrance of the sulcus or pocket and left *in situ* for 15 s, then transferred into microtubes and stored at −20°C until analysed.

The following gingival crevice fluid components were assessed as previously described (3): MRP8/14, MRP14 and total protein. In brief, on the day of analysis, 100 μL of 10 mM Tris–HCl (pH 7.4) was added to each sample. The tubes were gently vortexed for 30 s to elute the gingival crevice fluid components, and centrifuged at 4°C for 7 min at 2000*g*, with the strips held in place at the collar of the tube by its cap. After centrifugation, the strips were removed, and the supernatant used for analysis. Both MRP8/14 and MRP14 were quantitatively determined by ELISA (BMA; Biomedicals, Augst, Switzerland). Total protein content was determined according to the Bradford method. The quantities of MRP8/14, MRP14 and total protein were expressed as total amount (ng) per 15 s sample (3).

Data analysis

In the statistical analyses, two sources of correlation were taken into account, namely multiple sites per person and repeated measurements in patients, using generalized estimating equations (GEE) models. We clustered the observations by person, and used the 'exchangeable' correlation matrix, which means that the correlation between sites from the same person was considered constant. Means and standard deviations were calculated using the usual formulas. The *p*-values were obtained from GEE models, which allow analysis of non-independent data.

To evaluate the diagnostic capacity of MRP8/14, MRP14 and protein to discriminate between patients with periodontitis and healthy subjects, mean values of all PP and PH sites of each periodontitis patient were compared with mean values of all HH sites of each healthy subject. Receiver operating characteristic curves were drawn, and the area under the curve was calculated. The area under the curve can be interpreted as the probability that a randomly chosen periodontitis patient will have a higher biomarker value than a randomly chosen control subject; a value of 0.5 corresponds to a score with no predictive power (predictive ability equivalent to chance) and 1.0 to a marker that discriminates perfectly. Values of 0.7 and more are often found clinically useful.

The level of significance was set at $p < 0.05$. SPSS 15 for Windows (SPSS Inc., Chicago, IL, USA) was used for all calculations and graphs.

Results

Baseline comparisons

At baseline, among periodontitis patients, the PP sites had greater pocket probing depth than PH sites (by definition), but were also considerably more likely to bleed on probing (Table 1). In contrast, PP and PH sites had similar levels of plaque (Table 1) and similar levels of gingival crevice fluid markers MRP8/14, MRP14 and total protein (Table 2).

The capacity of MRP8/14, MRP14 and protein to discriminate between patients with periodontitis and healthy subjects, expressed as areas under receiver operating characteristic curves for mean biomarker values measured at baseline, is shown in Table 3.

Longitudinal changes

In periodontitis patients, all clinical indicators of periodontal disease activity (plaque score, pocket depth and bleeding on probing) decreased after treatment, as anticipated (Table 1).

The changes in biomarker values over time are shown in Tables 2 and 4. Levels of MRP8/14 decreased significantly following therapy in periodontitis patients, for both PP and PH sites, from about 4000 ng/15 s sample at baseline to about 1500 ng/15 s sample at 6 mo, but even at that point the levels exceeded values measured in healthy subjects by about fourfold, and this residual

Table 1. Clinical parameters in patients with periodontitis

	Baseline	3 mo	6 mo	*p*-value[a] 1	*p*-value[a] 2
Plaque score (%)					
Diseased sites (PP)	34 (48)	23 (43)	5 (23)	0.58	0.004
Healthy sites (PH)	43 (51)	5 (22)	0	0.04	0.025
p-value[a] (PP vs. PH)	0.56	0.091	—	—	—
Probing pocket depth (mm)					
Diseased sites (PP)	6.0 (1.2)	3.1 (1.0)	2.8 (0.9)	< 0.001	< 0.001
Healthy sites (PH)	2.8 (0.4)	2.9 (1.2)	2.5 (0.5)	0.58	0.013
p-value[a] (PP vs. PH)	< 0.001	0.37	0.039	—	—
Bleeding on probing (%)					
Diseased sites (PP)	61 (49)	7 (26)	9 (29)	< 0.001	< 0.001
Healthy sites (PH)	14 (36)	5 (22)	0	0.40	0.18
p-value[a] (PP vs. PH)	0.010	0.70	—	—	—

Comparison of healthy and diseased sites at baseline and at 3 and 6 mo. Comparison between baseline and 3 mo (*p*-value 1) and between baseline and 6 mo (*p*-value 2).
PP, $n = 59$; PH, $n = 21$. Values are means (SD).
[a]GEE models.

Table 2. Mean values of MRP8/14, MRP14 and protein (expressed as ng/15 s sample), in gingival sites of patients with periodontitis (at three points in time) and in sites of healthy subjects (measured once)

	Baseline	3 mo	6 mo
MRP8/14			
Periodontitis: diseased sites (PP)	4118 (3880)	2582 (2696)	1500 (1976)
Periodontitis: healthy sites (PH)	4296 (4416)	1515 (2082)	1468 (3127)
p-value[a] (PP vs. PH)	0.75	0.65	0.95
Healthy subject sites (HH)	374 (436)	—	—
p-value[a] (Periodontitis vs. HH)	< 0.001	0.002	0.014
MRP14			
Periodontitis: diseased sites (PP)	2.21 (1.27)	2.06 (1.82)	0.90 (0.60)
Periodontitis: healthy sites (PH)	2.93 (2.52)	1.78 (1.71)	0.52 (0.39)
p-value[a] (PP vs. PH)	0.55	0.27	0.003
Healthy subject sites (HH)	1.36 (0.88)	—	—
p-value[a] (Periodontitis vs. HH)	0.054	0.32	<0.001
Protein			
Periodontitis: diseased sites (PP)	16551 (15797)	13990 (15971)	14656 (15444)
Periodontitis: healthy sites (PH)	17613 (13767)	7689 (5681)	8749 (5543)
p-value[a] (PP vs. PH)	0.77	0.037	0.060
Healthy subject sites (HH)	5507 (3879)	—	—
p-value[a] (Periodontitis vs. HH)	0.002	0.047	0.024

PP, $n = 59$; PH, $n = 21$; HH, $n = 91$.
[a]GEE models.

Table 3. Capacity of MRP8/14, MRP14 and protein (mean values of all gingival sites within each person) to discriminate between patients with periodontitis ($n = 19$) at baseline and healthy subjects ($n = 11$)

	Area under receiver operating characteristic curve	95% confidence interval	*p*-value
MRP8/14	0.93	0.83–1.00	< 0.001
MRP14	0.70	0.51–0.90	0.072
Protein	0.81	0.64–0.98	0.006

Table 4. Mean changes in biomarker values (expressed as ng/15 s sample) over time in periodontitis patients

	Baseline to 3 mo	*p*-value[a]	Baseline to 6 mo	*p*-value[a]
MRP8/14				
Diseased sites (PP)	−1774 (5337)	0.039	−2599 (4497)	0.007
Healthy sites (PH)	−2841 (3652)	0.067	−2842 (5210)	0.001
All	−2049 (4956)	0.027	−2658 (4644)	< 0.001
MRP14				
Diseased sites (PP)	−0.19 (1.82)	0.47	−1.31* (1.43)	< 0.001
Healthy sites (PH)	−1.26 (2.56)	0.035	−2.42* (2.61)	< 0.001
All	−0.47 (2.08)	0.24	−1.60 (1.86)	< 0.001
Protein				
Diseased sites (PP)	−3434 (17942)	0.30	−2132 (19116)	0.59
Healthy sites (PH)	−11114 (13996)	0.029	−7038 (9601)	0.027
All	−5300 (17293)	0.15	−3259 (17460)	0.35

Differences between changes in PP and PH sites are non-significant except for MRP14 between baseline and 6 mo (*$p = 0.045$).
PP, $n = 59$; PH, $n = 21$.
[a]GEE models.

difference between patients and healthy subjects remained statistically significant. The MRP14 values also decreased over time following treatment in periodontitis patients, so much that at 6 mo these levels were significantly below those of healthy subjects. Total protein decreased very little in diseased (PP) sites, and the difference from baseline values was non-significant both at 3 and at 6 mo. In contrast, protein levels decreased by about half in non-diseased (PH) sites, both at 3 and at 6 mo, and these decreases were statistically significant. Nevertheless, as was seen for MRP8/14, total protein levels did not reach the levels observed in healthy subjects.

Discussion

The present study assessed whether gingival crevice fluid levels of MRP8/14, its subunit MRP14, or total protein were able to discriminate periodontitis sites from healthy sites in periodontitis patients, and diseased subjects from healthy subjects. The biomarker levels were expressed as total amounts (ng per 15 s sample); as has been discussed in the literature, in samples of small volume the total amounts are more appropriate than concentrations, which are more prone to error (31). Our results showed that the gingival crevice fluid markers were able to discriminate diseased subjects from healthy subjects, whereas within periodontitis subjects the diseased sites (PP sites) could not be discriminated from clinically healthy sites (PH sites). In the perspective of a potential diagnostic utility, it has been mentioned that biomarkers should preferably characterize the patient's situation, rather than the local conditions of a sampled site only (1), which was the case for the present material. Concerning subjects, our data corroborate earlier findings reported by Kido *et al*. (27), who compared periodontitis patients with healthy subjects. Regarding differences between sites, they differ from those reported by Kaner *et al*. (29), who noted higher amounts of calprotectin in deep vs. shallow pockets. To understand this discrepancy, one should recall that Kaner's study was carried out in subjects with a clinical diagnosis of generalized aggressive periodontitis and started after completion of a hygiene phase, whereas our subjects were diagnosed with chronic periodontitis, and no

treatment whatsoever was carried out before collection of baseline gingival crevice fluid samples. Further studies indicate a similarity in gingival crevice fluid protein concentration (32) or elastase levels (33,34) in sites with various degrees of disease in the same periodontitis patient. A recent study comparing gingival crevice fluid biomarkers from periodontally diseased and healthy subjects also indicated that healty sites in diseased subjects showed higher levels of biomarkes than healthy sites in healthy subjects (35).

Subjects were not matched for age because patients with manifest chronic periodontitis tend to be older than average periodontally healthy adults. A cross-sectional study comparing periodontally healthy and diseased subjects of the same age range also found significantly higher MRP8/14 levels in diseased than healthy subjects (27). A study comparing younger with older periodontally healthy adults found a difference with regard to prostaglandin E_2 but not to migration inhibitory factor (36). With regard to smoking (six of the diseased but none of the healthy subjects were smokers), one could argue that if smoking reduced inflammation, the difference between PP and HH would have been even larger if none of the patients smoked. Owing to the low numbers of smokers, the influence of smoking could not be tested statistically.

The present study also measured the levels of MRP8/14 longitudinally over 6 mo in subjects with chronic periodontitis. We confirmed clinical improvements after scaling and root planing, as anticipated, and documented a significant decrease of MRP8/14 levels in diseased sites both at 3 and at 6 mo after treatment. This is consistent with our previous findings showing a decrease of MRP8/14 after non-surgical periodontal therapy (26). In the previous study, significant changes were noted even after 10 d, when non-surgical therapy was supplemented with systemic antibiotics. The improvements of biochemical parameters obtained in the present trial also compare favourably with previous reports on the effect of periodontal treatment on other gingival crevice fluid components. These studies usually evaluated the short-term effect of scaling and root planing on the level of gingival crevice fluid components, which in most cases declined significantly in parallel with the clinically visible resolution of inflammation at the same sites (5,29,34,37–40). In one study by our research group, clinical improvement after scaling and root planing was associated with highly significant reductions in elastase and interleukin-8 levels (41). However, these biomarkers could not predict the individual clinical response to treatment. Interestingly, in the present study a similar decrease was observed even in the PH sites, which were not subjected to scaling and root planing. This may further reinforce the notion that calprotectin in gingival crevice fluid reflects an improvement of health on the level of the subject rather than merely a local reaction at a treated site. Our observation is consistent with findings of similar kinetics, in sites with or without bone loss, of gingival crevice fluid levels of prostaglandin E_2 and interleukin-1 (42), suggesting that the entire dentition is affected by periodontal disease, rather than only a few sites. They are also in line with previous findings indicating that interactions between the microbiota and the host must be considered on the subject level (43). The influence of periodontal therapy in chronic periodontitis patients on MRP8/14 levels in gingival crevice fluid in uninstrumented sites has so far not been shown.

In conclusion, our data indicate that MRP8/14 does not differentiate between clinically diseased and healthy sites in patients with chronic periodontitis. However, since this marker was elevated in diseased compared with healthy subjects, and its values decreased following therapy, MRP8/14 may be used to monitor the response to treatment on the subject level. To further substantiate a utility to identify sites at risk for further disease, longitudinal observations extending beyond 6 mo will be required.

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