J Clin Periodontol 2014; 41: 348–356 doi: 10.1111/jcpe.12223

Journal of Clinical Periodontology

Matrix metalloproteinases and myeloperoxidase in gingival crevicular fluid provide site-specific diagnostic value for chronic periodontitis

Jussi M. Leppilahti[1], Patricia A. Hernández-Ríos[2], Jorge A. Gamonal[2,3], Taina Tervahartiala[1,6], Romina Brignardello-Petersen[4,5], Paivi Mantyla[1], Timo Sorsa[1,6] and Marcela Hernández[3,4]

[1]Institute of Dentistry, University of Helsinki, Helsinki, Finland; [2]Department of Conservative Dentistry, Faculty of Dentistry, University of Chile, Santiago, Chile; [3]Laboratory of Periodontal Biology, Faculty of Dentistry, University of Chile, Santiago, Chile; [4]Department of Pathology, Faculty of Dentistry, University of Chile, Santiago, Chile; [5]Institute of Health Policy, Management and Evaluation, University of Toronto, Toronto, Canada; [6]Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital, Helsinki, Finland

Leppilahti JM, Hernández-Ríos PA, Gamonal JA, Tervahartiala T, Brignardello-Petersen R, Mantyla P, Sorsa T, Hernández M. Matrix metalloproteinases and myeloperoxidase in gingival crevicular fluid provide site-specific diagnostic value for chronic periodontitis. J Clin Periodontol 2014; 41: 348–356. doi: 10.1111/jcpe.12223.

Abstract

Aim: To identify the diagnostic accuracy of gingival crevicular fluid (GCF) candidate biomarkers to discriminate periodontitis from the inflamed and healthy sites, and to compare the performance of two independent matrix metalloproteinase (MMP)-8 immunoassays.

Materials and Methods: *Cross sectional study*. GCF ($N = 58$ sites) was collected from healthy, gingivitis and chronic periodontitis volunteers and analysed for levels of azurocidin, chemokine ligand 5, MPO, TIMP-1 MMP-13 and MMP-14 by ELISA or activity assays. MMP-8 was assayed by immunofluorometric assay (IFMA) and ELISA. Statistical analysis was performed using linear mixed-effects models and Bayesian statistics in R and Stata V11.

Results: MMP-8, MPO, azurocidin and total MMP-13 and MMP-14 were higher in periodontitis compared to gingivitis and healthy sites ($p < 0.05$). A very high correlation between MPO and MMP-8 was evident in the periodontitis group ($r = 0.95$, $p < 0.0001$). MPO, azurocidin and total levels of MMP-8, MMP-13 and MMP-14 showed high diagnostic accuracy (≥ 0.90), but only MMP-8 and MPO were significantly higher in the periodontitis *versus* gingivitis sites. MMP-8 determined by IFMA correlated more strongly with periodontal status and showed higher diagnostic accuracy than ELISA.

Conclusions: MPO and collagenolytic MMPs are highly discriminatory biomarkers for site-specific diagnosis of periodontitis. The comparison of two quantitative MMP-8 methods demonstrated IFMA to be more accurate than ELISA.

Key words: biomarkers; chronic periodontitis; gingival crevicular fluid; matrix metalloproteinases; myeloperoxidase; sensitivity; specificity

Accepted for publication 21 December 2013

Conflict of interest and source of funding statement

This work was supported by grant from the National Fund for Scientific and Technological Development, Chilean Government (1120138), the Finnish Dental Society Apollonia and the Research Foundation of Helsinki University Central Hospital. The authors declare that they have no competing interests. Timo Sorsa is an inventor of US-patents 5652227, 5736341, 5866432 and 6143476.

Periodontitis is the most common bacterial infection worldwide. It results from the interaction of periodontopathogenic bacteria and host immune-inflammatory response that finally leads to the loss of the tooth supporting tissues (Houri-Haddad

et al. 2007, Bhavsar et al. 2007, Graves 2008) and enhanced susceptibility to other systemic diseases, such as cardiovascular disease, diabetes and preterm birth, among others (Pussinen et al. 2007).

Determination of the periodontal diagnosis, disease severity and treatment response is classically based on an array of clinical and radiographic assessments (Reddy et al. 1997). However, the provided information is restricted to the past periodontal disease (Armitage 2004, Offenbacher et al. 2007). On the other hand, individual variations in the inflammatory response profiles are proposed to impact the susceptibility, severity and outcome of the disease (Offenbacher et al. 2007, Kinane et al. 2011). Overall, periodontal disease assessment should ideally be based on clinical and complementary biological phenotype determinations (Page & Kornman 1997). Up to now, several components in oral fluids have been proposed as possible biomarkers for chronic periodontitis, but most of them appear to have limited usefulness, because they rather reflect inflammation than periodontal support loss (Loos & Tjoa 2005, Buduneli & Kinane 2011, Kinane et al. 2011).

Neutrophils (PMN) play a protective role in periodontal homoeostasis, but periodontal destruction has been associated with PMN hyperresponsiveness (Kinane et al. 2011). PMN chemoattractants, such as the CXC chemokine ligand (CXCL) 5, induce PMN recruitment, activation and degranulation, releasing azurocidin, matrix metalloproteinase (MMP)-8 and myeloperoxidase (MPO), among others. Because these mediators play a role in PMN-mediated periodontal tissue destruction, they might represent interesting candidate biomarkers (Buduneli & Kinane 2011, Choi et al. 2011, Lappin et al. 2011).

On the other hand, leucocyte and resident cell-derived collagenolytic MMPs, particularly MMP-8, MMP-13 and more recently MMP-14, have widely been reported to play a central role in disease pathogenesis and represent promising candidate biomarkers in oral fluids, such as saliva (Hernandez Rios et al. 2009, Gursoy et al. 2010, 2011, Hernandez et al. 2010, 2011a,b, Sorsa et al. 2010, 2011, Choi et al. 2011, Lappin et al. 2011). However, the diagnostic potential to discriminate between chronic periodontitis and periodontal inflammation needs to be assessed in a site-specific approach to identify attachment loss in specific sites. We aimed: i) to identify the diagnostic accuracy of inflammatory mediators and collagenolytic MMPs as candidate biomarkers in gingival crevicular fluid (GCF) to discriminate periodontitis from inflamed and healthy sites determined by clinical and radiographic assessment and ii) to compare the diagnostic potential of two MMP-8 immunoassays, immunofluorometric assay (IFMA) and commercial ELISA.

Methods

Patients and clinical measurements

In this cross-sectional clinical study, moderate to severe chronic periodontitis, gingivitis and healthy volunteers referred to the Center of Diagnostics and Treatment of Northern Metropolitan Health Services, Santiago, were prospectively enroled during March to December 2010 as previously described, based on their presenting symptoms and signs (Hernandez et al. 2010). Briefly, the criteria for entry were a minimum of 14 natural teeth, excluding 3rd molars and including at least 10 posterior teeth, at least five to six teeth had sites with probing depth (PD) $\geq$ 5 mm with attachment loss $\geq$3 mm and detectable bone loss in radiography (>50% of support tissues involved, according to a classification system of periodontal disease severity based on the location of the alveolar crest) and had never received previous periodontal treatment at the time of examination. The diagnosis was based on the classification of periodontal diseases (Armitage 1999). The gingivitis group included volunteers with gingival inflammation and bleeding on probing with no signs of attachment loss; and the healthy group consisted of volunteers that exhibited PD < 3 mm and no clinical attachment loss (CAL), clinical inflammation, gingival bleeding or radiographic evidence of bone loss.

All the individuals had no history of systemic disorders, such as diabetes mellitus, osteoporosis, pregnancy or lactating females and medications known to influence periodontal tissues, within the past 6 months period prior to the study. The study protocol was approved by the ethics committee of the Faculty of Dentistry, Universidad de Chile, and procedures were undertaken with the understanding and written consent of each subject and according to ethical principles, including the World Medical Association Declaration of Helsinki.

One calibrated periodontist (J. G.) monitored the patients, fulfilled the clinical reports and collected GCF samples. PD and CAL were examined at six sites in each tooth: mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual, using a North Carolina manual probe (Hu-Friedy, Chicago, IL, USA). A total of 58 GCF samples were subsequently obtained from the healthy (nine individuals, 20 sites), gingivitis (six individuals, 19 sites) and moderate to severe periodontitis patients (eight individuals, 19 sites) for the posterior immunobiochemical analyses.

GCF samples

GCF samples were consecutively collected with paper strips (ProFlow, Amityville, NY, USA), placed into the pocket until mild resistance was sensed, and left in place for 30 s as previously reported (Hernandez Rios et al. 2009). GCF was extracted from the strips by centrifugation at 18,000 *g* for 5 min. at 4°C in 80 μl of elution buffer containing 50 mM Tris-HCl pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$ and 0.01% Tritón X-100. Immunoassays were performed and read blindly by a trained research associate (T. T.).

Levels of azurocidin, CXCL5, MPO, MMP-8 and TIMP-1

The levels of azurocidin, CXCL5, MPO, MMP-8 and TIMP-1 were determined by the following commercial ELISA kits, according to manufacturer's recommendations: azurocidin (Cusabio Biotech Co. Ltd, Wuhan, China), CXCL5 (R&D Systems, Minneapolis, MN, USA), MPO (Immundiagnostik, AG, Bensheim, Germany) and TIMP-1 (Biotrak ELISA system, GE Healthcare, Amersham, Slough, Berkshire, UK).

Levels were obtained from a standard curve and expressed per ml of eluted GCF. MMP-8 levels were measured by a time-resolved IFMA, as previously described (Hanemaaijer et al. 1997), and also with a commercial Biotrak ELISA system (GE Healthcare, Amersham).

MMP-14 activity assay

MMP-14 was measured by using an MMP-14 Biotrak activity assay system (GE Healthcare, Amersham), following manufacturer's recommendations as described previously (Hernandez et al. 2010). The concentrations of active MMP-14 in the samples were determined by interpolation from a standard curve and expressed as ng/ml of eluted GCF. Total enzyme and endogenous levels of free active MMP-14 in the samples were detected with and without adding aminophenylmercuric acetate (APMA) (Sigma, St. Louis, MO, USA), respectively.

MMP-13 activity assay

Aliquots of GCF samples were assayed by the "Fluorokine E" activity fluorescent assay (R&D Systems, Inc. Minneapolis, USA), according to the manufacturer's recommendations, as described previously (Hernandez et al. 2006). Total and endogenous enzyme activity levels were measured with and without adding APMA, respectively, and expressed as ng of fluorescent product per ml of eluted GCF.

Statistical analyses

Comparison of the demographic and clinical parameters between study groups was performed using unpaired *t*-test and chi-squared test. The nested nature of the data was considered when appropriate.

To determine whether there were differences in the biomarkers' levels among healthy, gingivitis and periodontitis groups, generalized mixed regression models were used with the level of the marker of interest as the outcome, the health status as a fixed effect and patient as a random effect. It was assumed that the mean concentration of the markers was normally distributed in each group. When it was not the case, the log of the markers was taken for modelling purposes and after the model was fitted, the marker concentration was converted back to its original unit. The analyses were done using the packages "nlme" and "lmerTest" in R (http://www.R-project.org/). An ANOVA F test and a post hoc *t*-test with a Bonferroni correction for an alpha level of 0.05 were used for comparing individuals belonging to the healthy, gingivitis and periodontitis groups among each other.

To assess the discrimination properties of the biomarkers, Bayesian analyses in which the clustering of the data was accounted for by treating the patients as random effects were done. Patients were grouped in healthy and gingivitis *versus* periodontitis groups for all analyses, except for the analysis of MPO and MMP-8 measured by ELISA, in which data from gingivitis and periodontitis groups was compared because of the low detection frequencies observed for the healthy group. The accuracy of each marker was evaluated constructing a receiver operating characteristic (ROC) curve and calculating the area under the curve (AUC). Sensitivity and specificity were calculated for each of the points within the range of the marker value. Positive and negative predictive values were calculated using the prevalence of sites with periodontitis of the sample (32% when the full sample was considered, 51% when only patients with gingivitis and periodontitis were considered). The cut-off level for each biomarker was defined as the values for which sensitivity and specificity were as equal as possible, assuming that false positives or false negatives were equally important. All these analyses were done using the software JAGS, run using R. Three MCMC chains were used, and the burn-in phase was extended until convergence was met, as determined by the Gelman–Rubin statistic. The posterior distributions were calculated from a total of 10,000 samples.

The correlations between the markers and with health status were calculated using Spearman's correlation.

Results

Demographic data and clinical parameters of the study participants are presented in Table 1. No differences were found regarding age, gender and smoking status among healthy, gingivitis and chronic periodontitis groups. PD and CAL were higher for periodontitis group ($p < 0.05$).

The frequency of detection of the biomarkers in GCF from healthy, gingivitis and chronic periodontitis sites is presented in Table 2. Most of the markers were detected in the majority of the samples, except for MPO and MMP-8 determined by ELISA, which were seldom detected in the healthy group. TIMP-1 on the other hand, was undetected in most healthy and gingivitis samples. Based on their detection frequencies, ELISA determinations of MMP-8 and MPO were compared only between gingivitis and periodontitis groups, whereas quantitative analysis of TIMP-1 between study groups was not performed.

The quantitative analysis of GCF biomarkers (Fig. 1) showed a tendency for most biomarkers to increase progressively from healthy and gingivitis controls to chronic periodontitis. Statistically significantly elevated levels of MPO ($p = 0.0001$), and MMP-8 measured by both IFMA, and ELISA ($p < 0.0001$), were found

Table 1. Demographic and clinical parameters of study individuals

Paremeter	Healthy $n = 9$	Gingivitis $n = 6$	Periodontitis $n = 8$	p
Age (years)	48.2 ± 11.3	35.7 ± 15.4	46.0 ± 5	0.32
Females	3	5	4	0.22
Non-smokers	5	3	3	0.51
PD (mm)	1.68 ± 0.57	1.82 ± 0.31	3.67 ± 1.55*	**0.001**
CAL (mm)	0.61 ± 0.28	0.66 ± 0.32	2.85 ± 0.82*	**< 0.0001**

Results expressed as means ± SD or frequencies. Probing depth and clinical attachment loss Periodontitis *versus* gingivitis and healthy *$p < 0.05$ (Chi-squared or ANOVA and Bonferroni post hoc test). Bold text highlights statistically significant p values.
CAL, clinical attachment loss; PD, probing depth.

Table 2. Frequencies of positive site-specific GCF biomarker detection

Biomarker	Sites			*p**
	Healthy $n = 20$	Gingivitis $n = 19$	Periodontitis $n = 19$	
Azurocidin	13 (65%)	18 (94.7%)	19 (100%)	**0.003**
CXCL5	18 (90%)	17 (89.5%)	18 (94.7%)	0.241
MPO	6 (30%)	17 (89.5%)	19 (100%)	**< 0.0001**
MMP-8 IFMA	20 (100%)	19 (100%)	19 (100%)	–
MMP-8 ELISA	7 (35%)	18 (94.7%)	19 (100%)	**< 0.0001**
MMP-13	20 (100%)	18 (94.7%)	19 (100%)	0.361
MMP14	20 (100%)	18 (94.7%)	19 (100%)	0.361
TIMP-1	2 (10%)	2 (10.5%)	13 (68.4%)	**< 0.0001**

*chi-squared test.
Values are expressed as absolute and relative frequencies (%).
Bold text highlights statistically significant *p* values.
CXCL5, chemokine ligand 5; GCF, gingival crevicular fluid; IFMA, immunofluorometric assay; MMP, matrix metalloproteinase; MPO, myeloperoxidase.

in periodontitis in comparison to gingivitis and healthy sites. Azurocidin and total MMP-14 levels were significantly higher in periodontitis than in healthy sites ($p = 0.003$ and 0.048, respectively), whereas total MMP-13 was significantly higher in periodontitis compared to gingivitis sites ($p = 0.0006$). Finally, no statistically significant differences were found for CXCL5 level and MMP-14 activity between the groups, and MMP-13 activity was found to be elevated in the healthy sites when compared to gingivitis ones ($p = 0.024$).

Although both methods, IFMA and ELISA, represent quantitative immunoassays for MMP-8, IFMA was more sensitive. IFMA detected MMP-8 in all the study samples, whereas ELISA failed to detect MMP-8 in most healthy sites (Table 2). The correlation analysis between MMP-8 levels and each diagnostic category resulted in strong and moderate Spearman's correlation coefficients for IFMA and ELISA, respectively (0.74 for IFMA and 0.68 for ELISA, data not shown).

Spearman's correlation analysis between all biomarkers was performed and those showing significant associations ($p < 0.05$) within each study

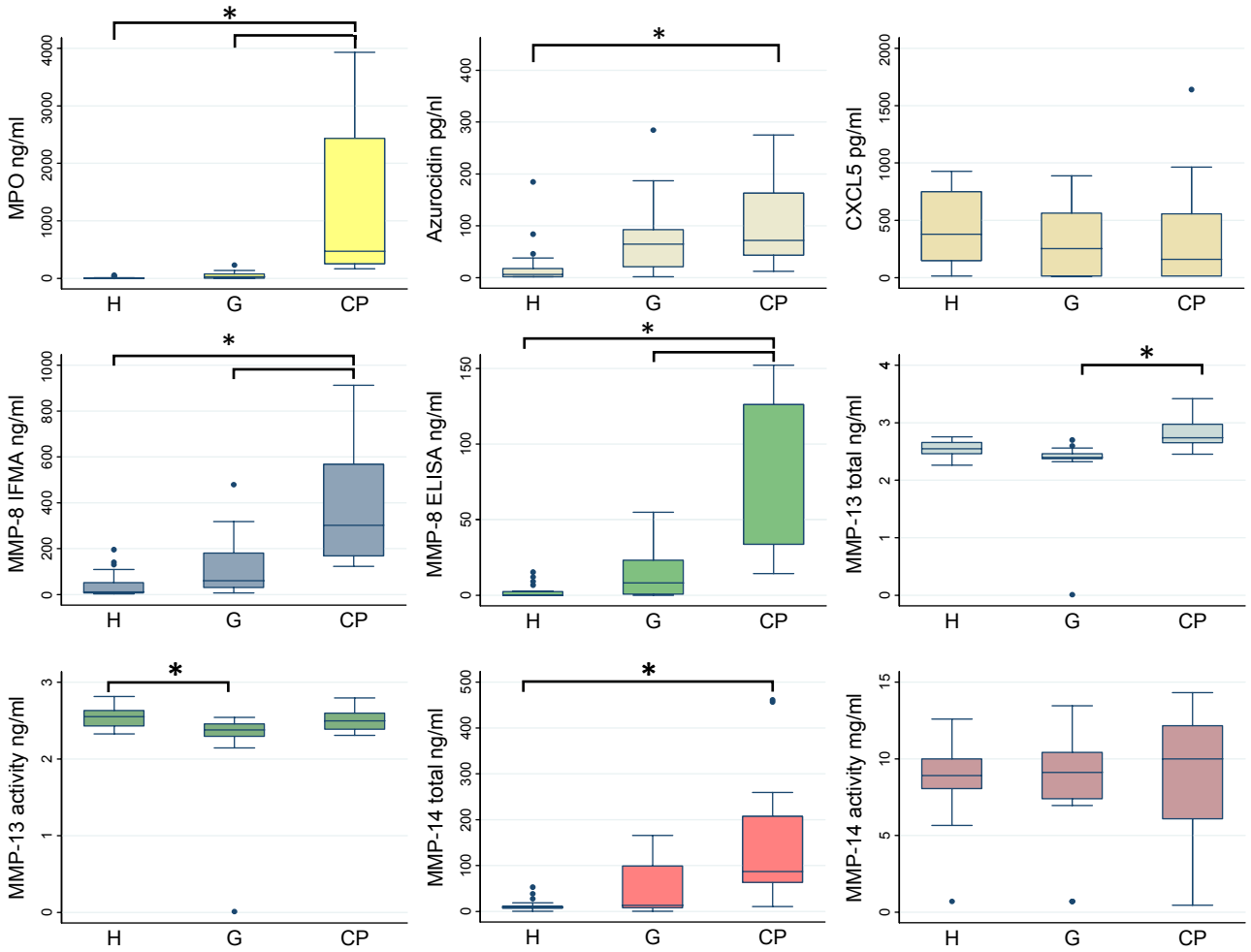

Fig. 1. Gingival crevicular fluid biomarker levels in healthy (H), gingivitis (G) and periodontitis sites (CP). *Global $p < 0.05$ and $p < 0.016$ for pairwise comparisons.

group are shown in Table 3. In the healthy sites, a moderate positive correlation was found between azurocidin and MMP-8 measured by IFMA ($\rho = 0.69$). In the sites with gingivitis, moderate positive correlations were found for active MMP-13 with CXCL5 and azurocidin ($\rho = 0.59$, both). Also strong positive correlations were seen for total MMP-14 and MMP-8 measured with ELISA ($\rho = 0.74$), and active MMP-14 with azurocidin ($\rho = 0.74$), whereas a moderate positive correlation was found between active MMP-14 and total MMP-13 ($\rho = 0.64$). Moderate to strong positive correlations were found between collagenases in periodontitis group. Both, active and total MMP-14 associated with total MMP-13 ($\rho = 0.71$ and $\rho = 0.56$, respectively), as well as MMP-8, measured by ELISA ($\rho = 0.60$ and $\rho = 0.67$, respectively). In turn, both total and active MMP-13, correlated with MMP-8 measured by IFMA ($\rho = 0.55$ and $\rho = 0.59$) and ELISA ($\rho = 0.66$). Noteworthy, a very strong positive correlation was identified between MPO and MMP-8 determined with IFMA ($\rho = 0.95$) and a strong positive correlation with ELISA ($\rho = 0.86$, $p < 0.0001$). No statistically significant correlation was found for TIMP-1 (not shown).

The diagnostic performance of GCF biomarkers is illustrated with ROC curves (Fig. 2). Most of the individual GCF biomarkers discriminated between chronic periodontitis *versus* gingivitis and healthy sites with a high accuracy (AUC $\geq$ 0.90, Table 4), except for CXCL5 [AUC = 0.49, 95% confidence interval (CI) 0.0001–0.999], MMP-13 activity (AUC = 0.70, 95% CI 0.293–0.973) and MMP-14 activity

Table 3. Correlation matrix between biomarkers in GCF

(A) Healthy and gingivitis groups

Periodontal status	Healthy ($n = 10$)	Gingivitis ($n = 12$)		
Marker	Azu	MMP13A	MMP14T	MMP14A
MPO	–	−0.36	0.35	0.21
Azu	1.00	0.59*	−0.28	0.74*
CXCL5	−0.04	0.59*	0.08	−0.15
MMP8I	0.69*	−0.38	0.29	0.04
MMP8E	–	0.18	0.74*	0.25
MMP13T	−0.24	0.41	0.35	0.64*
MMP13A	−0.42	1.00	−0.17	0.28
MMP14T	0.45	−0.17	1.00	0.19
MMP14A	0.02	0.28	0.19	1.00

(B) Periodontitis group

	Periodontitis ($n = 13$)								
Marker	MPO	Azu	CXCL5	MMP8I	MMP8E	MMP13T	MMP13A	MMP14T	MMP14A
MPO	1.00	–	–	–	–	–	–	–	–
Azu	0.41	1.00	–	–	–	–	–	–	–
CXCL5	0.04	0.13	1.00	–	–	–	–	–	–
MMP8I	0.95*	0.37	−0.01	1.000	–	–	–	–	–
MMP8E	0.86*	0.40	0.02	0.91*	1.00	–	–	–	–
MMP13T	0.51	0.32	0.42	0.55*	0.66*	1.00	–	–	–
MMP13A	0.40	0.13	0.17	0.59*	0.52	0.50	1.000	–	–
MMP14T	0.45	−0.03	−0.05	0.48	0.67*	0.56*	0.39	1.00	–
MMP14A	0.20	0.07	0.30	0.37	0.60*	0.71*	0.49	0.61*	1.00

*$p < 0.05$.
Values expressed as coefficient of correlation (ρ).
A, active; Azu, Azurocidin; CXCL5, chemokine ligand 5; GCF, gingival crevicular fluid; MMP, matrix metalloproteinase; MPO, myeloperoxidase; T, total.

Table 4. Diagnostic value of GCF biomarkers for chronic periodontitis

Marker	Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy (AUC, CI)
MPO (ng/ml)	2520.6	0.96	0.95	0.96	0.96	0.98 (0.778–1)
Azurocidin (pg/ml)	299.3	0.87	0.84	0.73	0.94	0.90 (CI 0.709–0.995)
MMP-8 IFMA (ng/ml)	754.1	0.95	0.94	0.90	0.98	0.97 (CI 0.851–0.999)
MMP-8 ELISA (ng/ml)	135.9	0.89	0.87	0.88	0.90	0.90 (CI 0.539–1)
MMP-13 Total (ng/ml)	2.69	0.90	0.86	0.78	0.95	0.94 (CI 0.724–1)
MMP-14 Total (ng/ml)	357.8	0.94	0.89	0.82	0.97	0.95 (CI 0.779–1)

AUC, area under the curve; CI, confidence interval; GCF, gingival crevicular fluid; IFMA, immunofluorometric assay; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NPV, negative predictive value; PPV, positive predictive value.

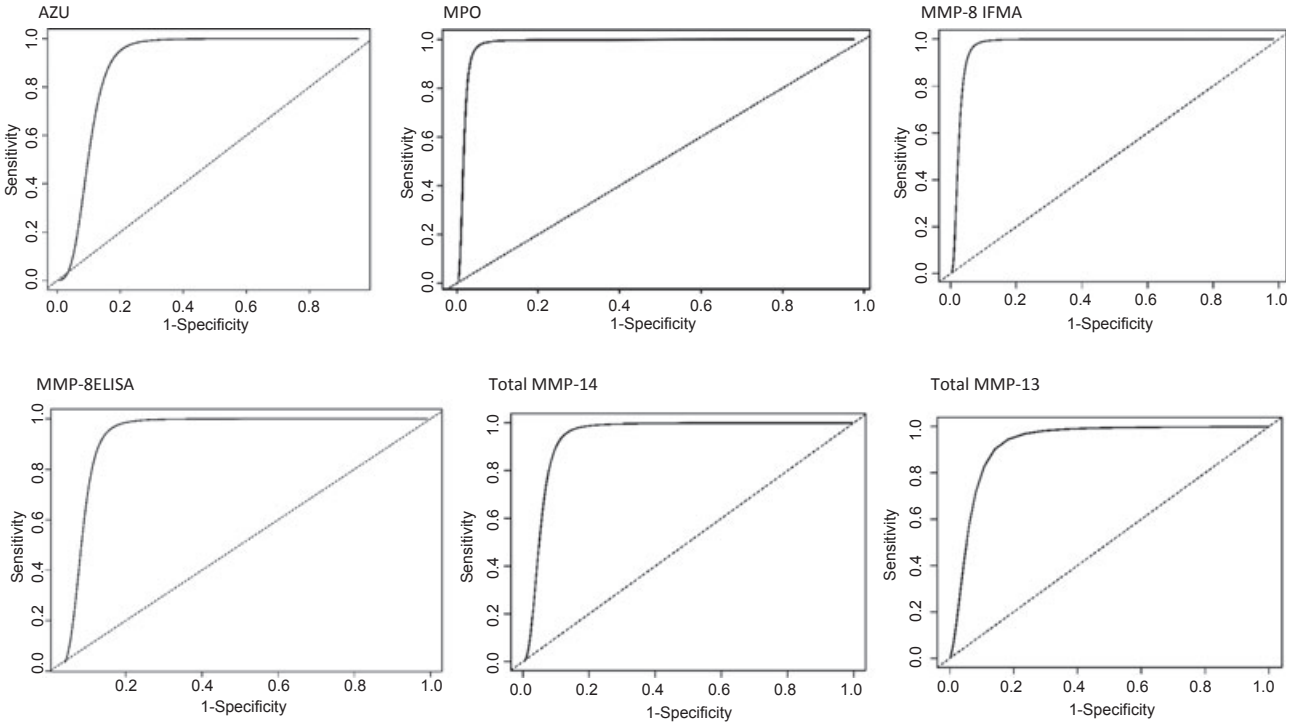

Fig. 2. ROC curves for biomarker discrimination of chronic periodontitis-affected sites in gingival crevicular fluid. ROC-curve analyses are shown in Table 4.

(AUC = 0.53, 95% CI 0.0012–0.999; data not shown). MPO was the marker with the highest AUC, followed by MMP-8 measured by IFMA, total MMP-14, total MMP-13 and azurocidin [AUC and 95% CI 0.98 (0.778–1), 0.97 (0.851–0.999), 0.95 (0.779–1), 0.94 (0.724–1) and 0.90 (0.709–0.995), respectively]. The accuracy of MMP-8 levels measured by ELISA was lower than IFMA's (AUC = 0.90, 95% CI 0.539–1).

The highest diagnostic performances for GCF markers to discriminate sites with chronic periodontitis from gingivitis and healthy ones, according to the chosen cut-off values corresponded to MPO with a sensitivity of 0.96, specificity of 0.95, positive predictive value of 0.96, and negative predictive value of 0.96, followed by MMP-8 determined by IFMA with a sensitivity of 0.95, specificity of 0.94, positive predictive value of 0.90, and negative predictive value of 0.98.

Discussion

The need for biomarker assessment is reinforced by the developing concept that variations in patients' inflammatory profiles underlying a similar clinical phenotype can impact disease susceptibility, severity and outcome (Offenbacher et al. 2007, Kinane et al. 2011). In this study, we identified high diagnostic accuracy biomarkers of inflammation and connective tissue degradation in GCF for site-specific diagnosis of chronic periodontitis. In addition, the comparison of two laboratory methods to quantitatively assess MMP-8 demonstrated IFMA to be more sensitive and accurate than ELISA.

Elevated collagenolytic MMPs and MPO in GCF have previously been associated with periodontitis severity and treatment response, however, basal physiological levels are necessary for the maintenance of periodontal tissue homoeostasis (Tervahartiala et al. 2000, Mantyla et al. 2006, Hernandez Rios et al. 2009, Kuula et al. 2009, Marcaccini et al. 2010, Hernandez et al. 2010, Reinhardt et al. 2010, Sorsa et al. 2010). Up to now, systematic reviews and consensus reports of putative biomarkers for periodontitis conclude that they rather reflect inflammation than periodontal disease and currently no single biomarker can disclose periodontal tissue destruction adequately (Loos & Tjoa 2005, Buduneli & Kinane 2011, Kinane et al. 2011). Although most previous studies analyse markers' levels in periodontitis and healthy groups, only few of them incorporate gingivitis controls to evaluate the influence of inflammation without the occurrence of attachment loss on analyte levels (Mantyla et al. 2003, Bostanci et al. 2007a,b, Rai et al. 2008, Xu et al. 2008). As chronic periodontitis, gingivitis encompasses inflammation and bacterial challenge, but differs in the occurrence of periodontal attachment and bone loss (Armitage 1999). Recently, it has been proposed that gingivitis might even represent a resistant disease phenotype (Garlet et al. 2012). Herein, we report that GCF levels of MPO, MMP-8, and total MMP-13 to some extent, discriminate between both inflammatory conditions in periodontal sites, whereas azurocidin and total MMP-14 levels might be more influenced by gingival inflammation. In addition, Receptor activator of NF-kB

ligand and osteoprotegerin also demonstrated different levels and expression patterns in periodontal diseases in comparison to gingivitis and healthy periodontium (Bostanci et al. 2007a,b), suggesting that bone resorption and matrix degradative enzymes represent interest candidate biomarkers to identify periodontitis sites. Although we did not evaluate the effect of the smoking status, all study groups showed no evidence of being unbalanced regarding this or demographic parameters. These results are reinforced by the fact that the sites' level statistic analysis accounted for intra-individual and between-subject variations.

Our results demonstrated that the association profiles between the study biomarkers varied according to the periodontal health status. In healthy sites, azurodicin correlated positively with MMP-8 levels. In gingivitis sites, MMP-14 correlated with MMPs-13 and -8, evidencing an initial association among collagenases. In addition, collagenases correlated with CXCL5 and azurocidin. Noteworthy, all collagenases associated with each other in chronic periodontitis, involving active and total levels of MMP-14, MMP-13 and MMP-8. Interestingly, a highly strong positive correlation between MMP-8 and MPO, confirmed by both immunoassays, was evident in periodontitis.

MMP-8, MPO and Azurocidin are stored in PMN granules, and thus, a putative association might be explained on the basis of either their coordinated release from PMN or by a functional interaction. LIX and its human analogue CXCL5, are potent chemoattractants for neutrophils (Choi et al. 2011) and have already been described as a target substrate of MMP-8 (Tester et al. 2007, Hernandez et al. 2011b). Azurocidin on the other hand is an antibacterial protein with proinflammatory properties that might also inhibit osteoclastogenesis (Choi et al. 2011), but no functional interaction with MMPs has been previously reported.

The MMP association profiles, on the other hand, might reflect either a cooperative collagenolytic effect and/or the assembly of collagenase activation cascades taking part in the loss of periodontal tissue homoeostasis. This observation is supported by the previous finding that MMP-14 can activate MMP-8 and -13 in vitro (Knauper et al. 1996, Holopainen et al. 2003), suggesting proteolytic activation to occur in periodontitis in vivo (Hernandez Rios et al. 2009, Hernandez et al. 2010). The identification of a strong association between MPO and MMP-8 ex vivo might be explained by in vitro studies describing MPO-mediated activation of MMP-8 through hypochlorous acid (Saari et al. 1990). This proposal is reinforced by the previously reported association in GCF between MPO and both active MMP-8 isoenzymes, released from different cell sources, involving PMN and mesenchymal cells (Hernandez et al. 2010).

The fact that the association between MPO and MMP-8 became so evident in chronic periodontitis suggests that it might reflect disease severity and even attachment loss (Hernandez et al. 2011a). Furthermore, MMP-8/MPO association could reflect the persistence of MMP-8 activation and, consequently, the need for further treatment and follow-up (Hernandez et al. 2011a). Overall, gingival inflammation might potentiate the assembly of collagenolytic MMP activation cascades and the establishment of periodontitis might potentiate this mechanism through MPO-mediated MMP-8 activation. Despite some of the non-significant correlations might become evident by increasing the sample size, the present results reveal the most striking enzyme interactions associating with periodontal status.

GCF diagnostics in periodontal disease has long been debated and several host-derived mediators proposed (Loos & Tjoa 2005, Buduneli & Kinane 2011). Nevertheless, the diagnostic value analyses and the inclusion of adequate controls to evaluate the impact of gingival inflammation will contribute to define their usefulness for the development of point of care tests with impact on periodontal care (Gursoy et al. 2010, Garlet et al. 2012, Ebersole et al. 2013).

This is the first study analysing the diagnostic value in GCF of an array of biomarkers involved in inflammation and periodontal tissue loss, namely MPO, Azurocidin, CXCL5, MMP-8, MMP-13 and MMP-14. In line with the previous results, the selection of the immunoassay and respective antibody influences both, the detection frequencies and levels, as a result of their different affinities and sensitivities (Hernandez Rios et al. 2009, Gursoy et al. 2010, Hernandez et al. 2010, Sorsa et al. 2010, Choi et al. 2011, Leppilahti et al. 2013). Overall, the accuracy of the tested biomarkers in this study was high, ranging from 0.90 to 0.98, except for CXCL5. Among these markers, MPO demonstrated the highest accuracy followed by MMP-8 measured by IFMA, total MMP-13, MMP-14, MMP-8 by ELISA and azurocidin. Noteworthy, our site-specific statistical approach accounted for the nested nature of patient's sites. Despite their presence is not specific for periodontitis, at a given cut-off, MPO and MMP-8 were able to discriminate not only between healthy, but also gingivitis sites from periodontitis ones with very high sensitivities, specificities, positive and negative predictive values. Altogether, these results support specially the usefulness of MPO and MMP-8, showing promise in side-GCF diagnostics. In addition, baseline levels of MMP-8 have been reported to predict treatment response in smokers (Leppilahti et al. 2013) and repeatedly elevated GCF MMP-8 levels indicate the sites at risk of periodontal attachment loss (Mantyla et al. 2006). Still, the GCF collection, elution and quantification methods need further standardization to define universal reference ranges.

In summary, MPO and collagenolytic MMPs represent highly discriminatory biomarkers for site-specific diagnosis of periodontitis. Particularly, MPO and MMP-8 discriminate between periodontitis and gingivitis. Furthermore, MPO and MMP-8 demonstrated a strong positive correlation in periodontitis sites. The comparison of two quantitative MMP-8 methods demonstrated IFMA to be more accurate than ELISA. These results support the applicability of these markers for point-of-care diagnostics, whereas their contribution for disease susceptibility, predictive, prognostic and therapeutic aims needs to be explored.

Acknowledgements

The authors are grateful to Leslie Henríquez, from Laboratory of

Periodontal Biology, Faculty of Dentistry, Universidad de Chile for her valuable technical assistance.

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Address:
Marcela Hernández Ríos
Faculty of Dentistry – University of Chile
Avenida Sergio Livingstone 943
Comuna de Independencia, Santiago – Chile
E-mail: mhernandezrios@gmail.com

Clinical Relevance

Scientific rationale for the study: Periodontal disease assessment should ideally be based on clinical and biological determinations, but currently no single biomarker can adequately differentiate periodontal tissue destruction from gingival inflammation. We evaluated the diagnostic accuracy of different hosts' analytes in GCF.
Principal findings: GCF MPO and collagenolytic MMPs were highly discriminatory biomarkers for site-specific diagnosis of periodontitis. MPO and MMP-8 discriminated between periodontitis and gingivitis. The comparison of two quantitative MMP-8 immunoassays demonstrated IFMA to be more accurate than ELISA.
Practical implications: These results support the applicability of these markers for the complementary diagnostic purposes in clinical practice.