CLINICAL INVESTIGATIONS

Periodontal Bacteria and Prediabetes Prevalence in ORIGINS: The Oral Infections, Glucose Intolerance, and Insulin Resistance Study

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Appendix

Methods

Periodontal Examination Reliability Studies

Three examiners conducted baseline exams: 73% were conducted by examiner 1, 21% by examiner 2, and 6% by examiner 3. All examiners were trained and calibrated by a study investigator (Dr. Papapanou).

Intraexaminer reliability data for the periodontal examination protocol implemented by these investigators have been published (Desvarieux et al. 2013), which include reliability data from the lead examiner in ORIGINS (Oral Infections, Glucose Intolerance, and Insulin Resistance Study), who conducted 73% of the examinations. Briefly, intraexaminer reliability studies on 14 participants yielded intraclass correlations of 0.97 and 0.94 between repeat measures of mean probing depth (PD) or attachment loss (AL), respectively. Mean absolute differences between repeat examinations for mean PD or AL were 0.09 and 0.17 mm.

Interexaminer reliability studies were performed on 8 ORIGINS participants (558 periodontal sites examined). Among 1,118 sites examined, the mean differences (SE) between repeat measurements for PD and AL were 0.5 (0.02) mm and 0.9 (0.04) mm, respectively. Among repeat PD measurements, 52% of measures were identical; 97% differed by ≤1 mm; and 99% differed by ≤2 mm. Among repeat AL measurements, 38% were identical; 79% differed by ≤1 mm; 95% differed by ≤2 mm; and 99% differed by ≤3 mm.

Subgingival Plaque Collection

In total, 1,188 subgingival plaque samples (4 samples from 297 participants) were collected from the most posterior tooth in each quadrant (excluding third molars) via sterile curettes and after removal of the supragingival plaque as previously described (Desvarieux et al. 2005). Plaque samples were transferred into Eppendorf tubes containing 200 mL of sterile T-E buffer (10mM Tris HCl, 1.0mM EDTA, pH 7.6) and were not pooled. Immediately after transfer to the laboratory, the plaque pellet was resuspended, vigorously vortexed, and 200 mL of a 0.5M-NaOH solution added.

Checkerboard DNA-DNA Hybridization

Digoxigenin-labeled whole genomic probes were prepared by random priming via the High-Prime Labeling Kit (Roche/Boehringer-Mannheim, Indianapolis, IN, USA) from the following microbial strains: *Aggregatibacter actinomycetemcomitans* ATCC 43718, *Porphyromonas gingivalis* ATCC 33277, *Tannerella forsythia* ATCC 43037, *Treponema denticola* ATCC 35404, *Prevotella intermedia* ATCC 25611, *Fusobacterium nucleatum* ATCC 10953, *Parvimonas micra* ATCC 33270, *Campylobacter rectus* ATCC 33238, *Eikenella corrodens* ATCC 23834, *Veillonella parvula* ATCC 10790, and *Actinomyces naeslundii* ATCC 49340. Further processing was carried out according to the checkerboard DNA-DNA hybridization method (Socransky et al. 1994; Papapanou et al. 2000). A

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LumiImager F1 Workstation (Roche/Boehringer-Mannheim) evaluated the chemiluminescence signal by comparing the obtained signals to known standards. Standard curves were generated for each species through LumiAnalyst software (Roche/Boehringer-Mannheim), and the obtained chemiluminescent signals were transformed into bacterial counts.

Laboratory Measures

Blood was collected following an overnight fast from all participants. Plasma glucose, serum lipids (triglycerides, total cholesterol, and high-density lipoprotein [HDL]), and hemoglobin A1c from whole blood were measured via standard methods using a Cobras Integra 400 Plus (Roche Diagnostics, Indianapolis, IN, USA). Low-density lipoprotein (LDL) cholesterol was determined through the Friedewald equation (Friedewald et al. 1972). Adiponectin was measured via ELISA (Millipore, Billerica, MA, USA), as were TNF-α (tumor necrosis factor α) and IL-6 (interleukin 6; R&D systems, Minneapolis, MN, USA); hsCRP (high-sensitivity C-reactive protein) was measured via the turbidimetric method with a Cobras Integra 400 Plus (Roche Diagnostics). TNF-α, IL-6, hsCRP, and adiponectin values were combined to create a summary inflammatory score (SIS; Behle et al. 2009). Briefly, individual biomarker values were natural log transformed and converted into a standard normal *z* score by subtracting the sample mean biomarker value among all participants from the participant-specific value and dividing by the sample standard deviation. Adiponectin *z* scores were multiplied by –1 due to its anti-inflammatory properties. The resulting biomarker *z* scores were summed to create a SIS for each participant.

Risk Factor Assessment

Cardiometabolic risk factors were measured by trained research assistants in dedicated clinical research space provided by a Center for Translational Science Award at Columbia University. Systolic and diastolic blood pressures were measured in triplicate, and the last two measurements were averaged. Participant body mass index (BMI) was calculated as kg/m^2 (weight/height) and classified as follows: normal (or underweight), BMI <25 kg/m^2 (only n = 4 participants had BMI <18.5 and were categorized with normals); overweight, BMI ≥25 and <30 kg/m^2; obese, BMI ≥30 kg/m^2. Risk factor questionnaires adapted from the Centers for Disease Control and Prevention's Behavioral Risk Factor Surveillance System and the National Health and Nutrition Examination Survey were administered to obtain the following key risk factor information: age, sex, race/ethnicity (non-Hispanic black, non-Hispanic white, Hispanic, other), educational level (high school completion, college or vocational training, advanced degrees), and cigarette smoking (current, former or never smoking, and duration/intensity of smoking among ever smokers to derive pack-years). Leisure-time physical activity (LTPA) was assessed and activities converted to metabolic equivalents (METs; Ainsworth et al. 1993). As previously described (Thai et al. 2014), METs categorized participants into 4 LTPA categories according to the 2008 Physical Activity Guidelines for Americans: no LTPA reported, low (0 to <500 MET min/wk), moderate (500 to <1,000 MET min/wk), or high (≥1,000 MET min/wk).

Statistical Analysis

SAS 9.4 was used for analysis.

The "bacterial burden score" is created by summing the standardized values of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, and *T. denticola* (Desvarieux et al. 2005; Demmer et al. 2010). This a priori determined definition was based on the following: 1) the 1996 World Workshop in Periodontics determination of species for which "strong evidence for etiology" existed (*A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*); 2) earlier studies of bacterial complexes demonstrating that *T. denticola* co-colonizes with *P. gingivalis* and *T. forsythia*; and 3) previous research linking this score to other cardiometabolic risk biomarkers (Desvarieux et al. 2005; Desvarieux et al. 2010; Desvarieux et al. 2013).

Analysis of variance and categorical analysis were used to obtain descriptive statistics according to bacterial levels and prediabetes status for important demographic, behavioral, dental, and cardiometabolic variables. Pearson correlation coefficients were used to assess associations among bacterial species.

Analysis of Periodontal Outcomes at the Periodontal Site Level

Mixed effects regression models examined the association between bacterial levels and clinical periodontal measures taken from sites adjacent to plaque collection as previously described (Demmer et al. 2008). Briefly, analyses were performed at the site level, and measures of periodontal inflammation (PD or bleeding on probing) were regressed on bacterial levels in mixed effects regressions. Participant was modeled as a random effect to account for within-person correlation of outcomes.

Multivariable Modeling Approach

All regression models were adjusted for the following potential confounders: age, sex, race/ethnicity, education, smoking status, BMI, systolic blood pressure, HDL cholesterol, and SIS. Clinical periodontal status was also included in multivariable models to examine whether bacterial relationships were independent of clinical status. We considered models with varying levels of intermediate adjustment to inform magnitude of confounding and different assumptions regarding causal structure that might contribute to observed associations (i.e., which variables might be confounders vs. mediators). We specifically hypothesized a priori that systolic blood pressure, HDL cholesterol, and inflammation might mediate associations between periodontal infections and prediabetes as previously described (Arora et al. 2014).

Finally, *A. naeslundii* was added to multivariable models as an internal bacterial "control" because this species is not considered as a periodontal pathogen

Appendix Table 1.
Participant Characteristics According to Tertiles of Bacterial Burden Score: Results among Participants Enrolled in ORIGINS, 2011–2013

	All (N = 300)	Tertile 1 (n = 99)	Tertile 2 (n = 99)	Tertile 3 (n = 99)	P Value
Age, y	34 ± 10	36 ± 1	34 ± 1	33 ± 1	0.04
Female	77	76	76	80	0.74
Race/ethnicity					
Hispanic	47	43	46	54	
Non-Hispanic white	23	19	30	19	0.14
Black	17	24	13	14	
Other	13	14	11	13	
Education					
<High school degree	11	16	10	6	
High school degree	22	19	20	26	0.18
Some college or vocational degree	67	65	70	68	
Bachelor degree	33	35	30	32	0.75
>Bachelor degree	67	65	70	68	
Smoking status					
Never	78	73	83	78	
Former	12	16	12	9	0.18
Current	10	11	5	13	
Pack-years of smoking	1 ± 3.6	1.8 ± 0.5	0.7 ± 0.3	0.5 ± 0.2	0.01
Activity level					
None	31	32	30	29	
Low	12	5	18	12	0.19
Moderate	16	18	17	14	
High	41	45	35	45	
Body mass index, kg/m^2	27.1 ± 6.1	27.6 ± 0.7	26.4 ± 0.6	27.1 ± 0.5	0.62
Normal	44	47	48	40	
Overweight	33	29	32	36	0.72
Obese	23	24	20	24	
Family history of diabetes	53	56	53	52	0.38
Mean blood pressure, mm Hg	117.7 ± 12.4	120.3 ± 1.4	115.9 ± 1.2	116.5 ± 1.1	0.03
Systolic	75.2 ± 9.6	77.8 ± 1.0	73.1 ± 0.9	74.5 ± 1.0	0.02
Diastolic	97.9 ± 27.8	100.9 ± 3.0	96.7 ± 2.8	96.7 ± 2.7	0.29
Cholesterol, mg/dL	59.2 ± 16.1	59.2 ± 1.6	58.5 ± 1.7	59.7 ± 1.6	0.83
LDL	77.4 ± 45.4	80.3 ± 4.2	79.1 ± 5.5	72.9 ± 3.7	0.25
HDL	2.0 ± 2.3	2.1 ± 0.3	2.0 ± 0.2	1.9 ± 0.2	0.59
Interleukin 6, pg/mL	1.6 ± 5.7	1.1 ± 0.1	2.4 ± 0.9	1.5 ± 0.4	0.64
C-reactive protein, mg/L	3.4 ± 8.5	2.8 ± 0.4	4.6 ± 1.4	3.0 ± 0.5	0.88
Adiponectin, ng/mL	9,342 ± 4850	9,906 ± 446	8,673 ± 509	9,397 ± 496	0.46
Inflammatory z score, standard normal units	–0.0008 ± 2.4	–0.2 ± 0.2	0.2 ± 0.2	–0.007 ± 0.3	0.53
Mean probing depth, mm	2.4 ± 0.3	2.4 ± 0.03	2.3 ± 0.03	2.4 ± 0.03	0.94
Mean attachment loss, mm	1.5 ± 0.6	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	0.07

(continued)

Appendix Table 1.
(continued)

	All (N = 300)	Tertile 1 (n = 99)	Tertile 2 (n = 99)	Tertile 3 (n = 99)	P Value
Periodontal bleeding on probing, % of sites/mouth	0.5 ± 0.3	0.5 ± 0.02	0.4 ± 0.02	0.5 ± 0.03	0.58
Tooth loss, *n*	4 ± 2.8	4 ± 0.3	4 ± 0.2	4 ± 0.3	0.43
Brushing frequency, %					
<1/d	1	1	0	1	
1/d	14	14	13	15	0.88
≥2/d	85	85	87	84	
Flossing frequency, %					
Never	8	11	2	12	
<Weekly	17	16	18	14	
Once a week	14	11	16	15	0.25
More than once a week	24	22	24	27	
Everyday	37	39	39	32	

Values presented in mean ± SD or percentages. Bacterial burden score is defined as the combined level of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. n = 2, missing for frequency of flossing or brushing; n = 6, missing values for activity level; n = 3, missing bacterial values due to inability to collect dental plaque (1 in each tertile)—therefore, 3 participants are excluded from all results compared across tertile of bacterial level; n = 1, missing clinical periodontal measures; n = 1, missing for LDL; n = 3, missing tumor necrosis factor α; n = 11, with undetectable C-reactive protein; n = 9, missing pack-years information.
HDL, high-density lipoprotein; LDL, low-density lipoprotein; ORIGINS, Oral Infections, Glucose Intolerance, and Insulin Resistance Study.

Appendix Table 2.
Association between Periodontal Microbiota and Bleeding on Probing: Results among 173 Participants (658 Sampled Periodontal Sites) with Moderate/Severe Periodontitis Enrolled in ORIGINS, 2011–2013

Species	Risk Ratio (95% CI)	P Value
Aggregatibacter actinomycetemcomitans	1.04 (0.99, 1.09)	0.16
Porphyromonas gingivalis	1.05 (1.00, 1.09)	0.04
Treponema denticola	1.07 (1.03, 1.11)	0.0002
Tannerella forsythia	1.05 (1.00, 1.09)	0.05
Campylobacter rectus	1.02 (0.97, 1.06)	0.44
Eikenella corrodens	1.01 (0.94, 1.07)	0.84
Fusobacterium nucleatum	1.00 (0.95, 1.05)	0.95
Prevotella intermedia	1.04 (0.99, 1.09)	0.12
Parvimonas micra	1.00 (0.95, 1.06)	0.91
Veillonella parvula	1.05 (0.99, 1.10)	0.08
Actinomyces naeslundii	0.95 (0.91, 0.99)	0.04

Moderate/severe periodontitis defined per the guidelines of the Centers for Disease Control and Prevention and American Academy of Periodontology. Risk ratios for all species, with the exception of *A. naeslundii*, are obtained from models that mutually adjust for *A. naeslundii* levels. The risk ratio for *A. naeslundii* is obtained from the model mutually adjusted for *P. gingivalis* level. Results were consistent for *A. naeslundii* across all models.
ORIGINS, Oral Infections, Glucose Intolerance, and Insulin Resistance Study.

(Desvarieux et al. 2005; Socransky and Haffajee 2005) but nevertheless positively correlates with the other measured species. *A. naeslundii* adjustment enables interpretation of results for other species in terms of ecologic balance.

Results

Generally, crude distributions of potentially confounding variables did not differ meaningfully across bacterial burden tertiles (Appendix Table 1). The strongest and most consistent trend a decrease in mean age and smoking intensity across increasing bacterial tertiles; mean age was ~3 y lower and smoking intensity ~1.5 pack-years lower among participants with third- vs. first-tertile bacterial levels.

The prevalence estimates of none/mild, moderate, and severe periodontitis were 42%, 52%, and 6%, respectively. Among 1,188 periodontal sites adjacent to dental plaque collection, 88% had PD ≤3 mm; 10% had PD = 4 mm, and 2% had PD >4 mm. The percentages of sites with 0, 1, 2, 3, 4, or ≥5 mm of AL were, respectively, 5%, 29%, 43%, 12%, 7%, and 4%; 72% of sites adjacent to plaque collection had bleeding on probing.

In crude analyses, full-mouth clinical periodontal measures were unrelated to

Appendix Table 3.
Multivariable Risk Ratios Summarizing Estimators of Prevalent Prediabetes: Results among 300 Participants Enrolled in ORIGINS, 2011–2013

	Crude RR (95% CI)	*P* Value	Multivariable-Adjusted RR[a] (95% CI)	*P* Value	Multivariable-Adjusted RR[b] (95% CI)	*P* Value
Age, 5-y increase	1.46 (1.32, 1.62)	<0.0001	1.43 (1.24, 1.65)	<0.0001	1.41 (1.28, 1.56)	<0.0001
Female	1.50 (0.77, 2.91)	0.23	1.86 (0.92, 3.75)	0.08	2.02 (1.02, 3.98)	0.04
Race/ethnicity (ref = Hispanic)	Ref	<0.0001	Ref	0.03	Ref	0.01
Black	1.30 (0.77, 2.21)	0.33	0.65 (0.36, 1.18)	0.16	0.70 (0.42, 1.17)	0.17
Other	0.79 (0.38, 1.66)	0.53	1.01 (0.46, 2.21)	0.98	1.05 (0.56, 1.97)	0.87
Non-Hispanic white	0.07 (0.01, 0.48)	0.007	0.13 (0.01, 1.23)	0.08	0.15 (0.02, 1.08)	0.06
≥Bachelor degree	0.33 (0.21, 0.54)	<0.0001	0.83 (0.47, 1.48)	0.54		
Smoking status (ref = never)	Ref	0.56	Ref	0.54	NA	NA
Former	1.45 (0.77, 2.75)	0.25	1.09 (0.54, 2.18)	0.80	NA	NA
Current	1.24 (0.57, 2.66)	0.59	0.69 (0.33, 1.45)	0.32	NA	NA
Activity level (ref = none)	Ref	0.008	Ref	0.30	NA	NA
Low	0.90 (0.45, 1.83)	0.78	1.31 (0.72, 2.36)	0.37	NA	NA
Moderate	0.84 (0.44, 1.62)	0.61	1.09 (0.59, 2.04)	0.78	NA	NA
High	0.36 (0.19, 0.70)	0.0026	0.61 (0.32, 1.17)	0.14	NA	NA
Body mass index (ref = normal)	Ref	0.002	Ref	0.61	NA	NA
Overweight	2.07 (1.08, 3.95)	0.03	0.77 (0.40, 1.48)	0.44	NA	NA
Obese	3.09 (1.65, 5.79)	0.0004	1.01 (0.53, 1.94)	0.98	NA	NA
Systolic blood pressure, 12 mm Hg	1.69 (1.41, 2.02)	<0.0001	1.52 (1.26, 1.85)	<0.0001	1.41 (1.21, 1.66)	<0.0001
HDL cholesterol, 16 mg/dL	0.64 (0.49, 0.85)	0.001	0.57 (0.41, 0.78)	0.0006	0.64 (0.48, 0.83)	0.001
Triglycerides, 45 mg/dL	1.16 (0.99, 1.37)	0.08	0.80 (0.62, 1.03)	0.08	NA	NA
Inflammatory score	1.38 (1.12, 1.70)	0.002	0.98 (0.74, 1.29)	0.87	NA	NA

ORIGINS, Oral Infections, Glucose Intolerance, and Insulin Resistance Study; ref, reference.
[a]Results when all variables are modeled simultaneously.
[b]Results including only statistically significant variables from model a.

bacterial levels, with the exceptions of *A. actinomycetemcomitans* and *T. denticola*, which had modest inverse associations with AL. This was due to the aforementioned age and smoking patterns, as analyses adjusted for age and smoking showed no relationship between bacteria and clinical periodontal variables (data not shown). Findings were unchanged in analyses restricted to only those sites from which dental plaque samples were collected. However, periodontal site-level analyses among participants with moderate/severe periodontitis (defined per the guidelines of the Centers for Disease Control and Prevention / American Academy of Periodontology) demonstrated that *P. gingivalis*, *T. denticola*, and *T. forsythia* were positively associated with periodontal inflammation, while these associations were inversely associated for *A. naeslundii* (Appendix Table 2).

Appendix References

Ainsworth BE, Haskell WL, Leon AS, Jacobs DR, Montoye HJ, Sallis JF, Paffenbarger RS. 1993. Compendium of physical activities: classification of energy costs of human physical activities. Med Sci Sports Exerc. 25(1):71–80.

Arora N, Papapanou PN, Rosenbaum M, Jacobs DR Jr, Desvarieux M, Demmer RT. 2014. Periodontal infection, impaired fasting glucose and impaired glucose tolerance: results from the Continuous National Health and Nutrition Examination Survey 2009–2010. J Clin Periodontol. 41(7):643–652.

Behle JH, Sedaghatfar MH, Demmer RT, Wolf DL, Celenti R, Kebschull M, Belusko PB, Herrera-Abreu M, Lalla E, Papapanou PN. 2009. Heterogeneity of systemic inflammatory responses to periodontal therapy. J Clin Periodontol. 36(4):287–294.

Demmer RT, Papapanou PN, Jacobs DR Jr, Desvarieux M. 2008. Bleeding on probing differentially relates to bacterial profiles: the Oral Infections and Vascular Disease Epidemiology Study. J Clin Periodontol. 35(6):479–486.

Demmer RT, Papapanou PN, Jacobs DR Jr, Desvarieux M. 2010. Evaluating clinical periodontal measures as surrogates for bacterial exposure: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). BMC Med Res Methodol. 10(2):1–7.

Desvarieux M, Demmer RT, Jacobs DR, Papapanou PN, Sacco RL, Rundek T. 2013. Changes in clinical and microbiological periodontal profiles relate to progression of carotid intima-media thickness: the Oral Infections and Vascular Disease Epidemiology Study. J Am Heart Assoc. 2(6):e000254.

Desvarieux M, Demmer RT, Jacobs DR Jr, Rundek T, Boden-Albala B, Sacco RL, Papapanou PN. 2010. Periodontal bacteria and hypertension: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). J Hypertens. 28(7):1413–1421.

Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs DR Jr, Sacco RL, Papapanou PN. 2005. Periodontal microbiota and carotid intima-media thickness: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). Circulation. 111(5):576–582.

Friedewald WT, Levy RI, Fredrickson DS. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem. 18(6): 499–502.

Papapanou PN, Neiderud AM, Papadimitriou A, Sandros J, Dahlen G. 2000. "Checkerboard" assessments of periodontal microbiota and serum antibody responses: a case-control study. J Periodontol. 71(6):885–897.

Socransky SS, Haffajee AD. 2005. Periodontal microbial ecology. Periodontol 2000. 38:135–187.

Socransky SS, Smith C, Martin L, Paster BJ, Dewhirst FE, Levin AE. 1994. "Checkerboard" DNA-DNA hybridization. Biotechniques. 17(4):788–792.

Thai A, Papapanou PN, Jacobs DR Jr, Desvarieux M, Demmer RT. 2014. Periodontal infection and cardiorespiratory fitness in younger adults: results from continuous national health and nutrition examination survey 1999–2004. PLoS One. 9(3):e92441.