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Serum antibodies to periodontal pathogens prior to rheumatoid arthritis diagnosis: A case-control study

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ABSTRACT

Objectives: 1) To quantify the association between anti-*Porphyromonas gingivalis* serum antibody concentrations and the risk of developing rheumatoid arthritis (RA), and 2) to quantify the associations among RA cases between anti-*P. gingivalis* serum antibody concentrations and RA-specific autoantibodies. Additional anti-bacterial antibodies evaluated included anti-*Fusobacterium nucleatum* and anti-*Prevotella intermedia*.

Methods: Serum samples were acquired pre- and post- RA diagnosis from the U.S. Department of Defense Serum Repository ($n = 214$ cases, 210 matched controls). Using separate mixed-models, the timing of elevations of anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* antibody concentrations relative to RA diagnosis were compared in RA cases versus controls. Associations were determined between serum anti-CCP2, ACPA fine specificities (vimentin, histone, and alpha-enolase), and IgA, IgG, and IgM RF in pre-RA diagnosis samples and anti-bacterial antibodies using mixed-effects linear regression models.

Results: No compelling evidence of case-control divergence in serum anti-*P. gingivalis*, anti-*F. nucleatum*, and anti-*P. intermedia* was observed. Among RA cases, including all pre-diagnosis serum samples, anti-*P. intermedia* was significantly positively associated with anti-CCP2, ACPA fine specificities targeting vimentin, histone, alpha-enolase, and IgA RF ($p<0.001$), IgG RF ($p = 0.049$), and IgM RF ($p = 0.004$), while anti-*P. gingivalis* and anti-*F. nucleatum* were not.

Conclusions: No longitudinal elevations of anti-bacterial serum antibody concentrations were observed in RA patients prior to RA diagnosis compared to controls. However, anti-*P. intermedia* displayed significant associations with RA autoantibody concentrations prior to RA diagnosis, suggesting a potential role of this organism in progression towards clinically-detectable RA.

Introduction

It has been hypothesized that rheumatoid arthritis (RA) may be initiated in mucosal tissues, including the periodontium [1]. Periodontitis (PD) is a biofilm-driven inflammatory disease of the soft and hard tissues in the oral cavity resulting from an interaction between the host immune response and a dysbiotic oral microbiota, ultimately leading to tooth loss [2]. Over the past few decades, there has been an increased awareness of the relationship between RA and PD. Both diseases share similar inflammatory pathways and risk factors [3]. Several studies have

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demonstrated PD as a risk factor for RA [4–6]. It has been speculated that this relationship may be mediated through the oral periodontal pathogen, *Porphyromonas gingivalis* [7].

P. gingivalis is a gram-negative anaerobe recognized as a keystone pathogen in the pathogenesis of PD [8,9]. Uniquely, it is the only prokaryote that can express a functional bacterial peptidyl arginine deiminase (PAD) enzyme (often termed PPAD) as a primary virulence factor, thus serving as a microbe of interest in its role with RA and PD [10]. The discovery of *P. gingivalis* PAD led to the hypothesis that *P. gingivalis* PAD-mediated protein citrullination at affected periodontal sites can launch a sequence of events that culminate in the generation of anti-citrullinated protein antibodies (ACPAs) and, eventually, in the clinical manifestation of RA [11]. However, while *P. gingivalis* is a periodontal pathogen implicated in RA pathogenesis, other bacterial species involved in PD, such as *Prevotella intermedia* and *Fusobacterium nucleatum*, may also influence development of RA [12,13].

We hypothesized that circulating concentrations of antibody to *P. gingivalis* would be higher in samples from individuals later developing RA compared to controls. Anti-bacterial serologies may be used as a surrogate of exposure to periodontal pathogens and we have previously reported associations between serum antibody to *P. gingivalis* and RA-related autoantibody expression among patients without clinically apparent RA, but with a higher risk of future disease [14]. Moreover, we postulated that, among those with RA, anti-*P. gingivalis* antibodies would be associated with the presence of RA-related autoantibodies prior to diagnosis. The purpose of this study was to: 1) quantify the association between anti-*P. gingivalis* serum antibody concentrations and the risk of developing RA, and 2) quantify the associations among RA cases between anti-*P. gingivalis* serum antibody concentrations and RA-specific autoantibodies. Additional anti-bacterial antibodies evaluated included anti-*P. intermedia* and anti-*F. nucleatum* to determine whether associations observed were specific to *P. gingivalis* or related to a broader dysbiosis that may be observed in PD.

Methods

Patient population

Study participants consisted of military personnel participating in the U.S. Department of Defense Serum Repository (DoDSR). Since 1996, DoDSR has been collecting serum samples to observe health history in the military population and further understand the risks of deployment concerning subsequent injuries or chronic illnesses [15].

Active-duty personnel with ≥2 RA diagnostic codes (≥1 from a rheumatologist) were screened from the military's electronic medical records [16]. The records were further examined to obtain the date of diagnosis and fulfillment of the 1987 American College of Rheumatology classification criteria [17]. Serum samples were acquired prior to and after RA diagnosis for up to four samples per case, a minimum of two samples and up to three samples from pre-diagnosis, collected at different time points, and one sample from post-diagnosis. This study utilized 214 RA cases who received a diagnosis of RA between 1995 and 2012. Out of these cases, 212 met the 1987 RA classification criteria and the other two cases were diagnosed by a board-certified rheumatologist. These RA cases were chosen because there was a clear date of RA diagnosis recorded, adequate information to evaluate the clinical course of their RA after diagnosis, and two or more pre-diagnosis and one post-diagnosis serum samples with adequate volumes available for analysis.

Controls were selected and matched to each case based on age (at time of RA diagnosis for their matched cases), sex, ethnicity, enlistment region, and duration of sample storage. Exclusions for the controls were a history of RA or other inflammatory arthritis [16].

Four of these controls were subsequently excluded due to insufficient information available to exclude inflammatory arthritis, leaving a total of 210 controls evaluable for the analysis. These cases and controls were included in earlier DoDSR studies by our group [16,18].

Clinical data collected included: age at time of diagnosis, sex, ethnicity, smoking status (those with missing data after chart review were imputed as never smokers), sample collection timing relative to RA diagnosis, follow-up time and RA medications received post-RA diagnosis, radiographic erosions, and number of samples tested [16].

Serum autoantibody assays

ACPA was determined using a commercially-available second-generation anti-CCP2 ELISA (Diastat, Axis-Shield Diagnostics, Dundee, Scotland); CCP2 positivity was based on the manufacturer's recommendation at a level of > 5 U/ml. Serum samples also were evaluated for 26 specific ACPAs using a bead-based multiplex antigen array that measures antibody reactivity to a panel of putative citrullinated autoantigens [19]. To reduce the chance of false discovery, analyses of antigen-specific ACPAs were limited to antibodies targeting citrullinated forms of vimentin, alpha-enolase, and histone, which are autoantigens consistently implicated in RA pathogenesis [20–22]. IgA rheumatoid factor (RF), IgG RF, and IgM RF concentrations (IU/ml) were determined using ELISA (Inova Diagnostics, San Diego, CA). RF positivity was based on concentrations for each isotype (IgA RF, IgG RF, and IgM RF) determined to be present in < 2% of controls.

Serum bacterial antibodies

Serum concentrations of IgG antibodies to outer membrane antigens (OMA) of *P gingivalis, P. intermedia,* and *F. nucleatum* were measured by ELISA, as described in a previous publication from our group [14].

Ethical considerations

The Institutional Review Boards approved the study protocol at the DoDSR, Walter Reed National Military Medical Center, and the University of Colorado Multiple Institutional Review Board.

Statistical analyses

Participant characteristics were compared between RA and control groups using chi-square tests, exact chi-square tests, t-tests, or Wilcoxon rank sum tests as necessary. Autoantibodies and bacterial antibodies were log (base 2) transformed for all analyses. The primary analysis investigated the associations between anti-*P. gingivalis* serum antibodies and RA diagnosis (i.e., case status). Anti-*P. intermedia* and anti-*F. nucleatum* were evaluated to determine whether associations observed were specific to *P. gingivalis* or conversely related to a broader dysbiosis observed in PD. Initial analyses compared anti-bacterial antibody concentrations and biomarkers (i.e., anti-CCP2, ACPA fine specificities targeting vimentin, histone and alpha-enolase, and RF isotypes) between groups in the pre-RA diagnosis sample that was closest to diagnosis, and the post-RA diagnosis sample using Wilcoxon rank sum tests. The timing of elevations in anti-bacterial concentrations were evaluated in RA cases versus controls in a manner previously described [16,18]. Briefly, we used mixed models for each bacterial concentration with a continuous time effect modeled using B-splines and assuming a multivariate normal distribution for random subject intercepts and slopes. At each month prior to diagnosis, we compared autoantibody concentrations to identify the first instance where concentrations differed significantly (p <0.05) between cases and controls. These multiple comparisons were using a stepdown Holm-simulated method. Correlations between anti-bacterial antibody concentrations were evaluated by Pearson correlation coefficient.

Secondary analyses examined potential associations between anti-*P. gingivalis*, anti-*P. intermedia,* and anti-*F. nucleatum* antibody concentrations and biomarkers (i.e., anti-CCP2, ACPA fine specificities targeting vimentin, histone and alpha-enolase, and RF isotypes) within the RA

group. These analyses were completed using both unadjusted and adjusted mixed-effects linear regression models with either RF or ACPA as the dependent variable, a fixed effect for each of the anti-bacterial antibodies in turn, and random subject intercepts. The adjusted models also included terms for age, sex, and smoking status.

All analyses were performed utilizing SAS v9.4 (SAS Institute, Cary, NC).

Results

Participant characteristics and autoantibody values

Patient characteristics and median autoantibody concentrations of the participants are shown in Table 1. RA cases were slightly more likely than controls to be ever smokers (32% vs. 23%, $p = 0.05$); however, when analysis was limited to non-missing data, RA cases and controls did not differ with respect to ever smokers ($p = 0.15$). Higher median serum concentrations of anti-CCP2, ACPA fine specificities targeting vimentin, histone, alpha-enolase, and IgA, IgG and IgM RF isotypes were observed for the immediate/closest pre-diagnosis sample and post-RA diagnosis sample in RA cases versus controls ($p<0.001$). Likewise, anti-CCP2 positivity and IgA, IgG, and IgM RF isotype positivity were significantly higher for the immediate/closest pre-diagnosis sample and post-RA diagnosis sample in RA cases versus controls ($p<0.001$) (Table 1).

Serum anti-bacterial antibodies in RA cases versus controls

Median anti-*P. gingivalis* serum antibody concentrations were not significantly different between RA cases and controls with respect to the immediate/closest pre-diagnosis or post-diagnosis samples (Table 2). Median anti-*P. intermedia* serum antibody concentrations were significantly higher in RA cases than controls for the immediate/closest pre-diagnosis sample ($p = 0.008$), but not in the post-diagnosis sample. In contrast, median anti-*F. nucleatum* serum antibody concentrations were lower in RA cases than controls in the immediate/closest pre-diagnosis sample ($p = 0.045$) but did not differ in post-diagnosis samples.

Association between pre-RA diagnosis serum anti-bacterial antibody concentrations and future RA case status

Temporal relationships of anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* serum antibody concentrations with RA cases and controls are shown in Fig. 1. No evidence of case-control divergence in anti-*P. gingivalis* and anti-*P. intermedia* was observed during the pre-RA diagnosis period. Anti-*F. nucleatum* displayed evidence of slight case-control divergence at 13 years, 7 months prior to diagnosis, with the controls having higher anti-bacterial antibodies than the cases, but values reconverged and were not significantly different at all later time points. Correlations among the anti-bacterial serum antibody concentrations were moderately strong and positive ($r = 0.46$–0.66; data not shown).

Autoantibody concentrations among RA cases and associations with anti-bacterial antibodies

In analyses limited to RA cases, using data from all pre-diagnosis observations, anti-*P. gingivalis* and anti-*F. nucleatum* serum antibody concentrations were not significantly associated with any of the RA autoantibodies in either unadjusted analyses or in multivariable models adjusted for age, sex, and smoking status (Table 3).

However, higher anti-*P. intermedia* serum antibody concentrations were significantly associated with higher concentrations of anti-CCP2, ACPA fine specificities targeting vimentin, histone, alpha-enolase, and IgA RF autoantibodies ($p<0.001$) in both unadjusted and adjusted analyses. Anti-*P. intermedia* serum antibody concentrations were also

Table 1
Patient characteristics and autoantibody values.

Characteristic	RA Cases $n = 214$	Controls $n = 210$	p-value
Age at time of diagnosis, mean (SD)	36.8 (7.9)	36.7 (8.0)	0.89[a1]
Sex, n (%)			
Female	102 (48)	101 (48)	0.93[a2]
Male	112 (52)	109 (52)	
Ethnicity, n (%)[b]			
White	123 (59)	122 (60)	
Black	58 (28)	55 (27)	
Hispanic	18 (9)	18 (9)	1.00[a3]
Asian	5 (2)	5 (2)	
American Indian	4 (2)	4 (2)	
Other	1 (0)	1 (0)	
Ever Smoker, n (%)[c]	68 (32)	49 (23)	0.05[a2]
Anti-CCP2, U/ml, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	59 (2, 216)	0.3 (0.1, 1.0)	<0.001[a4]
Post diagnosis sample	52 (3, 204)	0.4 (0.1, 0.9)	<0.001[a4]
Anti-CCP2, U/ml, n (% positive)[d]			
Immediate / closest pre-diagnosis sample	152 (72)	3 (1)	<0.001[a3]
Post diagnosis sample	153 (72)	0 (0)	<0.001[a3]
ACPA against vimentin, MFI, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	288 (93, 1725)	60 (47, 79)	<0.001[a4]
Post diagnosis sample	397 (86, 1849)	53 (47, 68)	<0.001[a4]
ACPA against histone, MFI, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	591 (133, 2563)	91 (71, 126)	<0.001[a4]
Post diagnosis sample	546 (122, 2478)	77 (62, 108)	<0.001[a4]
ACPA against alpha-enolase, MFI, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	310 (108, 4431)	82 (67, 103)	<0.001[a4]
Post diagnosis sample	406 (112, 3640)	78 (65, 98)	<0.001[a4]
IgA RF, IU/ml, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	5.8 (2.1, 27.1)	1.3 (0.9, 2.0)	<0.001[a4]
Post diagnosis sample	5.7 (1.8, 29.3)	1.2 (0.9, 2.0)	<0.001[a4]
IgA RF, IU/ml, n (% positive)[d]			
Immediate / closest pre-diagnosis sample	86 (41)	3 (1)	<0.001[a3]
Post diagnosis sample	86 (40)	4 (4)	<0.001[a3]
IgG RF, IU/ml, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	6.4 (4.7, 11.6)	4.5 (3.5, 5.7)	<0.001[a4]
Post diagnosis sample	6.7 (4.2, 11.7)	4.4 (3.3, 5.8)	<0.001[a4]
IgG RF, IU/ml, n (% positive)[d]			
Immediate / closest pre-diagnosis sample	40 (19)	5 (2)	<0.001[a3]
Post diagnosis sample	35 (16)	1 (1)	<0.001[a3]
IgM RF, IU/ml, median (IQ range)[d]			
Immediate / closest pre-diagnosis sample	30 (8, 105)	3.8 (2.1, 7.0)	<0.001[a4]
Post diagnosis sample	30 (8, 105)	3.6 (2.2, 7.7)	<0.001[a4]
IgM RF, IU/ml, n (% positive)[d]			
Immediate / closest pre-diagnosis sample	112 (53)	7 (3)	<0.001[a3]
Post diagnosis sample	112 (53)	4 (4)	<0.001[a3]
RA medications (Ever Used), n (%)			
Methotrexate	187 (88)	–	–
Anti-TNF inhibitor	157 (74)	–	–
Radiographic erosions, n (%)	95 (45)	–	–
Number of samples tested, per individual, n (%)			–
2	0 (0)	1 (0)	
3	3 (1)	102 (49)	
4	211 (99)	107 (51)	

(*continued on next page*)

Table 1 (*continued*)

Span of pre-RA samples in years, mean (SD)	−5.1 (5.7)	–	–
Span, oldest to newest sample, in years, mean (SD)[e]	12.8 (5.2)	12.2 (4.9)	0.30[a1]

ACPA = anti-citrullinated protein antibodies.
MFI = mean fluorescent intensity.
[a1]t-test. [a2]Pearson chi-square test. [a3]Exact Pearson chi-square test. [a4]Wilcoxon rank-sum test.
[b]Each ethnicity group was missing values for 5 cases and 5 controls.
[c]Data missing regarding 'ever smoking' in 5 cases and 89 controls (imputed as never smokers); when analysis limited to non-missing data, ever smoking observed in 33% of cases and 41% of controls ($p = 0.15$).
[d]Immediate pre-diagnosis samples available for 212 cases and 207 controls; post-diagnosis samples available for 214 cases and 112 controls.
[e]Among 214 cases and 112 controls with a post diagnosis/index date sample.

Table 2
Serum anti-bacterial antibodies in RA cases versus controls.

Serum anti-bacterial antibodies	RA Cases $n = 214$	Controls $n = 210$	p-value
Anti-*P. gingivalis*, ug/ml, median (IQ range)[a]			
Immediate / closest pre-diagnosis sample	47 (29, 83)	50 (30, 86)	0.633
Post diagnosis sample	50 (31, 84)	53 (30, 87)	0.913
Anti-*P. intermedia*, ug/ml, median (IQ range)[a]			
Immediate / closest pre-diagnosis sample	331 (258, 412)	313 (220, 379)	0.008
Post diagnosis sample	372 (289, 445)	371 (289, 423)	0.404
Anti-*F. nucleatum*, ug/ml, median (IQ range)[a]			
Immediate / closest pre-diagnosis sample	57 (34, 86)	61 (39, 105)	0.045
Post diagnosis sample	57 (33, 96)	67 (42, 97)	0.192

[a]Immediate pre-diagnosis samples available for 212 cases and 207 controls; post-diagnosis samples available for 214 cases and 112 controls.

significantly associated with IgG RF ($p = 0.047$, 0.049) and IgM RF ($p = 0.003$, 0.004) for unadjusted and adjusted values, respectively.

Discussion

This study shows serum anti-*P. intermedia* antibodies demonstrated significant associations with anti-CCP2, ACPA fine specificities targeting vimentin, histone, alpha-enolase, and IgA, IgG, and IgM RF autoantibody concentrations prior to RA diagnosis even after adjusting for age, sex, and smoking. In contrast, anti-*P. gingivalis* and anti-*F. nucleatum* serum antibody concentrations were not significantly associated with RA autoantibodies. Additionally, no longitudinal elevations of anti-*P. gingivalis*, anti-*P. intermedia*, and anti-*F. nucleatum* serum antibody concentrations were observed in RA patients prior to a diagnosis of RA compared to controls.

Prior studies have evaluated serum anti-*P. gingivalis* antibody concentrations in association with pre-RA case status [23–25]. Fisher et al. evaluated a southern European population prior to the onset of RA and reported the association between smoking and antibodies to *P. gingivalis* arginine gingipain (RgpB), and citrullinated PPAD peptides with the risk of RA and pre-RA autoimmunity [23]. Median timing from blood sampling to diagnosis in pre-RA cases was seven years. Their results showed that smoking was significantly associated with an increased risk of RA before clinical onset of disease and former smoking was associated with ACPA positivity in pre-RA cases. Antibodies to RgpB and PPAD peptides were not associated with risk of RA or with pre-RA autoimmunity. Similar to our study, *P. gingivalis* antibody was not associated with pre-RA autoimmunity or risk of RA and the authors suggested this organism may not play a role in the association between PD and RA in this cohort [23].

A study by Johansson analyzed a Northern Swedish population and investigated whether anti-*P. gingivalis* antibody levels pre-dated the onset of RA symptoms and ACPA production [24]. The median time blood samples pre-dated RA symptoms was approximately five years. In contrast to the Fisher et al. study [23], their data demonstrated significantly increased anti-RgpB IgG levels in pre-symptomatic patients and in RA patients compared with controls. Enhanced levels of antibodies to a citrullinated PPAD peptide (anti-CPP3) were also found in both pre-symptomatic and RA individuals. Interestingly, no significant association was noted between anti-RgpB and anti-CPP3 antibodies. This study supported a relationship between *P. gingivalis* and RA by

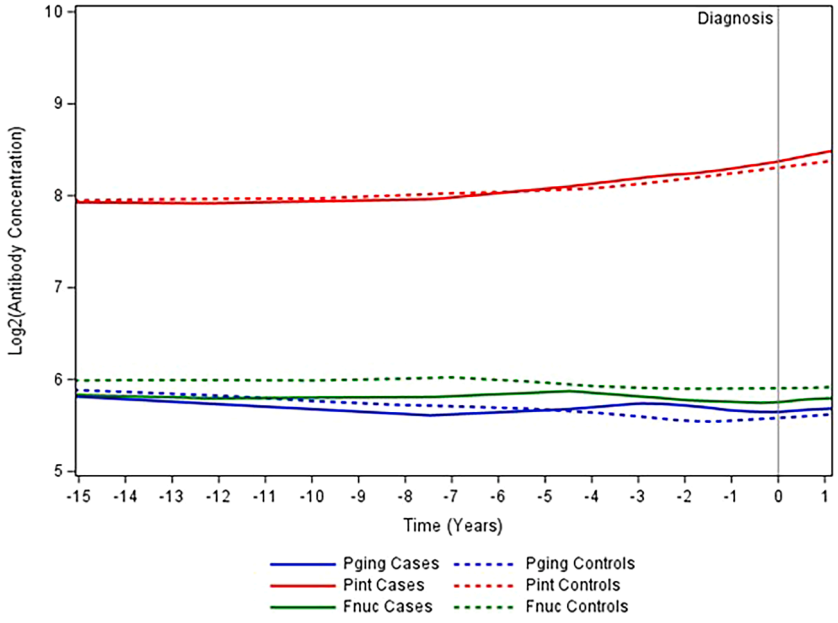

Fig. 1. Pre-rheumatoid arthritis diagnosis concentrations of serum anti-bacterial antibodies (RA cases shown with solid lines, controls shown with dashed lines).

Table 3
Associations of all pre-diagnosis autoantibody sample concentrations among RA cases with serum anti-bacterial antibodies.

	Unadjusted		Adjusted[a]	
Dependent Variable	Anti-*P. gingivalis* coefficient (95% CI)	p-value	Anti-*P. gingivalis* coefficient (95% CI)	p-value
Anti-CCP2	0.101 (−0.274, 0.475)	0.597	0.105 (−0.274, 0.484)	0.586
ACPA against vimentin	0.020 (−0.180, 0.220)	0.845	0.032 (−0.170, 0.233)	0.758
ACPA against histone	0.168 (−0.027, 0.363)	0.092	0.173 (−0.024, 0.371)	0.085
ACPA against alpha-enolase	0.073 (−0.132, 0.277)	0.485	0.094 (−0.111, 0.299)	0.367
IgA RF	−0.066 (−0.244, 0.112)	0.465	−0.060 (−0.239, 0.119)	0.510
IgG RF	0.038 (−0.057, 0.133)	0.434	0.040 (−0.056, 0.137)	0.411
IgM RF	0.019 (−0.158, 0.195)	0.835	0.030 (−0.144, 0.205)	0.733
Dependent Variable	**Anti-*P. intermedia* coefficient (95% CI)**	**p-value**	**Anti-*P. intermedia* coefficient (95% CI)**	**p-value**
Anti-CCP2	1.838 (1.210, 2.466)	<0.001	1.869 (1.235, 2.503)	<0.001
ACPA against vimentin	0.792 (0.457, 1.127)	<0.001	0.827 (0.490, 1.163)	<0.001
ACPA against histone	0.739 (0.410, 1.068)	<0.001	0.758 (0.426, 1.090)	<0.001
ACPA against alpha-enolase	0.790 (0.443, 1.136)	<0.001	0.840 (0.492, 1.187)	<0.001
IgA RF	0.525 (0.236, 0.814)	<0.001	0.536 (0.245, 0.827)	<0.001
IgG RF	0.163 (0.002, 0.325)	0.047	0.163 (0.001, 0.326)	0.049
IgM RF	0.454 (0.157, 0.750)	0.003	0.442 (0.146, 0.737)	0.004
Dependent Variable	**Anti-*F. nucleatum* coefficient (95% CI)**	**p-value**	**Anti-*F. nucleatum* coefficient (95% CI)**	**p-value**
Anti-CCP2	0.066 (−0.326, 0.459)	0.740	0.081 (−0.314, 0.476)	0.687
ACPA against vimentin	0.055 (−0.154, 0.264)	0.604	0.048 (−0.161, 0.256)	0.655
ACPA against histone	0.036 (−0.169, 0.241)	0.731	0.041 (−0.165, 0.247)	0.693
ACPA against alpha-enolase	0.179 (−0.034, 0.392)	0.100	0.181 (−0.032, 0.393)	0.095
IgA RF	−0.090 (−0.275, 0.094)	0.337	−0.081 (−0.266, 0.104)	0.388
IgG RF	0.080 (−0.020, 0.179)	0.117	0.084 (−0.016, 0.184)	0.098
IgM RF	−0.046 (−0.231, 0.138)	0.622	−0.024 (−0.206, 0.158)	0.799

ACPA = anti-citrullinated protein antibodies.
All measures in this table were log base 2 transformed.
[a] Models were adjusted for age, sex, and smoking.

demonstrating increased concentrations of anti-*P. gingivalis* antibodies in RA patients compared to controls, detectable years before symptom development [24].

Manoil et al. measured serum IgG antibodies against selected periodontal pathogens, including *P. gingivalis*, to determine whether they were associated with early symptoms or RA development [25]. This study did not find an association between serum IgG titers against individual periodontal pathogens and specific preclinical phases of RA development. However, the authors found an association between cumulative IgG titers against periodontal pathogens and ACPA-positivity. These data suggest that synergy among periodontal pathogens, rather than specific bacterial associations, may be associated with ACPA development [25].

Our results may differ from prior reports given differences in the populations studied. In the present study, antibody to *P. gingivalis* was directed against outer membrane antigens (OMA), rather than only to specific *P. gingivalis* virulence factors seen in the other two studies [23, 24]. Also, the majority of our study population was male and consisted of active United States military personnel compared to individuals residing in Northern [24] or Southern Europe [23]. Furthermore, the mean age of the RA cases in the European studies were around 50 years old and had a high percentage of ever smokers, ranging from 59% [23] to 67% [24], while our RA participants averaged 37 years old and had lower smoking prevalence of 32%. With the differences in age at disease onset, our younger cohort could suggest a high genetic burden for RA. That high genetic risk could potentially attenuate the importance of environmental factors in this population, such as smoking and bacterial infection leading to PD [26]. We did not determine HLA-SE in the current study, although a previous publication by our group found no evidence of an interaction of PD with HLA-DRB1 SE positivity [26].

In our previous study, relationship of *P. gingivalis* with RA autoantibodies in individuals at "high risk" for RA was examined [14]. Patients were considered autoantibody positive with one or more positive autoantibody tests and high-risk individuals were either ACPA-positive or were positive on two or more RF assays. No patients satisfied the 1987 American College of Rheumatology RA classification criteria [17]. Anti-*P. gingivalis* concentrations were higher in both the high-risk and autoantibody positive groups than in the autoantibody negative group. There were no differences between groups with respect to anti-*P. intermedia* or anti-*F. nucleatum*. The majority of this cohort was slightly older and predominantly female when compared to our younger, male population and could account for the different associations with serum anti-bacterial antibodies [14]. These contrasting conclusions suggest additional research is needed to further explore whether antibody concentrations to the pathogen *P. gingivalis* may be increased prior to onset of RA symptoms and linked to the development of RA.

Although *P. gingivalis* is the most studied periodontal microorganism in the pathogenesis of RA, it has been suggested that *P. intermedia* may also play a role in RA progression, albeit by a different mechanism. A study by Schwenzer et al. suggested that, since *P. intermedia* does not express a PAD, its ability to induce ACPA differs from *P. gingivalis* [12] potentially through a mechanism whereby degradation of neutrophil extracellular traps (NETs) by nucleases from *P. intermedia* leads to the release of PADs [27] and increases the pathogenicity of this organism [28]. Kimura et al. [29] evaluated synovitis and its association with periodontal pathogens and established biomarkers of RA. Greater *P. intermedia* antibody titer was observed in active RA patients and RA patients in clinical remission with subclinical synovitis, detected by ultrasound, compared to RA patients in clinical remission without subclinical synovitis. An association of *P. intermedia* antibody titer and disease activity of RA, specifically synovitis, was proposed. The mechanism suggested by the authors is activation of macrophages by *P. intermedia* which initiates production of IL-6 and TNF-α, inflammatory cytokines that play a role in periodontal and joint destruction. Of note, Scher et al. reported that *Prevotella* and *Leptotrichia* species were the only characteristic taxa in the oral microbiota in the new-onset RA group irrespective of PD status and were completely absent in the oral microbiota of controls [30]. While other investigators were unable to demonstrate a relationship between *P. intermedia* and RA [31,32], our study observed a strong association with anti-CCP2, certain ACPA specificities as well as several isotypes of RF and highlights a need to further explore the potential role of *P. intermedia* in RA pathogenesis and, in particular, the generation of these RA-related autoantibodies.

Limited studies exist evaluating anti-*F. nucleatum* antibody concentrations with RA. One study analyzed saliva samples of early RA patients and found microbiota rich in *F. nucleatum* when compared to healthy controls and proposed the oral microbiota may be useful in detecting

risk assessment for early onset of RA [13]. In looking at subgingival biofilm of RA patients, *F. nucleatum* was found in higher concentrations in aCCP-positive patients with RA versus controls, though this finding was not statistically significant [33]. In a separate study, *F. nucleatum* was found in the synovial fluid of RA patients derived from both native and prosthetic joints. Identical clones of the bacteria were found in the same patient's plaque sample, and it was proposed that *F. nucleatum* can translocate from the oral cavity to the synovial cavity [34]. In contrast, our data does not provide compelling evidence to support a role of *F. nucleatum* in RA development. In contrast to prior reports, our results demonstrated only a slight case-control divergence of anti-*F. nucleatum* prior to RA diagnosis; however, controls had initially higher concentrations that reconverged to no longer be statistically significant than concentrations in RA cases. When compared to *P. gingivalis and P. intermedia*, potential mechanisms linking *F. nucleatum* and RA risk remain poorly understood.

There are limitations in this study. The participants were military personnel with a relatively high proportion of men to women (52% vs. 48%, respectively) and a younger age of RA onset (37 years old). Thus, these results may not be generalizable to other RA populations [35]. A majority of RA cases utilized methotrexate and/or biologics (88% and 74%, respectively), which could have impacted these results. Furthermore, there were only 112 control patient samples available for post-RA diagnosis evaluations. The lack of a difference in anti-*P. intermedia* concentrations in RA cases versus controls post-RA diagnosis needs to be interpreted with caution in light of this smaller sample size available for analysis. In addition, most of the pre-RA serum samples were collected within 5 years of diagnosis, which could have limited our ability to detect earlier differences in anti-bacterial or autoantibody elevations [18]. In future studies, more frequent serum sample collection over more extended time periods would provide an even more comprehensive look at the autoantibody and anti-bacterial responses potentially leading to RA onset. PD status was not determined in this study and, therefore, we were unable to associate periodontal status with the patients' systemic response against the periodontal pathogens investigated. Moreover, the taxa could exert a local response without triggering a serum IgG response; therefore, null associations should be carefully considered. Finally, future studies also should focus on the plethora of inflammatory reactions occurring in the gingival tissues that have the potential to stimulate autoantibody production associated with RA.

Conclusion

In conclusion, no longitudinal elevations of serum anti-bacterial antibody concentrations were observed in RA patients prior to a diagnosis of RA compared to controls. However, anti-*P. intermedia* displayed a significant association with RA autoantibody concentrations prior to RA diagnosis, suggesting a potential role of this organism in progression towards clinically-detectable RA.

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Military disclaimer

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Data Availability

Data requests can be made to the authors although use is restricted based on Department of Defense guidelines.

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