Diagnostic accuracy for apical and chronic periodontitis biomarkers in gingival crevicular fluid: an exploratory study

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Abstract
Aim: The aim of this study was to assess the levels and diagnostic accuracy of a set of potential biomarkers of periodontal tissue metabolism in gingival crevicular fluid (GCF) from patients with chronic periodontitis (CP) and asymptomatic apical periodontitis (AAP).
Materials and Methods: Thirty one GCF samples from 11 CP patients, 44 GCF samples from 38 AAP patients and 31 GCF samples from 13 healthy volunteers were obtained ($N = 106$). Matrix metalloproteinases (MMPs) -2 and -9 were determined by zymography; levels of MMP-8 by ELISA and IFMA and MPO by ELISA. IL-1, IL-6, TNFα, DKK-1, Osteonectin, Periostin, TRAP-5 and OPG were determined by a multiplex quantitative panel. Statistical analysis was performed using linear mixed-effects models.
Results: The MMP-9 and MMP-8 were higher in CP, followed by AAP, *versus* healthy individuals ($p < 0.05$). ProMMP-2, MPO, IL-1, IL-6, PTN, TRAP-5 and OPG were significantly higher in CP when compared with AAP and healthy patients ($p < 0.05$). The highest diagnostic accuracies were observed for ProMMP-2, ProMMP-9, MMP-8 and TRAP-5 (AUC > 0.97) in CP, and for the active form of MMP-9 and MMP-8 (AUC > 0.90) in AAP.
Conclusion: Gingival crevicular fluid composition is modified by CP and AAP. MMP-9 and MMP-8 show diagnostic potential for CP and AAP, whereas MMP-2 and TRAP-5 are useful only for CP.

Chronic periodontitis (CP) and asymptomatic apical periodontitis (AAP) are the most common forms of chronic inflammatory diseases involving alveolar bone loss that affect the marginal and apical peri- odontium respectively (Takahashi 1998, Gamonal et al. 2010). If left untreated, both can lead to tooth

loss and eventually to systemic consequences, such as cardiovascular diseases (Paraskevas et al. 2008, Cotti et al. 2011). Both pathologies originate as a manifestation of a localized tissue injury with well-defined signs of chronic inflammation against a conspicuously dominant Gram (−) anaerobic biofilm.

Key pro-inflammatory bone-resorptive cytokines include interleukin 1 (IL-1), interleukin 6 (IL-6) and tumour necrosis factor (TNF)-α, which in turn, induce the expression of collagen/gelatin-degrading enzymes, such as matrix metalloproteinases (MMPs) -8, -9 and -2. Myeloperoxidase (MPO) is a microbicidal enzyme stored in primary granules from neutrophils and is also proposed to activate MMPs. Bone catabolism will result from the imbalance between bone protective factors, such as osteoprotegerin (OPG) and pro-resorptive factors, such as dickkopf-related protein 1 (Dkk-1), periostin (PTN) and tartrate-resistant acid phosphatase-5 (TRAP-5) and the release of other non-collagen matrix glycoproteins, such as osteonectin (ON). Accordingly, these mediators and products of periodontal tissue metabolism could reflect the health and disease state of periodontal tissues, and therefore, might contribute as an adjunctive diagnostic tool (Mose et al. 2003, Hernandez et al. 2006, 2011, Balli et al. 2014, Napimoga et al. 2014, Lim et al. 2015).

Up to now the diagnosis of CP and AAP is based on a clinical-radiographic evaluation, however, this reflects the accumulated damage from previous episodes of periodontal tissue destruction (Penesis et al. 2008, de Paula-Silva et al. 2009). Moreover, the clinical presentation does not reflect the underlying inflammatory response. In accordance, differences in disease severity involve qualitatively and quantitatively different inflammatory responses (Offenbacher et al. 2008). Gingival crevicular fluid (GCF) is a plasmatic extravasate obtained non-invasively from the gingival sulcus/pocket that reflects periodontal destructive processes, demonstrating a high diagnostic potential as a source of factors related to metabolic activity in marginal periodontal diseases, whereas it remains mostly unexplored in apical periodontitis (Belmar et al. 2008, Burgener et al. 2010, Buduneli & Kinane 2011, Dezerega et al. 2012, Leppilahti et al. 2014). Furthermore, the comparative extent in which CP and AAP can modify GCF is unknown. Bacterial products from the endodontic biofilm and/or inflammatory mediators might leak from the periapex through the periodontal ligament and local blood vessels and subsequently reach the crevice and even the general circulation eliciting a moderate inflammatory response (Burgener et al. 2010, Gomes et al. 2013). Nowadays, the challenge focuses on the necessity of designing innovative non-invasive chair-side point-of-care diagnostic methods, accounting for the biological profile as a complement to the existing clinical–radiographic assessment to contribute to the early detection of the disease, assess its severity, evaluate treatment outcome, as well as to identify sites at risk of further progression (Buduneli & Kinane 2011).

We propose that GCF reflects marginal and apical periodontal status; and that the levels of periodontal tissue metabolism markers have diagnostic potential for marginal and apical chronic periodontitis. The aim of this study was to assess and compare the levels and diagnostic accuracy of a set of potential GCF biomarkers of periodontal tissue metabolism, including MMP-2, MMP-9, MMP-8, MPO, IL-1, IL-6, TNFα, Dkk-1, ON, PTN, TRAP-5 and OPG, in GCF of patients with CP, AAP and healthy controls.

Materials and Methods

Patients and clinical–radiographic measurements

In this cross-sectional study individuals consulting at the clinics of Diagnostics, Periodontics and Endodontics from the Faculty of Dentistry, University of Chile Chile were examined between 2010 and 2013, and enrolled if had CP, AAP or were healthy. GCF samples were obtained for site-based analysis. Clinical and radiographic parameters were the gold standard for the diagnosis of CP and AAP. Patients with moderate to severe CP were included and defined as having at least 14 natural teeth excluding third molars, five or more sites with probing depth ≥5 mm (Van Den Steen et al. 2003), clinical attachment loss (CAL) ≥3 mm and extensive radiographic bone loss (Hernandez et al. 2007). Patients with concomitant AAP were excluded. Clinical diagnosis of AAP was defined by the presence of a radiographic apical lesion >3 mm in teeth with extensive caries and negative clinical tests of pulp sensitivity, according to previously defined criteria (Gutmann et al. 2009). Finally, healthy volunteers were included if showed a probing depth ≤3 mm in every site, bleeding on probing (BOP) <10%, and the absence of clinical diagnosis of AAP. Exclusion criteria for all subjects involved periodontal or endodontic treatment prior to clinical examination and the presence of systemic disorders or conditions, such as diabetes mellitus, osteoporosis, pregnancy, nursing and the intake of medications that influence periodontal tissues 3 months prior the beginning of the study. All the clinical exams and recordings were performed by a trained periodontist (MB) and endodontist (MG). The subjects background was recorded in a medical chart that included demographic variables, smoking habits and the assessment of periodontal clinical parameters including PD, CAL and BOP, using a calibrated periodontal probe (UNC-15, Hu-Friedy, Chicago, IL, USA). In addition, the radiographic variables bone level (BL) and apical lesion size (ALS) were, respectively, recorded in patients with CP and AAP, from conventional periapical retroalveolar radiographs. (BL) was obtained by calculating the difference between 100% and the percentage of bone loss from the cemento-enamel junction to the alveolar bone crest (Hajishengallis et al. 2011) and ALS, by calculating the area from average of the lesion's highest vertical and horizontal diameter (Dezerega et al. 2012). The study protocol was clearly explained to all the participants of this study, who signed an informed consent approved by the Ethics Committee of the Faculty of Dentistry of the University of Chile and the Ethics Committee of FONDECYT, according to the ethical standards of the Declaration of Helsinki.

GCF sampling and elution

Gingival crevicular fluid samples were obtained one week after establishing the clinical diagnosis of CP and AAP by placing paper strips (Periopaper®, ProFlow, Amityville, NY, USA) into the gingival sulcus or pocket for 30s by a trained periodontist (MB). In CP patients, GFC samples from the deepest probing depth sites were obtained from each subject. In patients with AAP, one pooled GCF sample per tooth was obtained from the whole crevice's perimeter (mesiovestibular, vestibular, distovestibular, distolingual, lingual and mesiolingual). Finally, samples from each mesiovestibular site from the first molars were obtained in healthy volunteers. Strips contaminated with blood or saliva were discarded. A total of 31 GCF samples from 11 patients with CP, 44 GCF samples from 38 patients with AAP, and 31 GCF samples from 13 healthy volunteers were obtained ($N = 106$; Fig. 1 and Table 1).

The collected fluid was subsequently eluted in a constant ratio of 80 μL buffer per strip for standardization purposes as previously reported (Hernandez et al. 2007), containing 50 mM Tris-HCl pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$ and 0.01% Triton X-100 with an EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH) and analysed.

Biomarker determinations

The gelatinolytic activity of MMP -2 and -9 were determined through gelatin zymography using 10% SDS-PAGE gels with 1 mg/ml of gelatin

Table 1. Number of sampled sites from participants satisfying the criteria for inclusion who underwent clinical and radiographic diagnosis and respective biomarkers' assays

Marker	CP	AAP	Healthy
MMP-2	31	31	31
MMP-9	31	31	31
MMP-8 ELISA	24	25	29
MMP-8 IFMA	31	26	31
MPO	26	25	29
IL-1	31	34	31
IL-6	31	34	31
TNFα	31	34	31
DKK-1	18	18	19
ON	18	18	19
PTN	18	18	19
TRAP-5	18	18	19
OPG	18	18	19

CP, chronic periodontitis; AAP, asymptomatic apical periodontitis.

(Belmar et al. 2008). A densitometry was performed through a GEL LOGIC 2200 PRO™ gel imager and the Carestream Molecular Imaging software (©CarestreamHealth, Rochester, NY, USA). Results were expressed in arbitrary densitometric units (au) per ml of eluted GCF. Activity ratio (AR) was reported as the ratio between the proforms and the total enzyme.

The MMP-8 levels were determined by ELISA (DENTOELISA MMP-8, DENTOGNOSTICS, GmbH, Jena, Germany), and time-resolved immunofluorometric method (IFMA) (Hanemaaijer et al. 1997) and MPO levels though ELISA kit (Immunodiagnostik, AG, Bensheim Germany). IL-1, IL-6, TNFα, Dkk-1, ON, PTN, TRAP-5 and OPG levels were determined through Multiplex detection panels (Millipore, St. Charles, MO, USA.), according to the manufacturer's instructions. Data were collected through a Luminex platform (Magpix, Millipore, St Charles, MO, USA), and analysed with the MILLIPLEX Analyst software (ViageneTech, Carlisle, MA, USA). Results were expressed per ml of elution.

Statistical analyses

Statistical analysis was performed considering the site as the unit of analysis and the analysis of the data was performed blindly. Group proportions were compared by the classical chi-square test, whereas mean values were compared with a one-way ANOVA and Bonferroni post hoc test at the individual's level. Since biomarkers frequencies and levels were measured at different sites within a patient, the presence of biomarkers was compared in three groups with multilevel logistic regression, whereas multilevel log-linear models were used for the biomarker levels. Bonferroni correction was used to prevent type I error inflation in multiple comparisons. The correlations between clinical variables and biomarker levels were also assessed through multilevel log-linear models in the two disease groups. The intraclass correlation coefficient (which compares the inter-individual variability to the total variability) was determined for each biomarker. The evaluation of the diagnostic accuracy of the biomarkers was performed through the construction of ROC curves, by calculating the area under the curve (AUC) and determining the optimal cut-off points to estimate the highest sensitivity and specificity altogether, as assessed by Youden's Index. Positive and negative predictive values were based on the sample prevalence of the sites (50% for CP and 59% for AAP). The AUC was determined using a Bayesian binormal parametric approach with uninformative priors. Random effects at the patient level were considered to account for the repeated measurements. Three MCMC chains were used and the burn-in phase was extended until convergence was met, as determined by the Gelman–Rubin statistic. Posterior distributions were based on a total of 10,000 iterations. Statistical analyses were performed
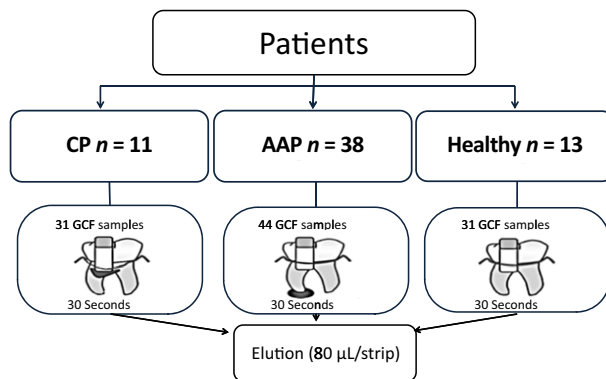

Fig. 1. Flow diagram of participants satisfying the criteria for inclusion and their respective gingival crevicular fluid samples.

on all available data. Missing data that went off the dynamic range of the assays were registered as the minimum value. Results were considered to be significant at the 5% critical level ($p < 0.05$). All calculations were performed using Jags 3.4.0 (Vienna, Austria), R 3.0.1 (Vienna, Austria) and SAS 9.2 (Cary, NC, USA) for Windows.

Results

Demographic data of the study participants are presented in Table 2. Sampled sites from chronic periodontitis patients presented higher PD, CAL and BOP than those with asymptomatic apical periodontitis and healthy individuals sites ($p < 0.0001$). Regarding radiographic variables, the sites from CP patients presented a BL (mean SD) of 43 (23) %, whereas AAP teeth presented a mean (SD) apical lesion size of 5.5 (2.3) % mm.

The frequencies of biomarker detection in GCF from CP, AAP and healthy sites are also presented in Table 2. Only ProMMP-9, MMP-8 (ELISA and IFMA), MPO and soluble TRAP-5 were identified in all the samples. Detection frequencies of proMMP-2, IL-1, IL-6 and PTN varied significantly among the study groups ($p < 0.05$), showing the highest frequencies in CP sites. No statistically significant differences were observed for TNFα, Dkk-1, ON and OPG detection rates among the study groups ($p < 0.05$).

Regarding GCF biomarker levels (Fig. 2), ProMMP-9, active MMP-9, MMP-9 activity ratio and MMP-8 levels measured by both, ELISA and IFMA, were higher in chronic periodontitis, followed by asymptomatic apical periodontitis, and were the lowest in healthy controls, with statistically significant differences among all groups ($p < 0.0001$). Significantly higher levels of ProMMP-2, MPO, IL-1$β$, IL-6, PTN, TRAP-5 and OPG were also observed in sites with chronic periodontitis in comparison to asymptomatic apical periodontitis and healthy sites ($p < 0.01$), but no differences were found between the later two groups. No statistical differences were found for TNFα, Dkk-1 and ON. The intra-class correlation coefficient (Table 3) varied between 0.26 for PTN and 0.66 for TRAP-5. Between 26% and 66% of the biomarkers

Table 2. Demographic, clinical and molecular parameters of study subjects with Chronic Periodontitis (CP), Asymptomatic Apical Periodontitis (AAP), Healthy controls and their respective sampled sites or teeth

Subjects	CP ($n = 11$)	AAP ($n = 38$)	Healthy ($n = 13$)	$p*$	CP/H	AAP/H	CP/AAP
Age (years, [mean, SD])	50.8 (13.9)	44.1 (14.1)	44.1 (13.5)	0.37	–	–	–
Females (%)	45.5	74.2	53.9	0.14	–	–	–
Smokers (%)	36.4	34.6	15.4	0.28	–	–	–
PD (mm, mean ± SD)	3.7 (0.5)	1.8 (0.3)	2.0 (0.3)	<0.0001	<0.0001	0.11	<0.0001
CAL (mm, mean ± SD)	4.3 (7)	1.6 (0.3)	1.6 (0.4)	<0.0001	<0.0001	1.00	<0.0001
BOP (%)	69.3 (11.6)	2.9 (1.5)	2.9 (1.6)	<0.0001	<0.0001	1.00	<0.0001
Sites/Clinical Parameters	CP ($n = 31$)	AAP ($n = 44$)	Healthy ($n = 31$)	$p**$	CP/H	AAP/H	CP/AAP
PD (mm, mean ± SD)	6.3 (2.0)	2.1 (0.5)	2.2 (0.4)	<0.0001	<0.0001	0.38	<0.0001
CAL (mm, mean ± SD)	7.5 (2.5)	1.8 (0.4)	1.7 (0.5)	<0.0001	<0.0001	0.47	<0.0001
BOP (%)	100	0	0D	–	–	–	–
BL (%,mean ± SD)	43 (22.6)	–	–	–	–	–	–
ALS (mm, mean ± SD)	–	5.5 (2.3)	–	–	–	–	–
Biomarker	CP	AAP	Healthy	$p***$	CP/H	AAP/H	CP/AAP
ProMMP-2	28 (90.3)	21 (67.7)	9 (29.0)	0.025	0.008	0.033	0.10
ProMMP-9	31 (100)	31 (100)	31 (100)	NA	NA	NA	NA
MMP-9a	31 (100)	31 (100)	28 (90.3)	NA	NA	NA	NA
MMP-9 AR	31 (100)	31 (100)	28 (90.3)	NA	NA	NA	NA
MMP-8 ELISA	24 (100)	25 (100)	29 (100)	NA	NA	NA	NA
MMP-8 IFMA	31 (100)	26 (100)	31 (100)	NA	NA	NA	NA
MPO	26 (100)	25 (100)	29 (100)	NA	NA	NA	NA
IL-1	28 (90.3)	12 (35.3)	27 (87.1)	0.011	0.68	0.0066	0.0064
IL-6	30 (96.8)	20 (57.1)	15 (48.4)	0.023	0.0067	0.50	0.011
TNFα	18 (58.1)	16 (47.1)	15 (48.4)	0.83	–	–	–
DKK-1	12 (66.7)	10 (55.6)	9 (47.4)	0.51	–	–	–
ON	8 (44.4)	14 (77.8)	9 (47.4)	0.28	–	–	–
PTN	13 (72.2)	4 (22.2)	5 (26.3)	0.010	0.011	0.77	0.0068
TRAP-5	18 (100)	18 (100)	19 (100)	NA	NA	NA	NA
OPG	17 (94.4)	15 (83.3)	14 (73.7)	0.32	–	–	–

PD, probing depth; CAL, clinical attachment level; BOP, bleeding on probing; BL, bone level; ALS, apical lesion size. Biomarker detections expressed as absolute frequencies (%). CP, chronic periodontitis; AAP, asymptomatic apical periodontitis; ProMMP-2, pro-form of MMP-2; ProMMP-9, pro-form of MMP-9; MMP-9a, active form of MMP-9; MMP-9 AR, activity ratio of MMP-9; MMP-8 ELISA, MMP-8 measured with enzyme-linked immunosorbent assay; MMP-8 IFMA, MMP-8 measured with immunofluorometric assay; MPO, Myeloperoxidase; IL-1, Interleukin-1; IL-6, Interleukin-6; TNFα, tumour necrosis factor alpha; DKK-1, Dickkopf-1; ON, osteonectin; PTN, periostin; TRAP-5, tartrate-resistant acid phosphatase-5; OPG, Osteoprotegerin. p *Obtained by ᴀɴᴏᴠᴀ and Bonferroni post hoc test; **Obtained by linear mixed model; ***Obtained by logistic linear mixed model; NA, not available.
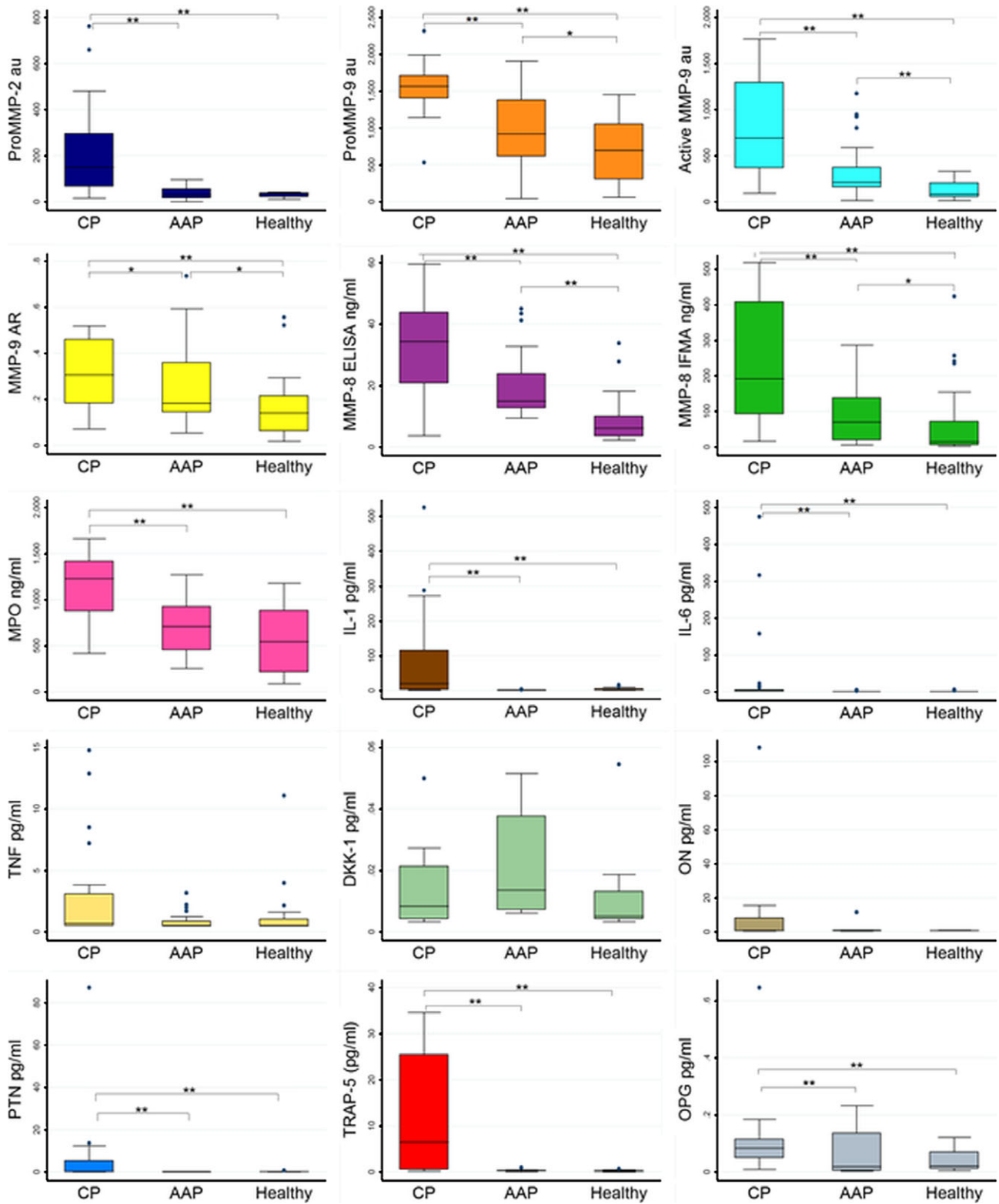
Fig. 2. Gingival crevicular fluid biomarker levels in Chronic Periodontitis (CP), Asymptomatic Apical Periodontitis (AAP) and Healthy Controls. ProMMP-2: Pro-form of MMP-2. ProMMP-9: Pro-form of MMP-9. MMP-9a: Active form of MMP-9. MMP-9 AR: Activation rate of MMP-9. MMP-8 ELISA: MMP-8 measured with enzyme-linked immunosorbent assay. MMP-8 IFMA: MMP-8 measured with immunofluorometric assay. MPO: Myeloperoxidase. IL-1: Interleukin-1. IL-6: Interleukin-6. TNFα: Tumour necrosis factor alpha. DKK-1: Dickkopf-1. ON: Osteonectin. PTN: Periostin. TRAP-5: Tartrate-resistant Acid Phosphatase -5. OPG: Osteoprotegerin. $*p < 0.05$, $**p < 0.01$ obtained with a F test in a linear mixed model.

variability was therefore attributable to variations between patients.

The linear mixed model on the logarithmic scale was used to assess correlations between markers and clinical variables (Table 4). In the CP group, a positive correlation was found between PD and the biomarkers ProMMP-2, active MMP-9, MMP-9 activation ratio, MMP-8 (measured with IFMA), MPO, IL-1 and TRAP-5 ($p < 0.05$). CAL positively correlated with the markers ProMMP-2, MMP-8 (ELISA) and TRAP-5, and negatively with PTN ($p < 0.05$). A significant negative correlation was observed between BL and proMMP-2, ON and TRAP-5 ($p < 0.01$). The asymptomatic apical periodontitis group showed no significant correlations between biomarkers and the lesion size ($p > 0.05$).

The diagnostic performance of GCF biomarkers is illustrated with ROC curves (Fig. 3A,B) and their respective values, in Table 5. The ROC curves that showed a high diagnostic accuracy for sites with CP (Fig. 3A and Table 5A), defined as AUC ≥ 0.9, corresponded to

Table 3. Biomarker intra-class correlation coefficients (ICC)

Biomarker	ICC
ProMMP-2	0.45
ProMMP-9	0.30
MMP-9a	0.42
MMP-9 AR	0.44
MMP-8 ELISA	0.42
MMP-8 IFMA	0.58
MPO	0.41
IL-1	0.45
IL-6	0.33
TNFα	0.50
DKK-1	NA
ON	0.43
PTN	0.26
TRAP-5	0.66
OPG	0.60

ProMMP-2, pro-form of MMP-2; ProMMP-9, pro-form of MMP-9; MMP-9a, active form of MMP-9; MMP-9 AR, activity ratio of MMP-9; MMP-8 ELISA, MMP-8 measured with enzyme-linked immunosorbent assay; MMP-8 IFMA, MMP-8 measured with immunofluorometric assay; MPO, myeloperoxidase; IL-1, interleukin-1; IL-6, interleukin-6; TNFα, tumour necrosis factor alpha; DKK-1, Dickkopf-1; ON, osteonectin; PTN, periostin; TRAP-5, tartrate-resistant acid phosphatase-5; OPG, osteoprotegerin; NA, not available.

Table 4. Correlation matrix between biomarkers and clinical variables in (A) chronic periodontitis and (B) asymptomatic apical periodontitis.

	Pro MMP2	Pro MMP9	MMP9a	MMP9 AR	MMP8 ELISA	MMP8 IFMA	MPO	IL-1	IL-6	TNF	DKK1	ON	PTN	TRAP5	OPG
(A)															
PD	**0.007** / 0.40	0.20	**0.011** / 0.19	**0.005** / 0.11	0.14	**0.034** / 0.15	**0.004** / 0.07	**0.0011** / 0.47	0.98	0.98	0.46	0.36	0.13	**0.008** / 0.27	0.12
CAL	**0.003** / 0.009	0.18	0.066	0.061	**0.040** / 0.060	0.95	0.97	0.088	0.86	0.61	0.55	0.58	**0.012** / −0.41	**0.046** / 0.21	0.23
BL	**0.0027** / −0.002	0.13	0.20	0.24	0.16	0.96	0.68	0.33	0.16	0.63	0.71	**0.0028** / −0.067	0.60	**0.0003** / −0.024	0.57
	Pro MMP2	Pro MMP9	MMP9a	MMP9 AR	MMP8ELISA	MMP8 IFMA	MPO	IL-1	IL-6	TNF	Dkk1	ON	PTN	TRAP5	OPG
(B)															
LS	0.40	0.92	0.32	0.37	0.76	0.66	0.62	NA	0.27	0.49	NA	NA	NA	NA	NA

Results expressed as *p*-values; bold: $p < 0.05$ and Estimate. PD, probing depth; CAL, clinical attachment level; BL, bone level; LS, lesion size; ProMMP2, pro-form of MMP-2; ProMMP9, pro-form of MMP-9; MMP9a, active form of MMP-9; MMP9 AR, activity ratio of MMP-9; MMP-8 ELISA, MMP-8 measured with enzyme-linked immunosorbent assay; MMP-8 IFMA, MMP-8 measured with immunofluorometric assay; MPO, myeloperoxidase; IL-1, interleukin-1; IL-6, interleukin-6; TNFα, tumour necrosis factor alpha; DKK1, Dickkopf-1; ON, osteonectin; PTN, periostin; TRAP5, tartrate-resistant acid phosphatase-5; OPG, osteoprotegerin; NA, not available.
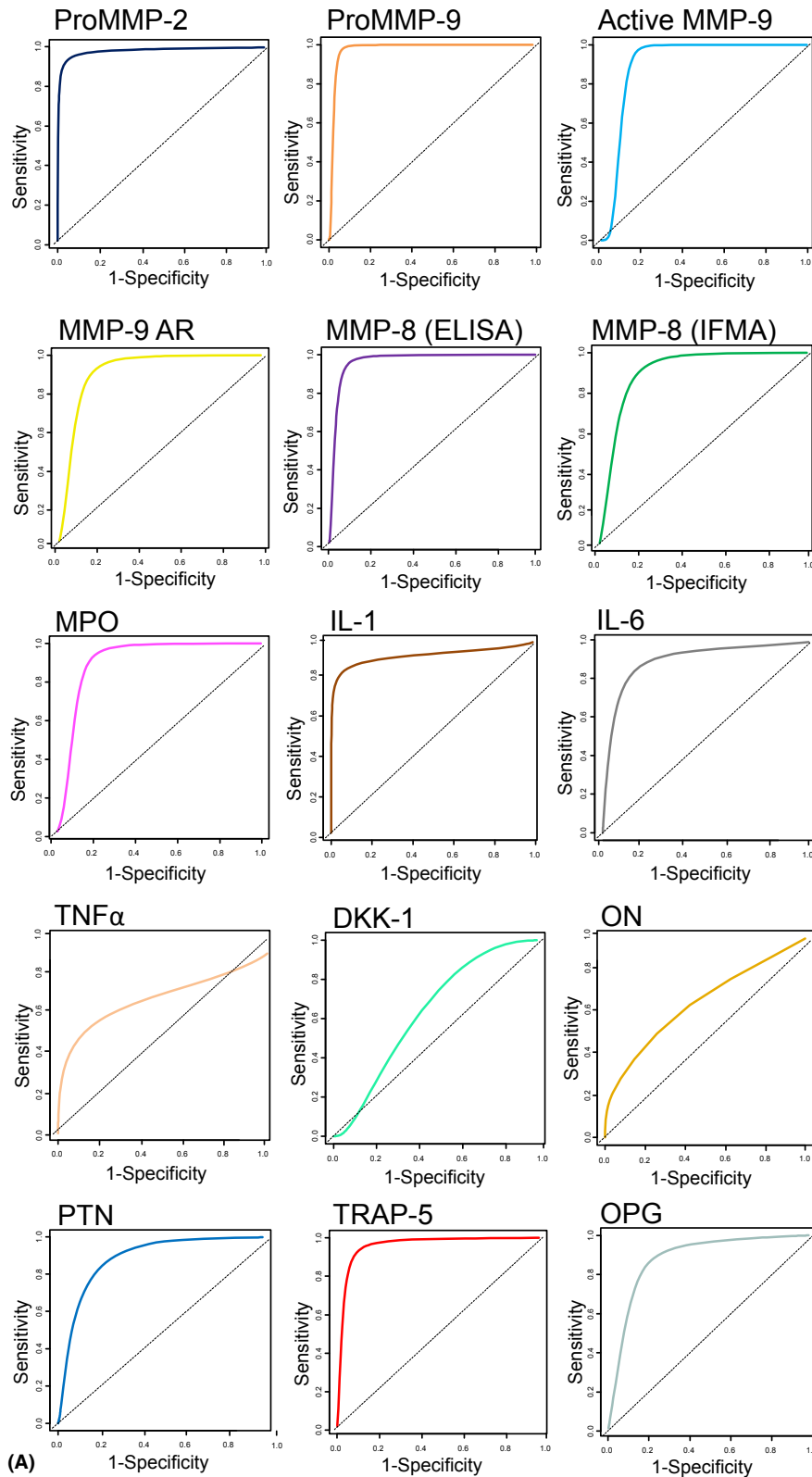
Fig. 3. The ROC Curves for the Diagnosis of (A) Chronic Periodontitis; (B) Asymptomatic Apical Periodontitis in gingival crevicular fluid. ProMMP-2: Pro-form of MMP-2. ProMMP-9: Pro-form of MMP-9. MMP-9a: Active form of MMP-9. MMP-9 AR: Activation rate of MMP-9. MMP-8 (ELISA): MMP-8 measured with enzyme-linked immunosorbent assay. MMP-8 (IFMA): MMP-8 measured with immunofluorometric assay. MPO: Myeloperoxidase. IL-1: Interleukin-1. IL-6: Interleukin-6. TNFα: Tumour necrosis factor alpha. DKK-1: Dickkopf-1. ON: Osteonectin. PTN: Periostin. TRAP-5: Tartrate-resistant Acid Phosphatase -5. OPG: Osteoprotegerin.
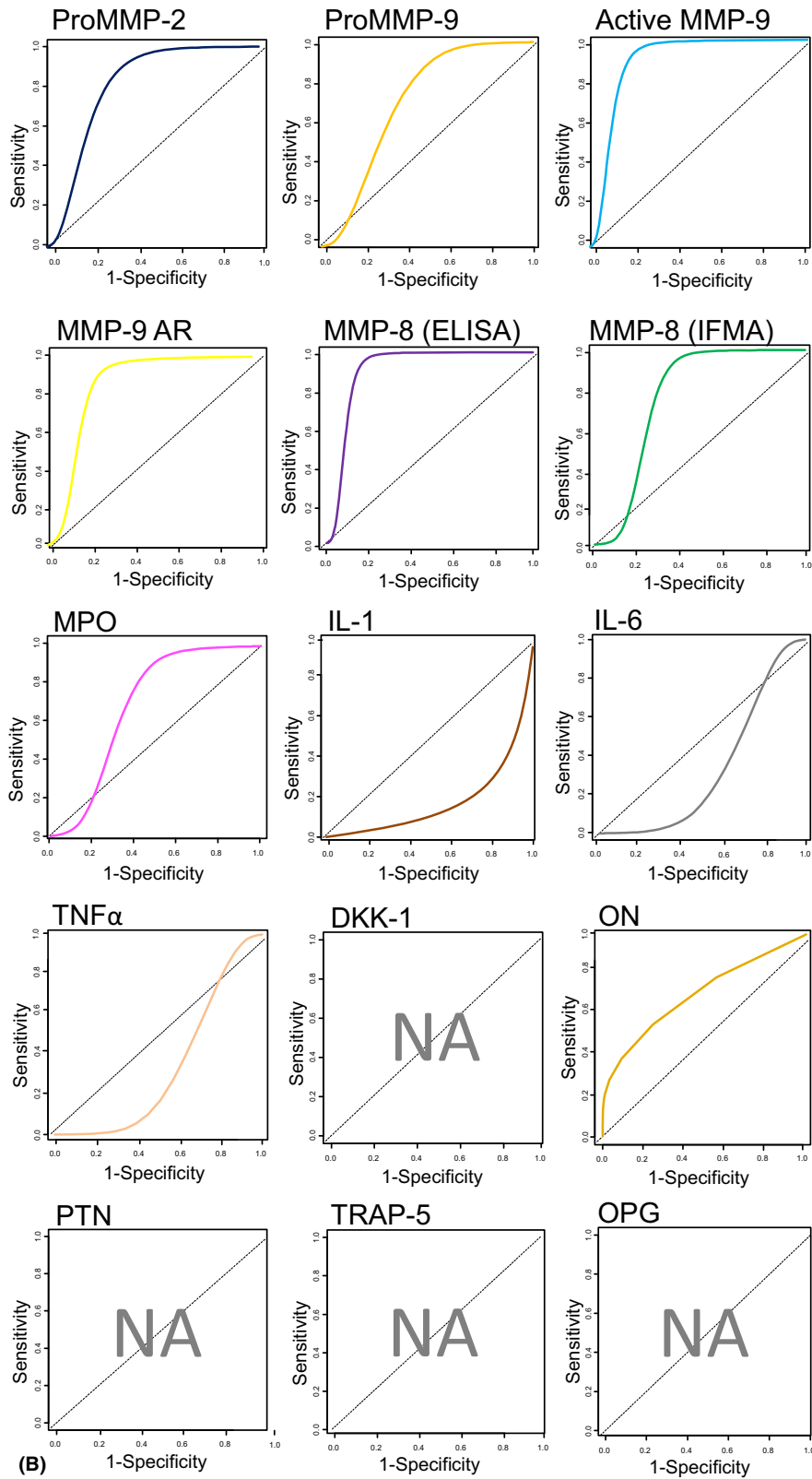
Fig. 3. Continued

proMMP-2 (AUC = 0.99, 95% CI 0.9–1.0), proMMP9 (AUC = 1.0, 95% CI 0.87–1.0), active MMP-9 (AUC = 0.91, 95% CI 0.73–0.98), MMP-9 activation ratio (AUC = 0.93, 95% CI 0.70–1.0), MMP-8 determined by ELISA (AUC = 0.98, 95% CI 0.84–1.0), and MMP-8 determined by IFMA

(AUC = 0.92, 95% CI 0.72–0.99), MPO (AUC = 0.90, 95% CI 0.67–1.0), IL-1 (AUC = 0.92 95% CI 0.68–1.0), IL-6 (AUC = 0.93, 95% CI 0.63–1.0), PTN (AUC = 0.95, 95% CI 0.31–1.0), TRAP-5 (AUC = 0.98, 95% CI 0.82–1.0) and OPG (AUC = 0.91, 95% CI 0.63–1.0). Conversely, TNFα (AUC = 0.64, 95% CI 0.37–0.86), DKK-1 (AUC = 0.73, 95% CI 0.13–1.0) and ON (AUC = 0.76, 95% CI 0.06–1.0) showed a low level of diagnostic performance for sites with chronic periodontitis. Optimal cut-off points were obtained for each marker by using Youden's index. ProMMP-9 showed the best performance, with a sensitivity of 98%, a specificity of 94%, and positive and negative predictive values of 95% and 98%, respectively, at a cut-off point of 1275 au (arbitrary units). It was followed by proMMP-2, with a sensitivity of 94%, a specificity of 96%, and positive and negative predictive values of 96% and 94%, respectively, at a cut-off point of 29 au. In the third place, the ELISA-measured MMP-8, with a sensitivity of 96%, a specificity of 91%, and positive and negative predictive values of 92% and 96%, respectively, at a cut-off point of 17 ng/ml; and fourthly, TRAP-5, with a sensitivity of 93%, a specificity of 90%, and positive and negative predictive values of 91% and 94%, respectively, at a cut-off point of 0.50 pg/ml.

In the construction of ROC curves for the diagnosis of asymptomatic apical periodontitis (Fig. 3B and Table 5B), active MMP-9 was the marker with the highest accuracy, followed by MMP-8 measured by ELISA. Both markers showed a high performance for the diagnosis of AAP *versus* healthy sites [AUC and 95% CI 0.93 (0.68–1.00) and 0.91 (0.72–1.00) respectively]. In turn, activation ratio of MMP-9 and ProMMP-2 showed an acceptable performance [AUC and 95% CI 0.85 (0.60–1.00) and 0.84 (0.51–1.00) respectively], whereas proMMP-9, MMP-8 measured by IFMA, ON and MPO showed a low performance for the discrimination of AAP *versus* healthy sites [AUC and 95% CI 0.77 (0.23–1.00); 0.77 (0.51–0.93); 0.75 (0.03–1.00) and 0.70 (0.30–0.98) respectively]. The rest of the markers were not able to dis-

criminate between AAP and healthy sites. Youden's index showed the highest performance of MMP-8 (ELISA) with a sensitivity of 96%, a specificity of 82%, and positive and negative predictive values of 88% and 94%, respectively, at a cut-off point of 13 ng/ml, followed by active MMP-9, with a sensitivity of 94%, a specificity of 80%, and positive and negative predictive values of 87% and 93%, respectively, at a cut-off point of 137 au.

Discussion

Despite clinical and radiographic-based parameters remain as the gold standard for the diagnosis of periodontal diseases, variations in the inflammatory profile of a similar clinical phenotype can impact disease susceptibility and severity. Thus, identifying a combined array of GCF biomarkers accounting for disease's biological profile is becoming a challenge to aid clinical diagnosis and short-term follow-up (Offenbacher et al. 2007, Kinane et al. 2011). At present, this is the first study that compares the changes in the GCF's biomarkers involving both, patients with CP and AAP. In this study, we report that both diseases manifest quantitative changes in GCF composition and an accurate set of GCF biomarkers of periodontal tissue metabolism were identified for the diagnosis of both, CP and AAP.

Comparing of GCF composition in sites with CP, AAP and healthy controls demonstrated changes in the detection frequency and levels of specific markers. MMP-9 and MMP-8 (measured by both, ELISA and IFMA), showed higher levels in sites with CP, followed by sites with AAP and healthy controls. In turn, proMMP-2, MPO, IL-1β, IL-6, PTN, TRAP-5 and OPG were significantly higher in CP in comparison to AAP and healthy sites.

In agreement with our results, broad evidence supports high levels of MMP-8, MMP-9, MMP-2, MPO, IL-1β, IL-6 and RANKL/OPG and reduced levels of PTN in CP patient's GCF (Mantyla et al. 2006, Marcaccini et al. 2010, Reinhardt et al. 2010, Sorsa et al. 2010, Buduneli & Kinane 2011, Balli et al. 2014); whereas limited evidence

reports higher gelatinolytic activity of MMP-2 and -9, and levels of MMP-8 and TNF-α in GCF from AP patients (Belmar et al. 2008, Burgener et al. 2010, Garrido Flores et al. 2011, Garrido et al. 2014). On the other hand, Dkk-1 and soluble TRAP-5 were determined for the first time in GCF. Dkk-1 is a negative regulator of Wnt-β-catenin signalling and has been reported to decrease the expression of osteogenic markers and to increase osteoclastic activity (Lim et al. 2015), nevertheless, it was hardly detected in GCF. TRAP-5, a marker of osteoclastic activity and bone resorption (Mose et al. 2003), was instead detected in all samples and emerges as an excellent novel candidate to discriminate the sites with CP. The results of this study support that destructive chronic inflammatory processes of the periodontium at both, marginal and apical levels modify GCF composition. MMP-8, MMP-9 levels can differentiate between healthy and apical periodontitis; and at higher levels, these markers along with proMMP-2 and TRAP-5, can discriminate between AAP and CP patients. In addition, MMP-8, proMMP-2 and TRAP-5 associated with clinical–radiographic parameters, including PD, CAL and BL, reflecting disease severity.

The use of GCF as a diagnostic tool in marginal periodontitis represents a widespread concept. Although the evidence available and the current results support that GFC composition is affected by apical periodontitis, the mechanisms remain unknown. Bacteria from the endodontic biofilm and/or their products might reach out the marginal periodontium through the same portals used for entering the sterile pulp, including root fractures, microcracks, exposed dentinal tubules and lateral canals (Nair 2004) and inflammatory mediators may diffuse through periodontal ligament (Burgener et al. 2010) or circulation. Substance P immunoreactive nerves have been found in the periodontal ligament. This neuropeptide might be released in response to pulp injury affecting the activity of immunoreactive cells, which can induce inflammation (Shin et al. 2011). Future morphologic studies assessing these issues need to be con-

Table 5. Accuracy of biological markers for the diagnosis of (A) chronic periodontitis and (B) asymptomatic apical periodontitis

Biomarker	Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy (AUC, CI)	Youden's Index
(A)							
ProMMP-2	28.5	0.94	0.96	0.96	0.94	0.99 (0.90–1.00)	0.89
ProMMP-9	1275	0.98	0.94	0.95	0.98	1.00 (0.87–1.00)	0.91
MMP-9a	514	0.97	0.82	0.84	0.96	0.91 (0.73–0.98)	0.79
MMP-9 AR	5.40	0.92	0.81	0.84	0.94	0.93 (0.70–1.00)	0.73
MMP-8 ELISA	17.1	0.96	0.91	0.92	0.96	0.98 (0.84–1.00)	0.86
MMP-8 IFMA	71.0	0.91	0.80	0.83	0.91	0.92 (0.72–0.99)	0.71
MPO	7.91	0.94	0.79	0.83	0.93	0.90 (0.67–1.00)	0.73
IL-1	5.95	0.81	0.95	0.95	0.84	0.92 (0.68–1.00)	0.76
IL-6	1.68	0.84	0.86	0.85	0.87	0.93 (0.63–1.00)	0.69
TNFα	1.26	0.45	0.89	0.83	0.62	0.64 (0.37–0.86)	0.34
DKK-1	0.0041	0.83	0.44	0.57	0.85	0.73 (0.13–1.00)	0.26
ON	0.71	0.47	0.79	0.46	0.71	0.76 (0.06–1.00)	0.26
PTN	0.019	0.87	0.78	0.76	0.91	0.95 (0.31–1.00)	0.65
TRAP-5	0.50	0.93	0.90	0.91	0.94	0.98 (0.82–1.00)	0.83
OPG	0.030	0.87	0.79	0.82	0.87	0.91 (0.63–1.00)	0.66
(B)							
ProMMP-2	12.4	0.91	0.67	0.78	0.90	0.84 (0.51–1.00)	0.58
ProMMP-9	608	0.88	0.51	0.69	0.84	0.77 (0.23–1.00)	0.39
MMP-9a	137	0.94	0.80	0.87	0.93	0.93 (0.68–1.00)	0.73
MMP-9 AR	0.15	0.91	0.74	0.83	0.89	0.85 (0.60–1.00)	0.65
MMP-8 ELISA	12.8	0.96	0.82	0.88	0.94	0.91 (0.72–1.00)	0.77
MMP-8 IFMA	33.1	0.94	0.63	0.79	0.91	0.77 (0.51–0.93)	0.57
MPO	5.21	0.90	0.51	0.71	0.85	0.70 (0.30–0.98)	0.41
IL-1	14.9	0	099	0.15	0.41	0.05 (0.00–0.30)	−0.0008
IL-6	1.75	0.96	0.12	0.61	0.88	0.34 (0.02–0.86)	0.088
TNFα	0.61	0.95	0.15	0.61	0.87	0.32 (0.03–0.80)	0.10
DKK-1						NA	
ON	0.67	0.60	0.68	0.47	0.65	0.75 (0.03–1.00)	0.28
PTN						NA	
TRAP-5						NA	
OPG						NA	

AUC, area under the curve; CI, confidence interval of 95%; PPV, positive predictive value; NPV, negative predictive value; ProMMP-2, pro-form of MMP-2; ProMMP-9, pro-form of MMP-9; MMP-9a, active form of MMP-9; MMP-9 AR, activity ratio of MMP-9; MMP-8 ELISA, MMP-8 measured with enzyme-linked immunosorbent assay; MMP8 IFMA, MMP-8 measured with immunofluorometric assay; MPO, myeloperoxidase; IL-1, interleukin-1; IL-6, interleukin-6; TNFα, tumour necrosis factor alpha; DKK-1, Dickkopf-1; ON, osteonectin; PTN, periostin; TRAP-5, tartrate-resistant acid phosphatase -5; OPG, osteoprotegerin; NA, not available.

ducted. Furthermore, it has been suggested that local changes in GCF might also reflect systemic inflammation (Sorsa et al. 2011), through direct extravasation of circulating inflammatory mediators. Accordingly, a potentially apical periodontitis-induced low-grade systemic inflammation (Gomes et al. 2013) might also be reflected in GCF.

Based on the fact that CP presents a site-specific pattern (Buduneli & Kinane 2011, Leppilahti et al. 2014) and apical periodontitis affects the whole tooth, GCF samples were obtained from deep periodontal pockets sites and whole crevice's perimeter of the tooth (Belmar et al. 2008, Dezerega et al. 2012) respectively. This potential comparison limitation derived from each disease's pattern was overcome by performing the sampling standard-

ization for 30 s and subsequent elution to the same volume per strip (80 μl of TCL buffer). In addition, the statistical approach accounted for the nested nature of the data, according to the site or tooth level of analysis. Overall, this represents an exploratory study comparatively analysing the diagnostic accuracy of GCF markers in AAP and CP that might set a base for future wider scale studies.

Recently, our group reported a high diagnostic accuracy and discriminating capacity for CP site-specific diagnosis, particularly for MMP-8 and MPO (Leppilahti et al. 2014). The current results reinforce these findings and provide further evidence for new accurate biomarkers for CP, involving MMP-2, MMP-9, IL-1β, IL-6, PTN, TRAP-5 and OPG showing AUC >0.9.

Among them, proMMP-2, proMMP-9, MMP-8 (ELISA) and TRAP-5 are easily detected in GCF, and showed the highest diagnostic accuracies (AUC > 0.97) and Youden's indexes.

On the other hand, MMP analysis in GCF demonstrated for the first time its diagnostic potential in teeth with AAP. Active MMP-9 and MMP-8 (ELISA) showed the highest diagnostic accuracy (AUC > 0.9) and Youden's index. AAP might remain undetected for long periods, whereas the radiographic study does not reliably show changes in the periradicular tissues in the short-term (Weiger et al. 1998, Penesis et al. 2008). Thus, AAP chair-side diagnostics might help to prevent its adverse complications, such as abscess formation, pain and swelling or adverse systemic effects (Endodontology, 2006) and follow-up

could impact the clinical outcome as immediate tooth rehabilitation is critical for the prognosis. Therefore, it would be worthy to further explore the potential utility of MMP-8 and MMP-9 as potential diagnostic biomarkers in AAP and for short-term follow-up. Nevertheless, it is important to bear in mind that the diagnostic potential reported for AAP might be limited to the absence of CP, since we did not evaluat how GCF might be modified by the concomitant presence of both diseases.

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Clinical Relevance

Scientific rationale for the study: Identifying a combined array of GCF biomarkers accounting for disease's biological profile is becoming a challenge to aid clinical periodontal diagnosis. The levels of GCF biological markers for mar-ginal and apical periodontium dis-criminated between health and disease states with high diagnostic accuracy and provide additional information about the patients' cur-rent biological status.

Principal finding: Gingival crevicu-lar fluid MMP-8 and MMP-9 levels reflect CP and AAP.
Practical implications: These results support the potential of these markers for the complementary diagnostic purposes in clinical prac-tice.