RESEARCH REPORTS
Clinical

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Saliva/Pathogen Biomarker Signatures and Periodontal Disease Progression

J Dent Res DOI: 10.1177/0022034511399908

APPENDICES

Clinical Measurements

All teeth except third molars were assessed for periodontal clinical measures by one of three calibrated examiners (JSK, TM, or CAR). Clinical parameters of the study including PD, CAL, and BOP were measured at 6 sites *per* tooth. Other clinical assessments included dichotomous measures of plaque accumulation (PI) and gingival redness index (GRI), as previously described (Haffajee *et al.*, 1983).

Standardized Radiographs

Standardized periapical digital radiographs (Schick Technologies, Long Island City, NY, USA) were taken in the posterior dentition of all participants by a parallel technique for the determination of alveolar bone height at baseline, 6, and 12 mos, and were analyzed by one calibrated examiner (LR) equipped with a computer software measurement tool (Emago®, Oral Diagnostic Systems, Amsterdam, Netherlands).

Examiner Training and Calibration

Clinical Measures

Intra- and inter-examiner calibration sessions were held at the beginning and middle time-points of the study. Intra-examiner correlation coefficients for FGM ranged from 0.98 to 0.99 at the beginning of the study and from 0.88 to 0.95 at the mid-point of the study. Intra-examiner correlation coefficients for PD ranged from 0.92 to 0.98 at the beginning of the study and from 0.94 to 0.95 at the mid-point of the study. Inter-examiner correlation coefficients for FGM were 0.98 at the beginning of the study and 0.94 at the mid-point of the study. Inter-examiner correlation coefficients for PD were 0.98 at the beginning of the study and 0.92 at the mid-point of the study.

Radiographic Measures

Intra- and inter-examiner calibration exercises were conducted prior to the radiographic analysis. The intra-examiner correlation coefficient was 0.99, and the inter-examiner correlation coefficients ranged from 0.87 to 0.99. The distance from the alveolar bone crest to the cemento-enamel junction or the restorative margin reference was recorded as the radiographic alveolar bone level (RBL).

Saliva Biomarker Analysis

Whole-saliva samples were analyzed in batches with corresponding standard curves. Protein biomarker levels were determined by colorimetric-based enzyme-linked immunosorbent assays (ELISAs), fluorescence-based protein microarrays, and radioimmunoassay (RIA), run according to manufacturer protocols. ELISAs (R&D Systems Inc., Minneapolis, MN, USA) were used for measurement of MMP-8 and -9, calprotectin, and osteoprotegerin (OPG). Detection of the cytokines interleukin (IL)-β, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ was accomplished with a protein microarray (Whatman Inc., Florham Park, NJ, USA). Pyridinoline cross-links of type I collagen (ICTP) were determined with an RIA (Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA).

Appendix Table 1. Inclusion and Exclusion Criteria

1. Inclusion criteria
   1.1. Participants with a minimum of 20 teeth
   1.2. Participants between 35 and 70 years of age
2. Exclusion criteria
   2.1. Periodontal treatment within 3 mos prior to study inclusion
   2.2. Long-term (over 2 wks) antibiotic therapy for medical or dental reasons within 3 mos prior to study inclusion
   2.3. Long-term use of drugs known to affect periodontal status, such as anti-inflammatory drugs
   2.4. Individuals on immunosuppressive therapies, including glucocorticoids and/or cyclosporine
   2.5. History of metabolic bone diseases, such as rheumatoid arthritis or post-menopausal osteoporosis
   2.6. Pregnant women

Serum Biomarker Analysis

C-reactive protein (CRP) was measured in serum by means of the latex immunoturbidimetric assay (Equal Diagnostics Inc., Exton, PA, USA) and read by turbidity at 550 nm on a Cobas Mira Chemistry Analyzer (Roche Diagnostics Corp., Indianapolis, IN, USA). IL-1β and IL-6 levels were measured with an Immulite Analyzer and reagent kits (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). TNF-α samples were analyzed on an Immulite Analyzer with a solid-phase chemiluminescent immunometric assay. Colorimetric-based ELISAs were used for detection of protein biomarker levels of calprotectin (Cell Sciences®, Canton, MA, USA), OPG (Alpco Diagnostics, Salem, NH, USA), MMP- 8 and -9, and ICTP levels were analyzed as described above.

Statistical Analysis

We used a two-level hierarchical clustering approach to categorize the participants into three clusters, which we believed was the largest number of clusters possible to yield a sufficient number of individuals in each cluster relative to our overall sample size, allowing for meaningful comparisons among the three clusters. In the first stage, participants were classified into one of three groups according to the change (from baseline to 6 mos) in: (1) clinical measures only, (2) biofilm pathogens only, (3) salivary biomarker levels only, and (4) serum biomarker levels only. In the second stage, we used hierarchical clustering with the four group classifications produced in the first stage to assign each participant to one of three final groups. The observed rate of disease progression in each group was then compared with the distribution of the patient values (Z-scores) used in the clustering, and the probability of correct classification for each group was summarized as the proportion of individuals whose true classification matched their group membership. Intermittent missing data were assumed to be missing completely at random, and no imputation methods were used. Statistical significance was defined as a p-value less than 0.05. All analyses were performed with the statistical package R (R Development Core Team 2008, Vienna, Austria).

APPENDIX REFERENCES

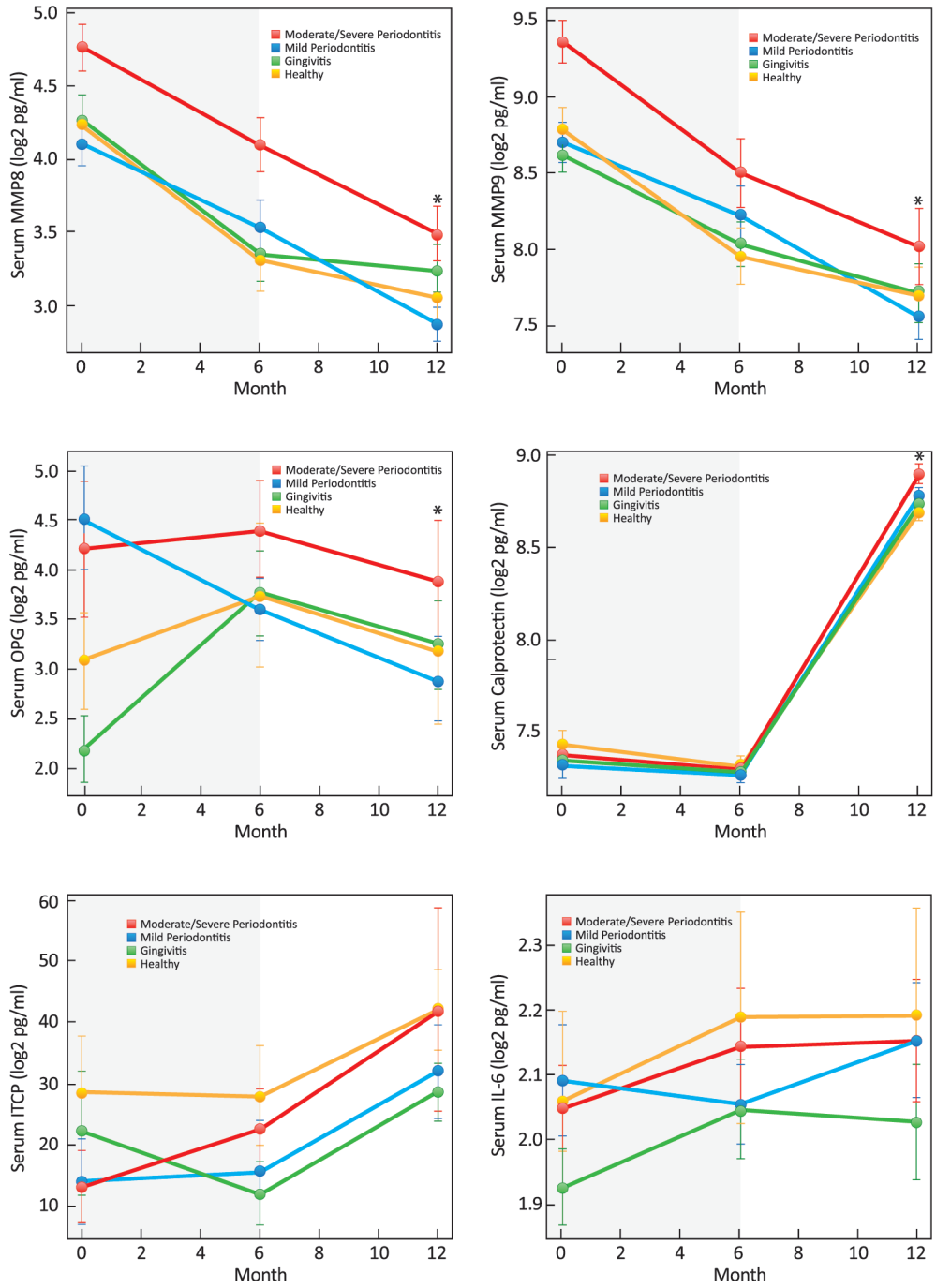
Haffajee AD, Socransky SS, Goodson JM (1983). Comparison of different data analyses for detecting changes in attachment level. *J Clin Periodontol* 10:298-310.

R Development Core Team (2008). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

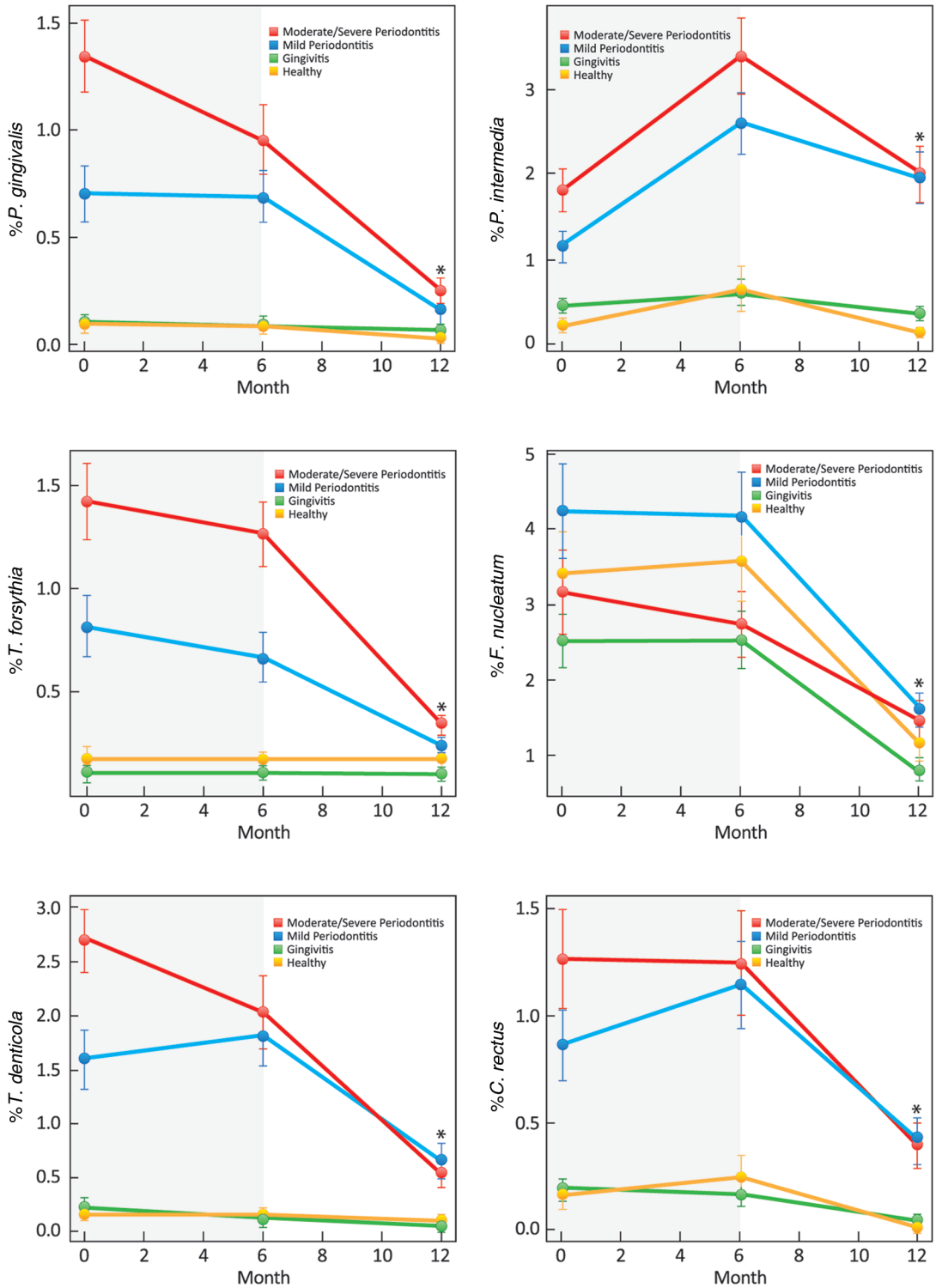
Appendix Table 2. Patient Demographics and Clinical Parameters Stratified by Level of Disease

	A	B	C	D	P-values Comparing A, B, C, & D		
	Healthy	Gingivitis	Mild Chronic Periodontitis	Moderate-to-Severe Chronic Periodontitis	Overall	Trend	P-value Comparing A,B, and C *vs.* D
Number of participants	15	24	24	20	n/a	n/a	n/a
% Male	60	42	46	35	0.514	0.218	0.325
% White	87	83	67	85	0.348	0.609	0.489
% Smoker	0	21	38	80	< 0.001	< 0.001	< 0.001
Mean number of teeth	28	27	26	25	< 0.001	< 0.001	0.001
Mean age	46	46	54	50	< 0.001	0.136	0.735
% BOP[a]	12	29	53	64	0.379	< 0.001	< 0.001
% Redness	13	23	51	57	0.461	< 0.001	< 0.001
% Plaque	24	29	58	61	0.533	< 0.001	0.001
Mean PD[b]	1.5	1.7	2.3	3.1	< 0.001	< 0.001	< 0.001
% Sites PD > 4 mm	0.0	0.1	8.0	21.1	0.899	< 0.001	< 0.001
Mean CAL[c]	0.6	0.8	1.7	3.0	< 0.001	< 0.001	< 0.001
Mean BL[d]	1.9	2.1	3.1	4.4	< 0.001	< 0.001	< 0.001

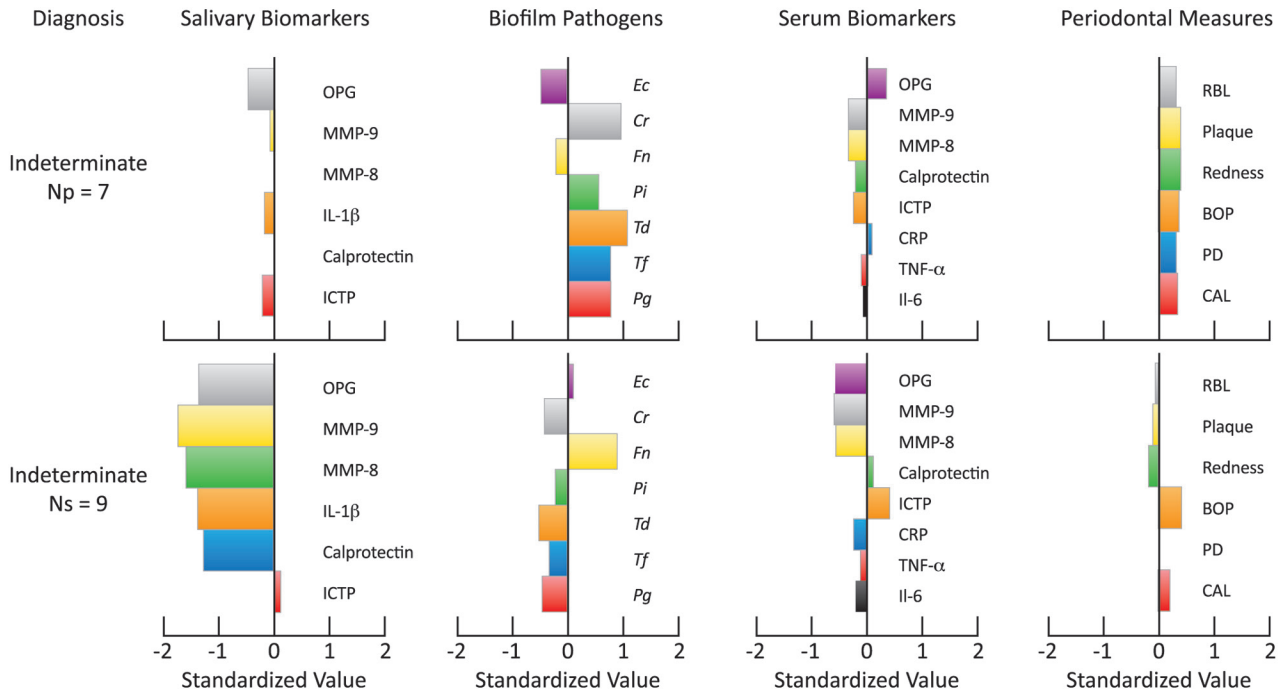
[a]Bleeding on probing.
[b]Periodontal probing depth.
[c]Clinical attachment level.
[d]Radiographic alveolar bone level.



Appendix Figure 1. Longitudinal plots of mean (± SD) serum biomarker levels stratified by initial category of periodontal health. Compared with baseline, all participants demonstrated significant reductions in MMP-8 and MMP-9 at 12 mos ($p < 0.05$). Compared with baseline, significant changes in OPG levels were seen in the mild periodontitis and gingivitis groups at 12 mos ($p < 0.05$). All participants had significant increases in calprotectin at 12 mos compared with baseline ($p < 0.05$). Serum ICTP and IL-6 levels did not reach statistical significance in any of the groups.



Appendix Figure 2. Longitudinal plots of mean (± SD) percentages of biofilm pathogens stratified by initial category of periodontal health. Compared with baseline, individuals in the moderate/severe periodontitis group had significant reductions in *P. gingivalis, T. forsythia, F. nucleatum, T. denticola,* and *C. rectus* at 12 mos; those in the mild periodontitis group had significant reductions in all periodontal pathogens at 12 mos; those in the gingivitis group had reductions in *F. nucleatum* and *C. rectus* at 12 mos; and those in the healthy group had reductions in *F. nucleatum* at 12 mos ($p < 0.05$).



Appendix Figure 3. Barplots of the indeterminate cluster, showing salivary biomarkers, biofilm, serum biomarkers, and periodontal measures of individuals with and without disease progression. Np = number of patients undergoing disease progression. Ns = number of patients demonstrating periodontal stability.