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Salivary Biomarkers of Periodontal Disease in Response to Treatment

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Abstract

Background—Salivary biomarkers of periodontitis were assessed longitudinally to determine response to therapy.

Methods—A 6-month case-controlled study of adults with chronic periodontitis was performed, with 33 participants receiving oral hygiene instructions (OHI) alone and 35 with scaling and root planing (SRP) combined with OHI. Saliva samples collected at week 0, 16 and 28 were analyzed for interleukin-1β (IL-1β), IL-8, macrophage inflammatory protein (MIP)-1α, matrix metalloproteinase-8 (MMP-8), osteoprotegerin (OPG) and tumor necrosis factor-α (TNF)-α. Clinical measures of periodontal disease were recorded at each visit.

Results—All parameters of periodontal health improved significantly in both groups by week 16 ($p<0.0001$) with the SRP group demonstrating greater benefit at week 16 and 28. Baseline OPG and TNF-α levels changed significantly at both follow-up visits ($p<0.03$), regardless of treatment group. IL-1β and MMP-8 levels decreased significantly from baseline ($p\leq0.04$) in the SRP group only. OPG, MMP-8 and MIP-1α were significantly reduced in responders compared with non-responders ($p=0.04$, 0.01, 0.05 respectively). In receiver operating characteristic analyses, MMP-8 produced the highest area under the curve (≥ 0.7; $p=0.01$).

Conclusion—Salivary levels of IL-1β, MMP-8, OPG and MIP-1α reflected disease severity and response to therapy suggesting their potential utility for monitoring periodontal disease status.

Keywords

Interleukin-1β; macrophage inflammatory protein (MIP)-1α; matrix metalloproteinase (MMP); osteoprotegerin (OPG); salivary biomarkers; biological markers; periodontal disease; therapy

Periodontal disease is a chronic microbial and inflammatory process characterized by the presence of sulcular pathogenic bacteria, impaired host immune response, and destruction of the connective tissue attachment. In affected tissues, biochemical signaling involving three biological phases (inflammation, connective tissue degradation, and alveolar bone turnover) contributes to the clinical morbidity observed. Circulating molecules in these biological phases have been detected at elevated levels in the gingival crevicular fluid and whole saliva of patients who have periodontal disease making them putative biomarkers of the disease

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Conflict of interest
The authors report no conflicts of interest related to this study.

(Sorsa et al. 1994, Lamster 1997, Faizuddin et al. 2003, Lamster et al. 2003, Miller et al. 2006, Goncalves Lda et al. 2010). Specifically, interleukin (IL)-1β, C-reactive protein, human macrophage inflammatory protein (MIP)-1α, matrix metalloproteinase (MMP)-8 and -9, osteoprotegerin (OPG), tumor necrosis factor (TNF)-α, receptor activator of nuclear factor-kappa B ligand (RANKL), and pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP) (Bertolini et al. 1986, Pederson et al. 1995, Gorska and Nedzi-Christodoulides et al. 2005, Gora 2006, Miller et al. 2006, Christodoulides et al. 2007, Ng et al. 2007, Scannapieco et al. 2007, Frodge et al. 2008, Tobon-Arroyave et al. 2008, Gursoy et al. 2009, Miller et al. 2010) show promise for aiding in the recognition of some aspects of periodontitis. In addition, data are accumulating in children and young adult populations (Ulker et al. 2008, Fine et al. 2009, Thomas et al. 2009, Heikkinen et al. 2010), as well as in patients with co-morbid systemic inflammatory disease (Costa et al. 2010, Mirrielees et al. 2010) as to the utility of these biomarkers for monitoring periodontal disease.

Saliva is appealing for use as a diagnostic fluid for point-of-care analysis for oral related disease because it is rapid, easy and non-invasive to collect, and generally readily abundant (Lamster 1990, Malamud et al. 2005, Christodoulides et al. 2007, Herr et al. 2007, Miller et al. 2010). However, few studies have longitudinally monitored salivary biomarker profiles in patients with respect to periodontal status (Henskens et al. 1996, Thomas et al. 2009) or determined if salivary biomarkers accurately represent periodontal disease status over time. If salivary biomarkers are to demonstrate clinical utility, then analyte concentrations should diminish in response to periodontal therapy. Thus, this investigation sought to test the hypothesis that salivary biomarkers reflect periodontal status over time in participants who received localized periodontal therapy. A secondary hypothesis was that salivary biomarkers reflect response to therapy.

Material and Methods

Participants

Sixty eight patients were enrolled in this longitudinal, case-controlled, clinical study. Participants were recruited from the general clinic population of the College of Dentistry as well as the surrounding counties by advertisement and had the diagnosis of chronic periodontitis based on the criteria defined by the American Academy of Periodontology (Armitage 1999, Armitage 2004). Inclusion criteria included ≥18 years of age who were in good general health, (excluding the case definition) and had ≥18 erupted teeth. Participants had to have five qualifying sites in two quadrants with a minimum of two affected teeth in each quadrant with each site having probing depth ≥ 5 mm, clinical attachment loss (CAL) of ≥ 3 mm, and bleeding upon probing (BOP) score of ≥ 2 (0= one, 1=pinpoint, 2=interdental bleeding, 3=spontaneous/heavy bleeding). Exclusion criteria were periodontal therapy in the past two years, a history of alcoholism; liver, kidney, or salivary gland dysfunction; inflammatory bowel disease; granulomatous diseases; diabetes or were undergoing or had undergone organ transplant or cancer therapy. Pregnancy or lactation, use of antibiotics or immunosuppressant medication within the last 6 months, need for antibiotics for infective endocarditis prophylaxis during dental procedures, symptoms of acute illness (i.e., fever, sore throat, body aches, and diarrhea), orthodontic appliances or presence of an oral mucosal inflammatory condition (e.g., aphthous, lichen planus, leukoplakia, and oral cancer) also were exclusion criteria. The study was performed at the University of Kentucky between August 2005 and August 2009 and was approved by the University Institutional Review Board. All subjects understood the study, provided written informed consent and received incentives (i.e., monetary compensation and a clinical examination) as part of the study protocol.

Clinical Evaluation and Therapy

Complete medical and dental histories were obtained from the patient's records and confirmed by interview. Periodontal health measures including BOP, probing depth (PD), and clinical attachment loss (CAL) were assessed as previously described (Frodge et al. 2008, Dawson et al. 2009) at baseline, week 16 and week 28. Participants were randomly assigned to receive either oral hygiene instructions (OHI) only or scaling and root planing (SRP) and OHI using a computer randomization schedule constructed prior to study initiation. OHI and SRP were performed by a registered dental hygienist during the first 30 days following the baseline visit. Patients returned to the clinic at week 8 as part of a separate study protocol for dental plaque sampling, and subjects had repeat OHI or SRP by the treatment hygienist following clinical examination at week 16. A single calibrated periodontist blinded to the participant group assignments, performed the clinical evaluations. Scaling and root planing therapy were performed as we have described previously (Preshaw et al. 2008). Response to therapy was defined based on published findings where non-surgical pocket therapy was expected to yield improvements in PD, CAL and BOP in the time frame examined (Cobb 1996, Kaldahl et al. 1996, Fleming 1999). Responders were defined as individuals who demonstrated 20% improvement in all four categories (%BOP sites, %CAL≥2mm sites, %PD≥4mm sites and %PD≥5mm sites) at both follow-up visits. This threshold was expected to underestimate the number of individuals who responded at individual sites at a single follow-up visit (*i.e.,* increased stringency for response to therapy).

Saliva Collection

Unstimulated whole expectorated saliva (5 mL) was collected from each subject between 9 and 11 a.m. according to a modification in the method described by Navazesh (Navazesh 1993). Subjects rinsed their mouth with tap water, then expectorated whole saliva into sterile tubes while seated in an upright position. Collected samples were placed immediately on ice and aliquoted prior to freezing at −80°C. Samples were thawed and analyzed within six months of collection.

Biomarker Analysis

Concentrations of salivary IL-1β, IL-8, MIP-1α and TNF-α were determined in duplicate using Luminex human cytokine/chemokine multiplex kits (Millipore, St. Charles, MO, USA) and salivary levels of OPG and MMP-8 were determined in duplicate for each subject using human quantikine enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's directions in the University of Kentucky General Clinical Research Center Core laboratory. Standards were included on all runs and all results are reported within the linearity of the assays.

Statistical Analysis

Demographic variables were compared between the OHI and SRP groups using a Chi square test or Fisher's exact test for categorical responses and a two sample t-test for interval level responses. Changes from baseline in the periodontal indices were compared between the treatment groups using analysis of covariance (ANCOVA) with adjustments for age, race and tobacco use. In these analyses, BOP was used as dichotomous score as previously described (Lang et al. 1990), with ≥2 being positive and < 2 being negative. Since the biomarker measurements were not normally distributed due to the presence of large values, the ranks of the biomarker levels (or change from baseline) became the dependent variables in the remaining analyses. The overall comparison of biomarker levels relied on linear mixed model for a two factor design: factor one being treatment group (OHI versus SRP) and factor two being visit (baseline, week 16 or week 28). At each follow-up visit, the comparison of biomarker levels relied on rank ANCOVA for a two factor design: factor one

being treatment group (OHI versus SRP) and factor two being responder status (yes or no) with age as a covariate. Correlations were based on Spearman's rank correlation coefficient. All analyses were performed using the PC SAS 9.1 (SAS Institute Inc., Cary, NC, USA) with statistical significance determined at the 0.05 level. Of note, in the presence of multiple comparisons, some of the results reported below could be due to chance alone.

Results

Sixty eight adults with chronic periodontitis ranging in age from 25 to 69 years were evaluated at baseline, week 16 and week 28 (Table 1). There were 33 in the OHI and 35 in the SRP groups. Participants were predominantly Caucasian and Hispanic, and male. The groups had a similar mean number of teeth, however the OHI group was 7 years older ($p=0.003$). There were no significant differences between groups in gender, race and smoking. Clinical measures of %CAL ≥2 mm, %PD sites ≥4 mm, %PD sites ≥5 mm and %BOP reflected the presence of generalized chronic periodontitis and were similar between the two groups ($p>0.05$).

Clinical Parameters and Response to Therapy

All parameters of periodontal health improved significantly in both groups by week 16 (Figure 1) compared with baseline ($p\leq0.001$). The improved levels persisted in both groups at week 28 ($p<0.0001$). Overall, SRP was more beneficial (37% to 56% reductions) than OHI (22% to 35% reductions) at both visits. As demonstrated in Figure 1, the percent change in means was significantly greater in the SRP group for %BOP ($P\leq0.005$), %PD sites ≥5 mm ($P\leq0.002$), and %PD sites ≥4 mm ($P\leq0.04$) than the OHI group at both follow-up visits.

Levels of several salivary biomarkers changed in response to therapy

Levels of salivary biomarkers were detected in all samples, had distinct ranges of distribution, and mean levels that were similar between the groups at baseline (Figure 2). The levels of MIP-1α and IL-8 did not vary from baseline at 16 or 28 weeks. The levels of OPG varied significantly from baseline at week 28 ($p<0.004$), regardless of treatment group. The levels of TNF-α varied significantly from baseline at both visits ($p<0.03$), regardless of treatment group. Similarly the levels of IL-1β varied significantly from baseline at both visits ($p<0.04$) in the SRP group, and only at week 28 for the OHI group ($p<0.05$). Levels of MMP-8 reduced significantly from baseline at both visits in the SRP group only ($p<0.0001$).

Correlates between biomarkers and changes in clinical measures of periodontal health

Correlations between each salivary biomarker and changes in clinical measures of periodontal health were calculated for each follow-up visit. In the week 16 analysis for the entire study population, MMP-8 and OPG showed statistically significant correlations with respect to the change in all four clinical measures of periodontal health ($r\geq0.25$, $p\leq0.04$). This relationship persisted for MMP-8 at week 28 for the entire population for PD≥4mm, PD≥5mm and positive BOP ($r\geq0.27$, $p\leq0.03$). OPG correlated with change in CAL≥2mm ($r=0.29$, $p=0.017$) at week 28 for the entire population. Also MIP-1α correlated with the change of PD≥4mm and positive BOP ($r\geq0.26$, $p\leq0.04$). In the OHI group alone, MMP-8 correlated with change in PD≥4mm ($r=0.40$, $p=0.025$) and positive BOP ($r=0.48$, $p=0.006$) at week 28. In the SRP group, MMP-8 showed a significant correlation with respect to the change in all four clinical measures of periodontal health ($r\geq0.34$, $p\leq0.05$) at week 16. MIP-1α correlated with the change of positive BOP ($r=0.44$, $p=0.01$) at week 16. MIP-1α also correlated with the change of PD≥5mm ($r=0.35$, $p=0.04$), CAL≥2mm ($r=0.43$, $p=0.01$) and positive BOP ($r=0.41$, $p=0.01$) in the SRP group at week 28.

Biomarkers and responders—We next investigated whether those who demonstrated a significant response to therapy displayed unique biomarker profiles from those who did not. In contrast to the correlation analyses used above that provided some evidence of salivary response and oral health, a responder in this analysis was defined as an individual who demonstrated 20% improvement in all four clinical categories (frequency of sites with positive BOP, CAL≥2mm, PD≥4mm and PD≥5mm) at both follow-up visits. Of the 68 participants, 51.5% were responders by week 16, with significantly more responders in the SRP (62.9%) than OHI group (27.1%; p=0.0005). OPG, MMP-8 and MIP-1α were significantly reduced in the responders compared with the non-responder (p= 0.008, 0.02, 0.02, respectively) at week 16. At completion of the study, OPG, MMP-8 and MIP-1α demonstrated (Figure 3) significant reductions in the responders compared to the non-responders (p=0.04, 0.01 and 0.05, respectively). Finally, receiver operating characteristic (ROC) analyses were performed to evaluate the change in each biomarker level with respect to response to therapy. As shown in Table 2, MMP-8 consistently produced the highest area under the curve (≥ 0.7; p=0.01) indicating it was the best biomarker for demonstrating response to therapy at weeks 16 and 28. The ROC score for OPG also suggested its usefulness for demonstrating response to therapy.

Discussion

In dental practice periodontal disease has been historically based on the clinical detection of BOP, PD, CAL, plaque index, and radiographic evidence of bone loss. The diagnostic utility of these clinical measures although reliable, are expensive with respect to time, cost and professional expertise required. Accordingly, the profession has searched for additional chair side measures to aid in early diagnosis and monitoring. Previous studies have shown that constituents present in saliva can provide important complimentary diagnostic information (Malamud 1992, Fox 1993, Kaufman and Lamster 2000, Kaufman and Lamster 2002, Giannobile et al. 2009), that have the potential for point-of-care use by dental professionals and the general public (Malamud et al. 2005, Christodoulides et al. 2007, Miller et al. 2010). However, most previous studies have investigated salivary biomarkers of periodontal disease in cross-sectional studies, and the behavior of various salivary biomarkers in longitudinal studies is limited (Henskens et al. 1996, Van Steijn et al. 2002, Hagewald et al. 2003, Gheren et al. 2008). Thus, a 6-month longitudinal study was designed to follow adults with chronic periodontitis who received either OHI or SRP to better understand the utility of contemporary biomarkers for monitoring periodontal disease and response to different therapies.

Salivary levels of IL-1β, IL-8, MIP-1α, MMP-8, OPG and TNF-α were determined in adults who had chronic periodontitis at baseline, week 16 and week 28 following OHI or SRP therapy. These six proteins are biochemically active for processes integrally involved in inflammation, connective tissue degradation, and osteoclast-modulated alveolar bone turnover (Bertolini et al. 1986, Sorsa et al. 1994, Choi et al. 2000, Engebretson et al. 2002, Silva et al. 2005, Emingil et al. 2005, Roodman 2006, Herr et al. 2007, Miller et al. 2010). All except IL-8 have been previously reported in saliva. IL-8 was included because of its role as a major mediator of the inflammatory response and chemoattractant for neutrophils (Zwahlen et al. 1993). We predicted that significant reductions in levels of these salivary biomarkers would occur in the SRP group compared with baseline and the OHI group. Our data supported the hypothesis that select salivary biomarkers reflected periodontal status over time in participants who received localized mechanical periodontal therapy. Further, the secondary hypothesis that select salivary biomarkers reflect response to therapy was demonstrated.

The two randomly assigned groups had similar levels of periodontal disease and similar baseline levels of each of the salivary biomarkers analyzed. The mean levels for OPG, MMP-8, IL-1β and TNF-α reflected the presence of generalized chronic periodontitis when compared with historical values reported for affected individuals (Miller et al. 2006, Ng et al. 2007, Scannapieco et al. 2007, Frodge et al. 2008). In contrast, salivary levels of MIP-1α and IL-8 have not been previously described in adults. Fine et al. reported MIP-1α levels in adolescents that were similar in concentration range as our adults (Fine et al. 2009), however adolescents who developed localized aggressive periodontitis in that study had much higher levels than our adult participants.

Our baseline clinical and biochemical data provided insight into the profile of salivary biomarker levels of adults who have chronic periodontitis, and allowed us to determine if these values can serve as a reference for monitoring response to therapy. The utility of these biomarkers for monitoring response to therapy was assessed in groups receiving either OHI or SRP. Both groups dramatically improved and progressed towards health (Fig. 1), with the SRP group responding significantly better (37% to 56% reductions) than the OHI group (22% to 35% reductions, $P \leq 0.04$). Of interest is the better than expected improvement in the OHI group that may have occurred in relation to the Hawthorne effect and the frequent dental visits employed in this study design. Within the picture of improving oral health, OPG and TNF-α declined significantly from baseline in both treatment groups while IL-1β and MMP-8 only showed significant changes from baseline in the SRP group. A plausible explanation for only these two biomarkers (OPG and TNF-α) changing significantly in the OHI group could be that significant amounts of disease remained in the OHI group at the follow-up visits (i.e., BOP ≥42%, PD≥4mm >18%, PD≥5mm 10%, CAL ≥21%) thus limiting the sensitivity of all of the biomarkers to distinguish the response within that group. Another possibility is that smoking, which was documented in 23% of the SRP group and 33% of the OHI group, influenced salivary biomarkers levels differentially (i.e., more for select biomarkers), as has been reported for OPG in a previous study (Buduneli et al. 2008). In any case, mechanical therapy was sufficiently effective in exceeding a biological threshold required to detect the changes in saliva in all four biomarkers (IL-1β, MMP-8, OPG, TNF-α) in the SRP group.

Analysis by responders allowed us to further explore the role of biomarkers and showed that several specific biomarkers have the potential utility for monitoring periodontal disease. OPG, MMP-8 and MIP-1α were early (week 16) salivary biomarkers of the responders. These biomarkers also identified the responders at week 28. Biomarker utility was further validated by performing ROC analyses with respect to change in biomarker level in responders vs. non-responders. Here, AUCs demonstrated that MMP-8 ≥ OPG > MIP-1α >IL-1β ranked as the best biomarkers indicative of response to therapy. The fact that MMP-8 was highly ranked is not a surprise. Salivary MMP-8 has been a consistent indicator of periodontal disease and connective tissue degradation in several studies (Miller et al. 2006, Ng et al. 2007, Scannapieco et al. 2007, Ramseier et al. 2009, Costa et al. 2010). In comparison, OPG and MIP-1α, have shown promise as biomarkers related to periodontal disease (Miller et al. 2006, Fine et al. 2009). However, their levels are predicted to correlate with osteoclast activity and bone turnover aspects of periodontal disease that may or not be present at any given time during the course of periodontal disease, and single time point measures may inadequately capture these events. Also IL-1β, although a marker of periodontal disease, can be elevated in both gingival and periodontal inflammation (Engebretson et al. 2002, Offenbacher et al. 2007), thus its sensitivity to periodontal tissue change may be insufficient to be used alone for monitoring periodontal disease.

Conclusions

In conclusion, this study is one of the first to examine the role of a panel of salivary biomarkers for monitoring periodontal health in a longitudinal study design. Our findings, although limited by study size, some potential statistical limitations and the rigidity of the inclusion criteria, show that MMP-8, OPG, MIP-1α and IL-1β have associations with biological aspects of periodontitis and reflect improved periodontal health as a result of localized therapy over a 6-month time period. Also, the findings suggest that select salivary biomarkers could have utility for monitoring periodontal health and response to therapy. To further advance the field, large validation studies are needed to confirm these data and investigate if salivary biomarkers are confounded by age, gender, race, smoking, presence of oral inflammatory conditions, or co-morbid conditions. We believe that validation studies could lead to their use in point-of-care devices that could have a major impact on periodontal care. One can envision that these devices could be used to assess periodontal health similar to the way a glucometer assesses blood glucose in diabetic subjects. Specific biomarkers (i.e., MMP-8, OPG, MIP-1α, IL-1β) levels above set thresholds might suggest immediate therapy would be beneficial, similar to how insulin is provided when glucose is elevated in a diabetic. In turn, individualized periodontal care based on salivary biomarker levels could be provided in the near future and these analyses could become an integral part of the assessment of periodontal health in dental and non-dental settings.

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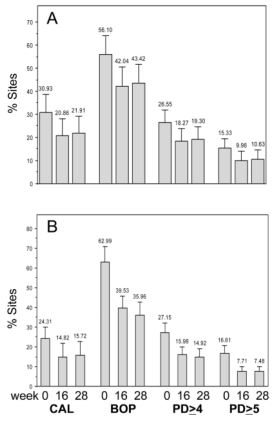

Figure 1.

Clinical periodontal measures by group and visit. A) OHI and B) SRP. Significant reductions ($p<0.0001$) occurred in each parameter compared to baseline within groups as determined by ANCOVA.

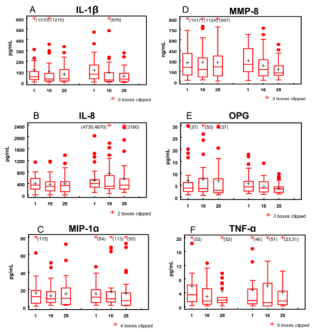

Figure 2.

Boxplots of biomarkers by group and visit. A) IL-1β, B) IL-8, C) MIP-1α, D) MMP-8, E) OPG, and F) TNF-α. Left three boxes correspond to OHI and right three boxes for SRP. Horizontal lines represent the 25th, 50th and 75th percentiles, plus sign represents the mean, and filled circles represent outliers and * indicates outliers clipped from boxplot. X-axis corresponds with weeks.

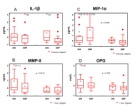

Figure 3.
Boxplots of change scores of biomarkers (log values) at week 28 by group and responder status. Left two boxes correspond to non-responder and right boxes to responders. Significant p-values for comparing responders to non-responders are indicated on the plots.

Table 1

Comparison of demographics and clinical characteristics between study groups at baseline.

	OHI (*n=33*)	SRP (*n=35*)	*P* Value
Age (years; mean +/− SD)	47.3 +/− 8.8	40.3 +/− 10.0	0.003*
Female (%)	36.4	25.7	
White (%)	42.4	40.0	
Hispanic (%)	21.2	37.1	
African American	27.3	11.4	
Asian (%)	9.1	11.4	
Current tobacco use (%)	33.3%	22.9%	
# Teeth	26.1 +/− 6.4	26.5 +/− 4.8	
Periodontal indices (% sites; mean +/− SD)			
BOP Sites	56.1 +/− 22.7	63.0 +/− 22.5	
PD Sites ≥4 mm	26.5 +/− 14.8	27.2 +/− 14.7	
PD Sites ≥5 mm	15.3 +/− 11.3	16.6 +/− 11.8	
CAL ≥2 mm	30.9 +/− 21.4	24.3 +/− 18.1	

*determined by t test.

Periodontal indices analyzed by ANCOVA

Table 2

Area under the curve (AUC) values for each biomarker at the follow up visits with respect to response to therapy.

Biomarker	AUC at week 16	P value at week 16	AUC at week 28	P value at week 28
MMP-8 change	0.70	**0.01**	0.72	**0.01**
OPG change	0.70	**0.01**	0.69	**0.02**
MIP-1α change	0.64	0.28	0.68	0.06
IL-1β change	0.62	0.31	0.62	0.47
IL-8 change	0.43	0.47	0.51	0.54
TNF-α change	0.54	0.25	0.49	0.40