

Porphyromonas gingivalis and Disease-Related Autoantibodies in Individuals at Increased Risk of Rheumatoid Arthritis

Ted R. Mikuls,¹ Geoffrey M. Thiele,¹ Kevin D. Deane,² Jeffrey B. Payne,³ James R. O'Dell,¹ Fang Yu,⁴ Harlan Sayles,⁴ Michael H. Weisman,⁵ Peter K. Gregersen,⁶ Jane H. Buckner,⁷ Richard M. Keating,⁸ Lezlie A. Derber,² William H. Robinson,⁹ V. Michael Holers,² and Jill M. Norris¹⁰

Objective. To examine the relationship of *Porphyromonas gingivalis* to the presence of autoantibodies in individuals at risk of rheumatoid arthritis (RA).

Methods. Study participants included the following: 1) a cohort enriched in subjects with HLA-DR4 and 2) subjects at risk of RA by virtue of having a first-degree relative with RA. None of the study subjects satisfied the American College of Rheumatology 1987 classification criteria for RA. Autoantibodies measured included anti-citrullinated protein antibody (ACPA; by second-generation anti-cyclic citrullinated peptide

antibody enzyme-linked immunosorbent assay [ELISA]) and rheumatoid factor (RF; by nephelometry or ELISA for IgA, IgM, or IgG isotype). Individuals were considered autoantibody positive ($n = 113$) if they had ≥ 1 RA-related autoantibody; individuals were further categorized as high risk ($n = 38$) if they had ACPA or positive findings ≥ 2 assays for RF. Autoantibody-negative individuals ($n = 171$) served as a comparator group. Antibody to *P gingivalis*, *P intermedia*, and *F nucleatum* were measured. Associations of bacterial antibodies with group status were examined using logistic regression.

Results. Anti-*P gingivalis* concentrations were higher in high-risk ($P = 0.011$) and autoantibody positive group ($P = 0.010$) than in the autoantibody negative group. There were no group differences in anti-*P intermedia* or anti-*F nucleatum* concentrations. After multi-variable adjustment, anti-*P gingivalis* concentrations (but not anti-*P intermedia* or anti-*F nucleatum*) were significantly associated with autoantibody-positive and high-risk status ($P < 0.05$).

Conclusion. Immunity to *P gingivalis*, but not *P intermedia* or *F nucleatum*, is significantly associated with the presence of RA-related autoantibodies in individuals at risk of RA. These results support the hypothesis that infection with *P gingivalis* may play a central role in the early loss of tolerance to self antigens that occurs in the pathogenesis of RA.

Periodontitis (PD) has emerged as a risk factor for rheumatoid arthritis (RA). Affecting more than 20% of the general population (1), PD is an inflammatory disorder initiated by bacterial infection, which has a detrimental impact on the integrity of several different oral tissues, including the gingiva, cementum, and periodontal ligament, ultimately leading to tooth loss. PD

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¹Ted R. Mikuls, MD, MSPH, Geoffrey M. Thiele, PhD, James R. O'Dell, MD: Omaha VA Medical Center and University of Nebraska Medical Center, Omaha; ²Kevin D. Deane, MD, Lezlie A. Derber, MSPH, V. Michael Holers, MD: University of Colorado, Aurora; ³Jeffrey B. Payne, DDS, MDentSc: University of Nebraska Medical Center, Lincoln; ⁴Fang Yu, PhD, Harlan Sayles, MS: University of Nebraska Medical Center, Omaha; ⁵Michael H. Weisman, MD: Cedars-Sinai Medical Center, Los Angeles, California; ⁶Peter K. Gregersen, MD: Feinstein Institute for Medical Research, Manhasset, New York; ⁷Jane H. Buckner, MD: Benaroya Research Institute, Seattle, Washington; ⁸Richard M. Keating, MD: University of Chicago, Chicago, Illinois; ⁹William H. Robinson, MD, PhD: VA Palo Alto Health Care System, Palo Alto, California, and Stanford University, Stanford, California; ¹⁰Jill M. Norris, MPH, PhD: University of Colorado, Denver.

Address correspondence to Ted R. Mikuls, MD, MSPH, Department of Medicine, University of Nebraska Medical Center, 986270 Nebraska Medical Center, Omaha, NE 68198-6270. E-mail: tmikuls@unmc.edu.

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and RA share several common disease attributes, including chronic tissue inflammation and, in severe cases, marked destruction of the underlying bone. Similarities between PD and RA extend beyond histopathologic and inflammatory features. Both PD and RA share common risk factors for susceptibility, most notably, HLA-DRB1 alleles and, importantly, cigarette smoking (2–9). Moreover, therapies used in RA have been reported to ameliorate the signs and symptoms of PD (10–12).

Several cross-sectional case–control investigations have corroborated the association of PD with RA, although these findings were not replicated in 2 recent studies (13,14). Compared to controls, RA patients experience more gingival bleeding, more missing teeth, twice as much loss of soft tissue attachment, and increased alveolar bone loss (15,16). In a recent study, patients with RA were almost twice as likely as those with osteoarthritis to have moderate-to-severe PD, an association that was independent of age, sex, race, and smoking history (17).

While most studies investigating the relationship between PD and RA have focused on shared inflammatory pathways, few have examined the associations of RA with the bacterial infections that initiate PD. A number of gram-negative oral pathogens have been implicated in PD, and several have garnered attention. Chief among the organisms of interest is *Porphyromonas gingivalis*. *P. gingivalis* has been reported to be the only prokaryote known to express peptidylarginine deiminase (PAD) (18,19), an enzyme responsible for the posttranslational modification of arginine into citrulline. Given the predominant role of citrullinated proteins in RA pathogenesis, it has been speculated that infection with *P. gingivalis* could facilitate autoantigen presentation and loss of tolerance in RA (19).

Investigations of *P. gingivalis* in RA have primarily involved studies examining RA patients with established disease. Based on these studies alone, it is not possible to know with certainty whether infection with *P. gingivalis* precedes RA onset or whether it occurs subsequent to RA disease incidence. Therefore, in the present study, we sought to examine the association of *P. gingivalis* infection with the presence of RA-related autoantibodies among individuals at increased risk of developing RA but without clinically evident disease. The existence of such an association in the absence of clinically apparent inflammatory arthritis would strongly support the hypothesis that infection precedes disease and is therefore not simply a consequence of established RA or its treatments. The existence of such an association would

also strongly support a central role of *P. gingivalis* in the initiation of RA.

SUBJECTS AND METHODS

Study population. Study subjects were participants in Studies of the Etiology of Rheumatoid Arthritis (SERA), an ongoing longitudinal cohort study. SERA is a multicenter prospective cohort study designed to investigate genetic and epidemiologic associations with RA-related autoimmunity during the preclinical period of RA development (20). SERA includes subjects at higher risk of developing RA, who were recruited from 2 populations: 1) a cohort enriched in the HLA-DR4 allele (the strongest genetic risk factor for RA), and 2) a cohort of first-degree relatives (FDRs; parent, full sibling, or offspring) of individuals with RA. Subjects were excluded from participation in SERA if they were <18 years of age, satisfied the American College of Rheumatology (ACR) 1987 classification criteria for RA (21), or had previously been diagnosed by a board-certified rheumatologist as having RA.

Individuals comprising the HLA-DR4-enriched cohort were parents of children enrolled in the Diabetes Autoimmunity Study in the Young (DAISY), a cohort of children with an increased risk of type 1 diabetes mellitus, through either the presence of HLA-DR4 or a family history of type 1 diabetes mellitus (22). DAISY parents have a prevalence of DR4 positivity that approaches 45% (22), which is higher than the background prevalence rates observed in populations of similar ancestry. FDRs of probands with RA were recruited by contact through the probands' rheumatologists from clinics at US academic centers, veterans hospitals, and private and public sector rheumatology clinics based in New York, Chicago, Omaha (as the center for the Rheumatoid Arthritis Investigational Network), Denver, Seattle, and Los Angeles (20). This study was approved by Institutional Review Boards at all study sites and all SERA participants provided informed written consent prior to study enrollment.

In addition to undergoing a blood draw and systematic examinations for evidence of early inflammatory arthritis, SERA participants provided enrollment information relevant to sociodemographic features (including age, sex, race/ethnicity, and education status) in addition to medical history and health-related behaviors (including comorbid diabetes mellitus and cigarette smoking status). Study participants were also asked about the presence of PD-related signs and symptoms at enrollment using the following questions (23): 1) Do your gums bleed after you brush your teeth? 2) Have you ever been told by a dentist or dental hygienist that you have gingivitis or gum disease? 3) Have you ever been told by a dentist or dental hygienist that you have deep gingival pockets? Formal dental/periodontal examinations were not included in the SERA study; thus, corresponding data were not available for these analyses.

Autoantibody testing and classification of subjects.

The measurement of RA-related autoantibodies was completed at the Clinical Research Laboratory of the University of Colorado Division of Rheumatology (SERA Coordinating Center). Anti-citrullinated protein antibody (ACPA) was measured using a second-generation anti-cyclic citrullinated peptide (anti-CCP-2; positive ≥ 5 units/ml) enzyme-linked

immunosorbent assay (ELISA) (Diastat; Axis-Shield). IgM, IgG, and IgA isotypes of rheumatoid factor (RF; in IU/ml) were measured by ELISA using Quanta Lite kits (Inova Diagnostics). RF was also measured by nephelometry (in IU/ml) according to the manufacturer's specifications (Dade Behring). For all RF assays (ELISA and nephelometry), positivity was defined as a serum concentration exceeding that observed in 95% of healthy controls according to the ACR criteria for RA (21).

For this study, a subset of SERA participants was examined based on a strategy of oversampling autoantibody-positive individuals with a random selection of visits from the remaining autoantibody-negative subjects. Analyses were limited to a single visit for each individual included in the study. SERA participants were classified as autoantibody positive if they had at least 1 RA-related autoantibody; a subset of these patients were further categorized as "high risk" if they were positive for ACPA or at least 2 of the 4 RF assays performed at a single visit. The high-risk definition is based on prior data suggesting that individuals with ACPA and/or RF have a substantially higher risk of developing RA (24–26). Furthermore, this classification scheme was based specifically on data from 980 SERA RA case probands and 200 healthy, randomly selected blood donors (controls), where positive findings on the ACPA assay or on at least 2 of the 4 RF assays was 96% specific to RA. For participants with the high-risk autoantibody profile who had multiple study visits, we examined the first study visit at which the patient satisfied the high-risk profile. For autoantibody-positive individuals not meeting the high-risk profile definition, we used the first study visit at which individuals were positive for a single RF test. Of note, in prior studies, a single RF isotype yielded a specificity for the future occurrence of RA in a range exceeding 93% (24–26). For autoantibody-negative individuals, a single random study visit was used. Autoantibody-negative FDRs and DAISY parents without positive test results for autoantibodies at all available study visits served as the referent group for analyses.

HLA-DRB1 genotyping. Participants were genotyped for HLA-DRB1 shared epitope (SE)-containing alleles as previously described (20). Briefly, complete subtyping for HLA-DR4 alleles was done via a modification of a real-time polymerase chain reaction (PCR) approach. HLA-DR4 subtypes that were considered SE positive included DRB1*0401, 0404, 0405, 0408, 0409, 0410, 0413, 0416, 0419, and 0421. A real-time low-resolution PCR analysis was also performed to identify the presence of SE-containing DR1 alleles, including *0101, 0102, 0104, 0105, 0107, 0108, and 0111.

Antibody to oral pathogens. Antibody to *P. gingivalis* (IgG anti-*P. gingivalis*) was measured at the Experimental Immunology Laboratory of the University of Nebraska Medical Center (27), as previously described. Briefly, strain 381 of *P. gingivalis* (ATCC) was grown in reducing broth (10 gm of yeast extract, 30 gm of trypticase soy broth, 1 gm of cysteine, 100 mg of dithiothreitol, 5 mg of hemin, and 2.5 mg of menadione in a 1-liter volume) (28). The cells were grown at 37°C for 24 hours, with constant low-speed shaking (150 revolutions per minute), to an optical density of 1.5 at 660 nm.

Outer membrane antigens from *P. gingivalis* were prepared as described elsewhere (29). Briefly, cells were pelleted from the broth culture medium at 6,500 rpm for 20 minutes in

200-ml bottles. The supernatant was removed, and the pellet was resuspended in 0.15M NaCl containing protease inhibitor 3 and frozen in a minimal volume overnight at -80°C. The next day, the suspension was thawed and washed twice more with 200 ml of 0.15M NaCl containing protease inhibitor 3, by centrifugation at 6,500 rpm for 20 minutes. The pellet was suspended in 50 ml of a buffer containing 0.05M sodium phosphate (with protease inhibitor), 0.15M NaCl, and 0.01M EDTA, pH 7.4. The suspension was sonicated on ice 5 times (1 minute each at 70W) using a Misonix XL-2000 instrument equipped with a one-quarter-inch microtip, cooling the probe between sonications. The sonicate was centrifuged at 12,000g for 20 minutes using a 50Ti rotor in a Beckman Ultracentrifuge. The pellet was discarded, the supernatant collected, and the protein concentration was determined using a Bio-Rad protein assay.

An ELISA was adapted from the procedure described by Engvall and Perlmann (30). *P. gingivalis* antigen was coated to ELISA plates using 100–200 ng/well of sonic extracts. Additionally, purified human IgG (Jackson ImmunoResearch) was serially diluted down the appropriate ELISA plates starting at 1,280 ng/ml, to construct a standard curve. Two-fold serum dilutions from patients (first dilution 1:100) were added to the plate, and the bound human IgG was detected with a peroxidase-conjugated, affinity-purified, Fc-specific anti-human IgG antibody (Jackson ImmunoResearch) and then developed using tetramethylbenzidine substrate (Becton-Dickinson). Absorbance was detected after stopping the reaction using 2N H₂SO₄ and reading at 450 nm using an MRX II Microplate Reader (Dynatech). Data were analyzed using Revelations Software (Dynatech), and the bacterial concentration (in pg/ml) was extrapolated from a standard curve using purified human IgG. Previous studies have demonstrated that several strains of *P. gingivalis*, including strain 381, express common outer membrane proteins (31). Furthermore, case-control studies involving patients with PD have demonstrated higher mean antibody reactivity among cases as compared to controls for a majority of outer membrane proteins identified (31,32).

To examine potential issues of assay cross-reactivity and whether associations were unique to *P. gingivalis*, we also measured antibody to *Prevotella intermedia* (IgG anti-*P. intermedia*) and *Fusobacterium nucleatum* using similar approaches. Similar to *P. gingivalis*, *P. intermedia* (formerly known as *Bacteroides intermedius*; ATCC) and *F. nucleatum* (ATCC) are gram-negative anaerobes that act as major oral pathogens in PD (33,34). *P. intermedia* is specifically known to frequently coaggregate with *P. gingivalis* in PD-related biofilms (35). All bacterial antibody concentrations (in µg/ml) were extrapolated from a standard curve and then log-transformed for analysis. The intraassay coefficients of variation for the bacterial ELISAs ranged from 11% to 13%. There were no changes in the measured bacterial antibody concentrations in a subset of 20 serum samples following RF depletion (performed using HeteroBlock; Omega Biologicals [36]).

Data analysis. Group characteristics (autoantibody-positive and high-risk groups versus autoantibody-negative group) and the prevalence of self-reported PD signs and symptoms were compared using the chi-square test for categorical variables and Student's *t*-test for continuous variables. Given their skewed distributions, bacterial antibody concentrations

were normalized using natural log transformation. Log-transformed bacterial serology values were compared by group using Student's *t*-test. The correlation of transformed anti-bacterial antibody concentrations was examined by Spearman's correlation.

Associations of bacterial antibody concentrations with autoantibody-positive and high-risk status (versus autoantibody-negative controls) were examined using logistic regression analysis, with autoantibody-positive and high-risk status assessed in separate models. Associations were examined by generating odds ratios (ORs) and 95% confidence intervals (95% CIs). Subsequent multivariable regression analyses were conducted to adjust for confounding. In addition to both bacterial serologies (anti-*P gingivalis* + anti-*P intermedia* modeled separately from anti-*P gingivalis* + anti-*F nucleatum*), variables examined in multivariable models included factors associated with RA and/or PD (37) (age, sex, race [Caucasian versus other], ever smoking, HLA-DRB1 SE status [1 or 2 alleles versus none], presence of diabetes mellitus, and education level [more than high school versus high school or less]). In sensitivity analyses, we also examined the associations of bacterial serology findings with high-risk status, using an alternative definition of high risk of RA that included either positivity for ACPA or positivity for 2 or more RF isotypes (eliminating RF nephelometry results from the definition). All analyses were completed using Stata v10.1 software (StataCorp).

RESULTS

There were 284 subjects included in the analyses, including 171 autoantibody negative and 113 autoantibody positive individuals. Of the 113 subjects categorized

as autoantibody positive, 38 were further categorized as high-risk based on the presence of ACPA or positive findings on ≥ 2 RF assays. When high-risk status was alternatively defined as positivity for ACPA or ≥ 2 of 3 RF isotypes (eliminating RF nephelometry from the definition), there were 33 individuals categorized as being high-risk. Group characteristics are summarized in Table 1. Compared to the autoantibody-negative subjects (mean \pm SD age 44 ± 14 years), both the autoantibody-positive group (48 ± 15 years of age; $P = 0.044$) and the high-risk group (51 ± 16 years of age; $P = 0.012$) were older at enrollment. No other significant differences between the autoantibody-positive or high-risk groups compared to the autoantibody-negative group were noted. Specifically, there were no group differences in the prevalence of known PD risk factors, including HLA-DRB1 SE positivity, smoking status, or diabetes mellitus (Table 1). Relative to the autoantibody-negative group, there were also no significant differences in the prevalence of self-reported signs and symptoms of PD, including gum bleeding, a diagnosis of gum disease or gingivitis, or the presence of deep periodontal pockets, in either the autoantibody-positive group or the high-risk group.

Rates of positivity for the different RA-related autoantibodies in the autoantibody-positive and high-risk groups are summarized in Table 2. Eight of the subjects were ACPA positive, comprising 7% of those

Table 1. Descriptive characteristics and frequency of self-reported signs and symptoms of periodontitis among SERA participants*

	Autoantibody negative (n = 171)	Autoantibody positive (n = 113)	High risk (n = 38)
Sociodemographic features and RA risk factors			
Age, mean \pm SD years	44 \pm 14	48 \pm 15†	51 \pm 16†
Female, %	69	73	76
Caucasian, %	77	82	82
Ever smoked cigarettes, %	37	31	29
Diabetes mellitus, %	5	4	5
More education than high school, %	79	79	73
HLA-DRB1 shared epitope positive, %	55	55	58
Signs and symptoms of periodontitis, %			
Gum bleeding	22	26	19
Gum disease or gingivitis	21	23	19
Deep periodontal pockets	15	18	20

* Autoantibody positivity was defined as the presence of at least 1 rheumatoid arthritis (RA)-related autoantibody. High risk was defined as the presence of anti-citrullinated protein antibody or positive findings on ≥ 2 assays for rheumatoid factor (by nephelometry or enzyme-linked immunosorbent assay for IgA, IgM, or IgG isotype). Autoantibody negativity was defined as the absence of all RA-related autoantibodies examined. All subjects classified as high risk are also included in the autoantibody-positive group (i.e., all subjects positive for ≥ 1 RA-related autoantibody). SERA = Studies of the Etiology of Rheumatoid Arthritis.

† $P < 0.05$ versus autoantibody-negative control group.

Table 2. Frequency of RA-related autoantibodies in study participants classified as autoantibody positive or high risk*

	Autoantibody positive (n = 113)	High risk (n = 38)
ACPA, no. (%)	8 (7)	8 (21)
RF, no. (%)		
Nephelometry	38 (34)	18 (47)
ELISA		
IgA	26 (23)	16 (42)
IgM	36 (32)	25 (66)
IgG	60 (53)	26 (68)

* Autoantibody positivity was defined as the presence of at least 1 rheumatoid arthritis (RA)-related autoantibody. High risk was defined as the presence of anti-citrullinated protein antibody (ACPA) or positive findings on ≥ 2 assays for rheumatoid factor (RF; by nephelometry or enzyme-linked immunosorbent assay [ELISA] for IgA, IgM, or IgG isotype). All subjects classified as high risk are also included in the autoantibody-positive group (i.e., all subjects positive for ≥ 1 RA-related autoantibody).

positive for at least 1 autoantibody and 21% of the high-risk subgroup.

Concentrations (log-transformed) of circulating IgG antibody to *P gingivalis*, *P intermedia*, and *F nucleatum* in each study group are shown in Figure 1. Anti-*P gingivalis* antibody concentrations were significantly correlated with both anti-*P intermedia* ($r = 0.60$, $P < 0.001$) and anti-*F nucleatum* ($r = 0.45$, $P < 0.001$). Log-transformed anti-*P gingivalis* concentrations were higher in those with at least 1 autoantibody than in those with no autoantibodies (mean \pm SD 4.89 ± 1.00 versus 4.59 ± 0.88 ; $P = 0.010$), a difference that was numerically greater in high-risk individuals (5.03 ± 1.11 versus 4.59 ± 0.88 ; $P = 0.011$).

In contrast, there were no significant differences in anti-*P intermedia* concentrations among those with at least 1 autoantibody (5.46 ± 0.70 ; $P = 0.564$) or in high-risk individuals (5.57 ± 0.48 ; $P = 0.191$) as compared to autoantibody-negative individuals (5.42 ± 0.66). Likewise, there was no difference in anti-*F nucleatum* antibody concentrations among autoantibody-positive individuals (4.49 ± 0.82 , $P = 0.59$) or high-risk individuals (4.48 ± 0.90 , $P = 0.80$) compared to autoantibody-negative individuals (4.44 ± 0.84) (Figure 1). There were no significant differences in the findings on any of the bacterial serologies, including anti-*P gingivalis*, in comparisons of ACPA-positive subjects ($n = 8$) with autoantibody-negative subjects (data not shown).

Univariate and adjusted associations of log-transformed anti-*P gingivalis*, anti-*P intermedia*, and anti-*F nucleatum* concentrations with the presence of at least 1 RA-related autoantibody and with high-

risk status (versus autoantibody-negative controls) are summarized in Table 3. In unadjusted analyses, anti-*P gingivalis* concentrations (per log unit increase) were significantly associated with autoantibody-positive status ($OR_{unadj} 1.41$ [95% CI 1.08–1.85], $P = 0.011$), but anti-

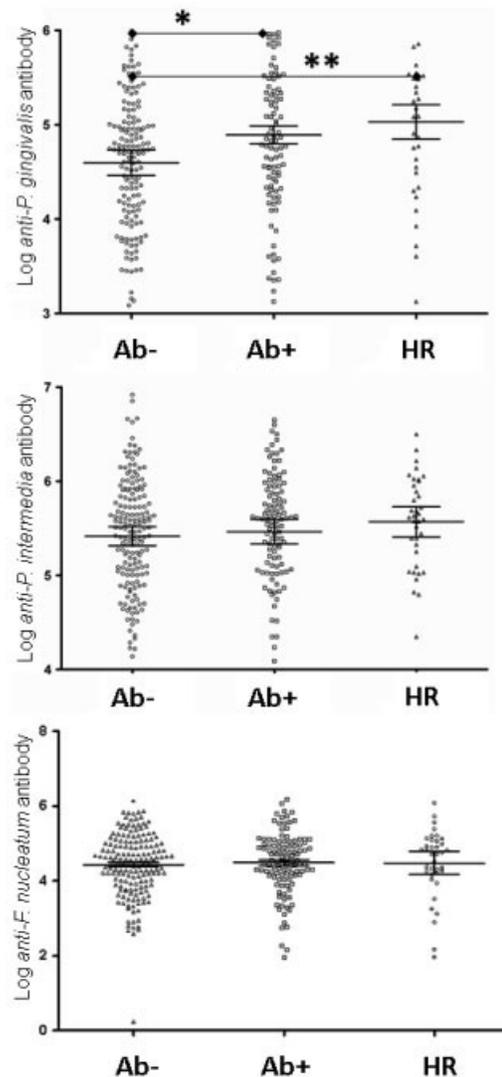


Figure 1. Log-transformed concentrations of antibody to *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum* based on autoantibody-positive (Ab+; $n = 113$), high-risk (HR; $n = 38$), or autoantibody-negative (Ab-; $n = 171$) status. Autoantibody-positive subjects were those positive for ≥ 1 rheumatoid arthritis-related autoantibody. High-risk subjects were those positive for anti-citrullinated protein antibody or for rheumatoid factor on ≥ 2 assays (by nephelometry or enzyme-linked immunosorbent assay for IgA, IgM, or IgG isotype). Each data point represents a single subject; horizontal lines and error bars show the mean \pm SD. * = $P = 0.010$; ** = $P = 0.011$.

Table 3. Associations of antibodies to *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum* with the presence of RA-related autoantibodies among SERA participants*

Bacterial IgG antibody	Autoantibody positive versus control			High risk versus control		
	Univariate	Multivariable model A†	Multivariable model B†	Univariate	Multivariable model A†	Multivariable model B†
Anti- <i>P. gingivalis</i>						
OR (95% CI)	1.41 (1.08–1.85)	1.56 (1.10–2.22)	1.39 (1.00–1.92)	1.68 (1.12–2.52)	1.72 (1.01–2.95)	1.70 (1.05–2.74)
P	0.011	0.013	0.047	0.012	0.046	0.031
Anti- <i>P. intermedia</i>						
OR (95% CI)	1.11 (0.78–1.59)	0.76 (0.47–1.23)	–	1.51 (0.82–2.80)	0.97 (0.42–2.27)	–
P	0.563	0.268	–	0.188	0.953	–
Anti- <i>F. nucleatum</i>						
OR (95% CI)	1.08 (0.81–1.45)	–	0.98 (0.67–1.43)	1.06 (0.69–1.62)	–	1.01 (0.57–1.76)
P	0.594	–	0.907	0.800	–	0.985

* Autoantibody positivity was defined as the presence of at least 1 rheumatoid arthritis (RA)-related autoantibody. High risk was defined as the presence of anti-citrullinated protein antibody or positive findings on ≥ 2 assays for rheumatoid factor (by nephelometry or enzyme-linked immunosorbent assay for IgA, IgM, or IgG isotype). All subjects classified as high risk are also included in the autoantibody-positive group (i.e., all subjects positive for ≥ 1 RA-related autoantibody). Associations were determined using log-transformed bacterial IgG antibody concentrations ($\mu\text{g/ml}$). SERA = Studies of the Etiology of Rheumatoid Arthritis; OR = odds ratio; 95% CI = 95% confidence interval.

† Adjusted for age, sex, race, ever smoker, shared epitope status, diabetes mellitus status, education level, and alternative bacterial serology findings (model A includes anti-*P. gingivalis* and anti-*P. intermedia*, and model B includes anti-*P. gingivalis* and anti-*F. nucleatum*).

P. intermedia (OR_{unadj} 1.11 [95% CI 0.78–1.59], $P = 0.563$) and anti-*F. nucleatum* (OR_{unadj} 1.08 [95% CI 0.81–1.45], $P = 0.594$) concentrations were not. Unadjusted associations of anti-*P. gingivalis* with high-risk status (OR_{unadj} 1.68 [95% CI 1.12–2.52], $P = 0.012$) were also significant, whereas the associations of anti-*P. intermedia* with high-risk status were not significant (OR_{unadj} 1.51 [95% CI 0.82–2.80], $P = 0.188$). Anti-*F. nucleatum* antibody showed no association with high-risk status, and these results did not change following multivariable adjustment.

The findings of the univariate analyses were not substantially changed following multivariable adjustment referent to anti-*P. gingivalis* concentrations, which remained significant. In contrast, associations of anti-*P. intermedia* concentrations with high-risk status were completely attenuated following multivariable adjustment (OR_{adj} 0.97 [95% CI 0.42–2.27], $P = 0.953$) (Table 3).

In multivariable analyses that included results from all 3 bacterial serologies in addition to the aforementioned covariates, the association of anti-*P. gingivalis* with autoantibody positivity (OR_{adj} 1.51 [95% CI 1.04–2.20], $P = 0.032$) and high-risk status (OR_{adj} 1.64 [95% CI 0.94–2.89], $P = 0.083$) did not change substantially, although the association did not reach statistical significance for high-risk status. Of the covariates examined, including age, sex, race, smoking, HLA-DRB1 SE status, diabetes mellitus, and education, only older age was significantly associated with autoantibody-positive and high-risk status in multivariable models (data not

shown). Associations of anti-*P. gingivalis* antibody with high-risk status were also unchanged and remained significant when an alternative definition of high-risk status, consisting of positivity to either ACPA or 2 of the 3 RF isotypes, was used (data not shown).

DISCUSSION

Although associations between RA and PD have been noted for several decades (38), the mechanisms underpinning this relationship have not been clearly elucidated. Recent studies, including one from our group (27,39,40), have suggested that infection with *P. gingivalis* is a cofactor in the development and progression of RA. We previously observed that antibodies to *P. gingivalis* are found in significantly higher concentrations among patients with RA than among healthy controls recruited from the community (27). Furthermore, in patients with RA, anti-*P. gingivalis* antibody concentrations have been shown to correlate with the presence of ACPA (27,40). Of 11 different oral bacteria examined in a recent study (41), *P. gingivalis* was shown to be the only organism capable of endogenously citrullinating both fibrinogen and enolase. This is important because autoantibodies directed against the citrullinated form of these antigens are highly specific for RA and have been speculated to play a pathogenic role in disease progression (42). It has been hypothesized that the dual expression of gingipains (lysine- and arginine-specific proteases expressed by several oral bacteria) and PAD by *P. gingivalis* acts in concert in RA, the former produc-

ing a carboxy-terminal arginine residue that then serves as a target for bacterially expressed PAD (18). Citrullination by bacterial PAD appears to be distinct from mammalian PAD that more efficiently citrullinates internal arginine residues (43).

In addition to associations with ACPA, *P. gingivalis* along with other oral pathogens have also been speculated to play a role in RF expression. Patients with PD are more likely than controls to be RF-seropositive, and RF has been identified in the gingival tissue and subgingival plaque of patients with PD (44). Because lysine and arginine residues exist in the Fc region of IgG (45), it has been suggested that modification of these domains by bacterially expressed gingipains leads to targeting and binding with RF (19). Whether the association of *P. gingivalis* with RA operates directly through effects on RF or ACPA generation in the oral cavity or in related lymphatic structures remains unknown and needs further study.

The timing and development of RF and ACPA in relationship to each other in the development of RA are also unknown; some studies suggest that ACPAs are generated prior to RF (25,26), although conversely, a study using samples from the US Department of Defense Serum Repository showed that RF positivity often precedes ACPA expression (46). This area of study is particularly relevant when trying to understand the mechanisms by which PD and infection with *P. gingivalis* may lead to the development of RA-related autoimmunity. For example, given the association of PD with elevations of RF levels in the oral cavity and the association in our study of elevated levels of antibody to *P. gingivalis* in subjects that predominantly had only circulating elevations of RF, *P. gingivalis* infection may initially lead to the development of RF, followed by the generation of ACPAs. Investigations into this area are planned in further studies of longitudinal samples from the SERA project.

Hitchon and colleagues (39) recently examined the association of *P. gingivalis* antibody with the presence of RA-related autoantibodies in a North American native (NAN) population, a study that included patients with RA, unaffected relatives (FDRs), and controls. That study showed that antibodies specific to *P. gingivalis* were found in higher concentrations in both ACPA-positive RA patients and ACPA-positive FDRs as compared to ACPA-negative RA patients and ACPA-negative FDRs, respectively. In addition to important differences in the target populations and the techniques used for the measurement of bacterial serology values (a lipopolysaccharide-based assay in that study versus

whole lysate-based approach in our study), other aspects distinguish these efforts. ACPA positivity was relatively infrequent in our study population (2.8%), precluding meaningful analyses of this subgroup. This rate of ACPA positivity is much lower than the prevalence of 19% reported among NAN FDRs. While this may relate to differences in the background prevalence of disease risk factors such as HLA-DRB1 SE (73% for NAN FDRs versus 55% in our study population), this discrepancy is largely attributable to the different definitions used for ACPA positivity. In the present study, individuals were considered to be ACPA positive based solely on results of the anti-CCP-2 ELISA, since previous reports demonstrated a substantially increased risk of developing RA in patients seropositive by this assay (25,26). In contrast, NAN RA cases and NAN FDRs in the study by Hitchon et al were considered ACPA positive if they were seropositive by either the anti-CCP-2 assay (5% positive) or any anti-CCP-2 isotype assay (IgG1-IgG4, IgA, or IgM). As pointed out by those authors (39), the prognostic implications of anti-CCP-2 isotype positivity among unaffected relatives remain unclear. In addition to ACPA positivity, our high-risk group included individuals seropositive on ≥ 2 RF assays, a definition that was not operant in the prior NAN investigation, but one that has been shown to portend disease risk in other unaffected populations. For example, positivity for ≥ 2 RF isotypes has been shown to have a specificity of 98% for the development of RA (24).

Our results complement and extend previous reports in several meaningful ways. For each log-fold increase in anti-*P. gingivalis* antibody concentration, individuals in our study were 40–70% more likely to be seropositive for RA-related autoantibodies. These associations were independent of all other RA and PD risk factors examined. To our knowledge, ours is the first study to simultaneously examine associations of *P. gingivalis* and alternative oral pathogens with autoantibody expression in individuals at increased risk of RA. Recognizing that *P. intermedia* and other oral bacteria frequently coaggregate with *P. gingivalis* in PD-related biofilms (35), the observed correlations between bacterial antibody concentrations were expected. Importantly, the results of multivariable regression that simultaneously included antibody to *P. gingivalis* and an alternative oral pathogen (in addition to antibodies to all 3 organisms examined) as independent variables showed that associations with the presence of RA-related autoantibodies were specific for *P. gingivalis* and did not extend to at least 2 other oral pathogens frequently implicated in PD. Similar rates of self-reported signs and symptoms

of PD reported across the study groups further support, but do not prove, the contention that infection with *P gingivalis* drives the observed associations with RA-related autoantibody expression, rather than disease-related autoantibody production being a consequence of nonspecific periodontal inflammation. Recognizing only modest sensitivities and moderate predictive values for self-reported PD (23), it would have been optimal to have had results from periodontal examinations. However, results from standardized oral assessments were not part of this study, although such assessments are planned for ongoing studies.

Additional studies with longer followup times and larger sample sizes will be needed to more clearly define the epidemiologic links between *P gingivalis* infection and RA onset. It will be essential that future efforts be designed to more precisely detail the temporal relationship of this infection with the immunologic responses that follow, including the formation of RA-related autoantibodies. Although our study included simultaneous examinations of *P gingivalis* and 2 other oral pathogens, additional efforts will be needed to examine the many other oral pathogens that have been identified but were not included in this study in addition to the complex interactions that are likely to exist between pathogens comprising oral and subgingival microbiomes. Regardless, these results demonstrate that associations of *P gingivalis* infection with RA-related autoantibody expression exist among individuals without clinically apparent RA but with a higher background risk of developing the disease. Furthermore, based on these observations, it is unlikely that established RA serves as the initiating event in this relationship. Specifically, these results refute speculation that PD (and infection with *P gingivalis*) simply represents a consequence of severe RA or an “opportunistic” product of immunosuppressive therapy. Importantly, these results provide insight into a potentially critical environmental trigger in the pathogenesis of RA, one that could be targeted in future interventions aimed at disease prevention.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Mikuls had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Mikuls, Thiele, Deane, O’Dell, Weisman, Derber, Holers, Norris.

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