Periodontal Disease and the Oral Microbiota in New-Onset Rheumatoid Arthritis

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Objective. To profile the abundance and diversity of subgingival oral microbiota in patients with never-treated, new-onset rheumatoid arthritis (RA).

Methods. Periodontal disease (PD) status, clinical activity, and sociodemographic factors were determined in patients with new-onset RA, patients with chronic RA, and healthy subjects. Multiplexed-454 pyrosequencing was used to compare the composition of subgingival microbiota and establish correlations between the presence/abundance of bacteria and disease phenotypes. Anti–Porphyromonas gingivalis antibody testing was performed to assess prior exposure to the bacterial pathogen P. gingivalis.

Results. The more advanced forms of periodontitis were already present at disease onset in patients with new-onset RA. The subgingival microbiota observed in patients with new-onset RA was distinct from that found in healthy controls. In most cases, however, these microbial differences could be attributed to the severity of PD and were not inherent to RA. The presence and abundance of P. gingivalis were also directly associated with the severity of PD and were not unique to RA. The presence of P. gingivalis was not correlated with anti–citrullinated protein antibody (ACPA) titers. Overall exposure to P. gingivalis was similar between patients with new-onset RA and controls, observed in 78% of patients and 83% of controls. The presence and abundance of Anaeroglobus geminatus correlated with the presence of ACPAs/rheumatoid factor. Prevotella and Leptotrichia species were the only characteristic taxa observed in patients with new-onset RA irrespective of PD status.

Conclusion. Patients with new-onset RA exhibited a high prevalence of PD at disease onset, despite their young age and paucity of smoking history. The subgingival microbiota profile in patients with new-onset RA was distinct from that found in healthy controls. In most cases, however, these microbial differences could be attributed to the severity of PD and were not inherent to RA. The presence and abundance of P. gingivalis were also directly associated with the severity of PD and were not unique to RA. The presence of P. gingivalis was not correlated with anti–citrullinated protein antibody (ACPA) titers. Overall exposure to P. gingivalis was similar between patients with new-onset RA and controls, observed in 78% of patients and 83% of controls. The presence and abundance of Anaeroglobus geminatus correlated with the presence of ACPAs/rheumatoid factor. Prevotella and Leptotrichia species were the only characteristic taxa observed in patients with new-onset RA irrespective of PD status.

The term “microbiome” was coined a decade ago (1) and implies the totality of microbes (commensal and pathogenic), their genomes, and environmental interactions in a defined biologic niche. In this symbiotic relationship, humans provide nutrients and an adequate environment for microorganisms that, in return, shape the human immune system, degrade polysaccharides,
and produce vitamins and other essential factors that would be otherwise impossible to obtain. In 2008, the National Institutes of Health Human Microbiome Project (2) embraced the notion that it is impossible to fully understand human health and disease unless this collective human-microbiome “superorganism” is better studied and defined.

Rheumatoid arthritis (RA) is a systemic, inflammatory autoimmune disorder. It is regarded as a complex multifactorial disease in which multiple genes and environmental factors act in concert to cause pathologic events (3). Despite recent advances in understanding of the molecular pathways involved, the etiology of RA is almost completely unknown. Although genes certainly contribute to RA susceptibility (4), genetic effects appear to be augmented by exposure to environmental factors (i.e., smoking, hormones, and infection), which might explain the differences in incidence of the disease (5).

Among the more intriguing environmental co-variates modulating autoimmunity is the bidirectional cross-talk between the human host and the oral and intestinal microbiomes. Multiple lines of investigation have suggested a link between oral microbes, periodontal disease (PD), and RA (6,7). However, most clinical studies implicating specific oral microorganisms as triggers for RA have relied only on serologic testing methods. Data describing the subgingival microbiota in patients with RA are virtually nonexistent.

In the present study, we aimed to determine the periodontal status of RA patients and healthy controls and to directly correlate, for the first time, the presence and abundance of subgingival microbiota with RA status, utilizing 16S ribosomal RNA (rRNA) pyrosequencing. Because we wanted to understand whether specific populations of oral microbiota are associated with the initiation of RA, we focused our attention on patients with new-onset RA who had never been treated with DMARDs (oral and/or biologic agents) and/or corticosteroids (ever). Chronic RA was defined as any patient meeting the criteria for RA whose disease duration was a minimum of 6 months since diagnosis. Most subjects with chronic RA were receiving DMARDs (oral and/or biologic agents) and/or corticosteroids at the time of enrollment. Healthy controls were age-, sex-, and ethnicity-matched individuals with no personal history of inflammatory arthritis.

The exclusion criteria applied to all groups were as follows: recent (<3 months prior) use of any antibiotic therapy, current extreme diet (e.g., parenteral nutrition or macrobiotic diet), known inflammatory bowel disease, known history of malignancy, current consumption of probiotics, any gastrointestinal tract surgery leaving permanent residua (e.g., gastrectomy, bariatric surgery, colectomy), or significant liver, renal, or peptic ulcer disease. This study was approved by the Institutional Review Board of New York University School of Medicine.

**Classification of PD.** All periodontal examinations were performed at the New York University College of Dentistry. Periodontal status was assessed by 3 examiners with documented similar assessment skills who were blinded with regard to each subject’s RA status. For the periodontal status, defined according to the American Academy of Periodontology guidelines (9), the following parameters were recorded: probing depth, clinical attachment level, and bleeding on probing. The periodontal status in all patients and controls was classified as follows: 1) healthy, no bleeding upon probing; 2) gingivitis, with bleeding upon probing; 3) slight chronic periodontitis (at least 1 periodontal site with an attachment level of 1–2 mm and probing depth of ≥4 mm); 4) moderate chronic periodontitis (at least 2 teeth with an attachment level of 3–4 mm or at least 2 teeth with a probing depth of ≥4 mm); or 5) severe chronic periodontitis (at least 2 teeth with an attachment level of ≥5 mm and 1 tooth with a probing depth of ≥5 mm). In all, 31 patients with new-onset RA, 34 patients with chronic RA, and 18 healthy controls were available for these analyses.

**Sample collection and DNA extraction.** Oral samples were obtained by collection of subgingival biofilm from the 6 sites with the greatest extent of PD in all patients. Oral samples were harvested using a Gracey curette (after removal and discard of supragingival biofilm, to avoid potential salivary contamination). All samples were pooled and directly suspended in MoBio buffer—containing tubes. DNA was extracted within 1 hour of sample collection using a combination of the MoBio Power Soil kit and a mechanical disruption (bead-beater) method based on a previously described protocol (10). Samples were stored at −80°C.

**Inclusion and exclusion criteria.** The criteria for inclusion in the study required that patients meet the American College of Rheumatology/European League Against Rheumatism 2010 classification criteria for RA (8), including seropositivity for rheumatoid factor (RF) and/or anti–citrullinated protein antibodies (ACPA) (assessed using an anti–cyclic citrullinated peptide enzyme-linked immunosorbent assay [ELISA]; Euroimmun), and that all subjects be age 18 years or older. New-onset RA was defined as a disease duration of a minimum of 6 weeks up to 6 months since diagnosis, and absence of any treatment with DMARDs or steroids (ever). Chronic RA was defined as any patient meeting the criteria for RA whose disease duration was a minimum of 6 months since diagnosis. Most subjects with chronic RA were receiving DMARDs (oral and/or biologic agents) and/or corticosteroids at the time of enrollment. Healthy controls were age-, sex-, and ethnicity-matched individuals with no personal history of inflammatory arthritis.

**PATIENTS AND METHODS**

**Study participants.** Consecutive patients from the New York University rheumatology clinics and offices were screened for the presence of RA. After informed consent was signed, each patient’s medical history (according to chart review and interview/questionnaire), diet, and medications were determined. A screening musculoskeletal examination and laboratory assessments were also performed or reviewed. All RA patients who met the study criteria were offered enrollment.

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Amplification of the V1–V2 16S rRNA region and 454 pyrosequencing. For each sample, 3 replicate polymerase chain reactions (PCRs) were performed to amplify the V1 and V2 regions of 16S rRNA, as described previously by Ubeda et al (11). PCR products were sequenced on a 454 GS FLX Titanium platform (Roche). Sequences were deposited in the NCBI Sequence Read Archive (accession no. SRA050292).

Sequence analysis. Sequence data were compiled and processed using mothur software (12) and converted to standard Fasta format. Sequences were trimmed and aligned to the V1–V2 region of the 16S gene, using the Silva reference alignment as template (13). Potentially chimeric sequences were removed using ChimeraSlayer (14). To minimize the risk of pyrosequencing errors leading to overestimation of microbial diversity (15), low-abundance sequences were merged to the high-abundance sequence, using the pre.cluster option in mothur. Sequences were grouped into operational taxonomic units (OTUs) using the average neighbor algorithm. Sequences with a distance-based similarity of ≥97% were assigned to the same OTU. For each sample, OTU-based microbial diversity was estimated by calculating the Shannon diversity index and the Simpson diversity index (16), and richness was estimated using the Chao index. The Yue and Clayton diversity measure and the principal coordinates of analysis measure were applied on the mothur platform. Phylogenetic classification was performed using the Bayesian classifier algorithm, with a bootstrap cutoff value of 60% (17).

Serum ELISA for anti–HtpG P18y peptide antibodies. Porphyromonas gingivalis HtpG peptides were prepared in the laboratories of Drs. D. G. Sweier and C. E. Shelbourne (University of Michigan School of Dentistry, Ann Arbor) in a manner that has been previously described (18), and loaded into the wells of microtiter plates. Twenty-five microliters of each serum sample was added to the wells, followed by incubation with goat anti-human IgG (γ-chain specific). Levels of P gingivalis antibodies in the serum were analyzed by ELISA, according to protocol.

Statistical analysis. To determine statistically significant differences between samples from the disease and healthy control groups, bacteria with a mean count of <5 in both conditions were removed from the analyses, and t-tests were applied to scaled log_2-transformed bacteria count data, with rescaling using the DESeq R package (19). To adjust for multiple hypothesis testing, we assessed the false discovery rate (FDR), utilizing the false discovery rate (FDR) utilizing the fdr.R package (20). The final results were filtered for a significance level of P < 0.05 and an FDR of ≤0.1.

For cross-sectional analyses of baseline characteristics, differences were evaluated using Student’s t-test, Mann-Whitney U test, or chi-square test, as appropriate. SPSS software (version 16.0) was used for these analyses. Two-tailed tests were used for significance testing, and P values less than 0.05 were considered significant.

RESULTS

Presence of advanced PD in patients with new-onset RA. Of the 31 patients with new-onset RA included in the study, 68% were female and the mean age was 42.2 years (Table 1). The mean disease duration was 3.4 months (median 2 months) and no patient had ever received steroids, oral DMARDs, or biologic drugs. The mean Disease Activity Score in 28 joints (DAS28) (21) was 5.8. In accordance with the inclusion criteria, all patients with new-onset RA were seropositive for ACPAs and/or RF (96% were positive for ACPAs, 92% were positive for RF). More than two-thirds of the study patients had never smoked tobacco (16% were current smokers) (Table 1). Healthy controls were subjects who were age-, sex-, and ethnicity-matched to the patients.

The chronic RA group had proportionally more female participants. The mean age of the patients in this group was 47.7 years (P not significant compared to patients with new-onset RA) and 88% were ACPA positive. The mean disease duration in patients with chronic RA was 62.9 months (median 34 months) and the mean DAS28 was 4.4, reflecting the presence of moderate disease that had been presumably altered by DMARD intervention. Among the patients with chronic RA, 70% had no history of smoking.

As shown in Table 1, >75% of the patients with either new-onset RA or chronic RA were found to have moderate-to-severe forms of PD, a significantly higher proportion when compared to healthy controls. The prevalence of PD in our healthy control group was consistent with the expected prevalence of extensive PD in the general population (30–40%) (22). An interesting finding was the presence of PD in several of our patients with new-onset RA who were younger than age 30 years and who did not have any risk factors for PD, such as smoking; typically, periodontitis would not be observed in such subjects.

Comparable richness and diversity of the oral microbiota in patients with new-onset RA, patients with chronic RA, and healthy controls. Overall, 83 oral samples were obtained from all participants, yielding a total of 206,378 16S rRNA high-quality sequences (mean number of sequences 2,037/sample among a range of 443–5,008 read samples; P not significant). Using a distance-based similarity of ≥97% for species-level OTU assignment, a total of 2,136 OTUs were identified (details available from the corresponding author upon request).

We first studied the impact of RA status on microbial diversity by using the Simpson and Shannon inverse indexes. Both take into account, when calculating the diversity of a sample, not only the number of OTUs (estimated number of species) present, but also the relative frequency of the different OTUs within that sample. A high index reflects a more diverse micro-
biota. Utilizing both calculations, no significant differences in microbial diversity were observed between the RA groups and controls (details available from the corresponding author upon request).

We then analyzed whether RA status had an impact on microbial richness. When the Chao index (which estimates how many OTUs constitute the microbiota of a specific sample) was applied, no significant differences were found among the groups (details available from the corresponding author upon request).

In an attempt to discriminate among study groups, we also performed clustering analyses at the various taxonomic levels. Although certain significant differences were found, no particular oral bacterial phylum, class, order, or family could be used to discriminate between the new-onset RA, chronic RA, and healthy control groups. In contrast, differences were evident when groups were classified by severity of PD. As has been previously described by others (23,24), our assessment showed that the periodontal microbiota of healthy control subjects was dominated by 7 phyla, including Bacteroidetes (21.3%), Firmicutes (10.9%), Actinobacteria (21.8%), Proteobacteria (16.9%), Fusobacteria (24%), Spirochaetes (2.5%), and TM7 (1.6%) (Figure 1A). Assessment of the samples exhibiting moderate-to-severe forms of PD revealed an increase in the relative abundance of Bacteroidetes, Spirochaetes, and TM7, and a concomitant decrease in Actinobacteria and Proteobacteria (Figure 1B).

To further analyze whether the oral microbiota in patients with new-onset RA was distinct from that in healthy controls, we applied the Yue and Clayton diversity measure (which compares the relative abundance of OTUs present in different samples). We then applied

| Table 1. Demographic and clinical features and severity of periodontal disease among patients with new-onset rheumatoid arthritis (RA), patients with chronic RA, and healthy control subjects* |
|-------------------------------------------------|-------------------|-------------------|-----------------|
| Characteristic                                  | New-onset RA      | Chronic RA        | Healthy controls|
| Age, mean (median) years (n = 31)                | 42.2 (40)         | 47.7 (48)         | 42.2 (39)       |
| Female, %                                       | 68                | 79                | 65              |
| Disease duration, mean (median) months (n = 31)  | 3.4 (2)           | 62.9 (34)         | –               |
| Disease activity parameter                      |                   |                   |                 |
| ESR, mean mm/hour                               | 37.8              | 31.2              | 14              |
| CRP, mean mg/liter                              | 26.7              | 9                 | 0               |
| DAS28, mean (median)                            | 5.8 (6.01)        | 4.4 (4.72)        | –               |
| Patient VAS pain, mean (median) mm (n = 31)     | 64.4 (55)         | 46.6 (50)         | –               |
| TJC28, mean (median)                            | 11.9 (12)         | 5.6 (4)           | –               |
| SJC28, mean (median)                            | 8.2 (8)           | 4 (3)             | –               |
| Autoantibody status                             |                   |                   |                 |
| IgM-RF positive, %                              | 92                | 88                | 10              |
| ACPA positive, %                                | 96                | 88                | 0               |
| IgM-RF and/or ACPA positive, %                  | 100               | 96                | 10              |
| IgM-RF titer, mean (median) kU/liter (n = 31)   | 377 (157)         | 169.6 (98)        | 4.4 (0)         |
| ACPA titer, mean (median) kAU/liter (n = 31)    | 114.6 (150)       | 97.4 (60)         | 0               |
| HLA shared epitope positive, %                  | 45                | 33                | 16              |
| Medication use, %                               |                   |                   |                 |
| Methotrexate                                    | 0                 | 79                | 0               |
| Prednisone                                      | 0                 | 45                | 0               |
| Biologic agent                                  | 0                 | 12                | 0               |
| Smoking status, %                               |                   |                   |                 |
| Current                                         | 16                | 6                 | 6               |
| Former                                          | 16                | 24                | 16              |
| Never                                           | 68                | 70                | 78              |
| Periodontal status, %                           |                   |                   |                 |
| Healthy gingiva                                 | 0†                | 6†                | 45              |
| Gingivitis                                      | 13                | 3                 | 11              |
| Slight PD                                       | 10                | 6                 | 5               |
| Moderate PD                                     | 16                | 32                | 17              |
| Severe PD                                       | 62†               | 53†               | 22              |

* ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = Disease Activity Score in 28 joints; VAS = visual analog scale; TJC28 = tender joint count (of 28 joints); SJC28 = swollen joint count (of 28 joints); IgM-RF = IgM rheumatoid factor; ACPA = anti–citrullinated protein antibody; PD = periodontal disease.

† P < 0.01 versus healthy controls, by analysis of variance.
principal coordinates of analysis to the quantified similarity distances between samples and clustered the coordinates along orthogonal axes of maximal variance. Two principal coordinates (PC1 and PC2) could explain most of the microbial variation observed between samples. Of note, no clustering due to RA status could be observed (Figure 1C). However, as shown in Figure 1D, PC1 did cluster a group of samples obtained from patients with severe or moderate periodontitis. This result suggests that differences at the species/OTU level could characterize the more advanced forms of PD, but do not represent a specific signature for the oral microbiota of RA.

Role of Prevotella and Leptotrichia as characteristic species of the oral microbiota of new-onset RA. We next sought to identify a bacterium or groups of bacteria responsible for the clustering that identified patients with advanced PD. By applying multivariate statistical analyses taking into consideration each group, we also sought to identify bacterial taxa that were significantly different in the new-onset RA group (i.e., showing either a significant increase or significant decrease in abundance) when compared to the other groups. At most taxonomic levels, the composition of the oral microbiota in patients with new-onset RA was not significantly different from that in the other groups (Table 2) (additional results available from the corresponding author upon request). However, the genera Corynebacterium and Streptococcus were underrepresented in patients with RA, which reflects the lack of a healthy microbiota; these findings are therefore consistent with the presence of PD. Interestingly, OTU60 (Prevotella species) and OTU87 (Leptotrichia species) were the only characteristic taxa observed to be present in the patients with new-onset RA (in 32.2% and 25.8% of patients, respectively) irrespective of PD status, and were completely absent in the oral microbiota of healthy controls.

High abundance of periodontopathic bacteria in patients with new-onset RA, compared to diminished presence in chronic RA. To directly survey the presence of bacteria associated with the development of PD in...
patients with RA, we next examined how these phylotypes differed in the early and late phases of RA. Interestingly, OTU members of the red complex bacteria (a triad of the most virulent periodontopathic bacteria, comprising *Tannerella*, *Treponema*, and *Porphyromonas*) (25) were more prevalent in the microbiota of patients with new-onset RA compared to that of patients with chronic RA (Figure 2).

Association of *Porphyromonas* and *P. gingivalis*-related OTUs with severity of PD, but without specificity in the RA microbiota. Although there was some variability in levels, the genus *Porphyromonas* was present almost universally in all participants (Figures 3A and C). When we analyzed the 2,136 different OTUs among all patient groups (including 59 OTUs within *Porphyromonas*), we found that OTU1, with 100% 16S rRNA sequence homology with *P. gingivalis*, was significantly more prevalent and abundant in patients with the severe form of PD compared to healthy gums. However, this finding had no direct correlation with the presence of RA (Figures 3B and D).

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\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Taxonomy} & \textbf{New-onset RA vs. healthy controls} & \textbf{New-onset RA vs. chronic RA} & \textbf{All RA vs. healthy controls} & \textbf{Periodontal disease vs. healthy control gingiva} \\
\hline
\textbf{Genus} & ↑ Anaeroglobus† & ↓ Unclassified Veillonellaceae‡ & ↑ Anaeroglobus§ & ↑ Anaeroglobus$x$
\textbf{Unclassified Prevotellaceae}$\dagger$
\textbf{Phocaeiola}†
\textbf{Corynebacterium}†
\textbf{Streptococcus}$\ddagger$
\hline
\textbf{Species (OTU)} & ↑ Anaeroglobus OTU99¶ & ↑ Porphyromonas OTU57† & ↑ Anaeroglobus OTU99§ & ↑ Anaeroglobus OTU99§
\textbf{Leptotrichia OTU87¶} & ↑ Selenomonas OTU231† & ↑ Prevotella OTU134¶ & ↑ Prevotella OTU628 & ↑ Prevotella OTU628
\textbf{Prevotella OTU60}‡ & ↑ Prevotella OTU26† & ↑ Prevotella OTU134¶ & ↑ Prevotella OTU628 & ↑ Prevotella OTU208
\textbf{Selenomonas OTU168}§ & ↑ Treponema OTU32† & ↑ Selenomonas OTU168† & ↑ Treponema OTU134¶ & ↑ Treponema OTU134¶
\textbf{Tannerella OTU13†} & ↑ Leptotrichia OTU12acciones & ↑ Selenomonas OTU168† & ↑ Treponema OTU134¶ & ↑ Selenomonas OTU168¶
\textbf{Porphyromonas OTU1}† & ↓ Leptotrichia OTU39¶ & ↓ Prevotella OTU39¶ & ↓ Leptotrichia OTU12acciones & ↓ Prevotella OTU39¶
\textbf{Porphyromonas OTU1}† & ↓ Capnocytophaga OTU74♣ & ↓ Leptotrichia OTU12acciones & ↓ Capnocytophaga OTU74♣ & ↓ Leptotrichia OTU12acciones
\textbf{Leptotrichia OTU12}¶ & ↓ Corynebacterium OTU4¶ & ↓ Capnocytophaga OTU74♣ & ↓ Corynebacterium OTU4¶ & ↓ Capnocytophaga OTU74♣
\textbf{Corynebacterium OTU4}¶ & ↓ Unclassified TM7 OTU58† & ↓ Capnocytophaga OTU74♣ & ↓ Capnocytophaga OTU74♣ & ↓ Capnocytophaga OTU74♣
\hline
\end{tabular}
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\textsuperscript{*}↑ = significant increase in patients with new-onset RA or participants with periodontal disease when compared to patients with chronic RA or healthy controls;
\textsuperscript{↓}↓ = significant decrease in patients with new-onset RA or participants with periodontal disease when compared to patients with chronic RA or healthy controls.

\textsuperscript{†}P < 0.05.
\textsuperscript{‡}P < 0.01.
\textsuperscript{§}P < 0.0005.
\textsuperscript{♣}P < 0.005.
P < 0.05) in patients with advanced PD compared to those without PD (details available from the corresponding author upon request). Many other OTUs known to be associated with PD showed similar elevations, although the differences were less pronounced (results not shown). Taken together, these data suggest that although colonization of P gingivalis was twice as common in RA patients compared to healthy controls, the difference could be explained by the higher prevalence of PD in the RA population.

Lack of a significant difference in exposure to P gingivalis among the groups. Because the absence of P gingivalis in the oral microbiota did not exclude the possibility of prior exposure to the organism, we tested

Figure 2. Abundance of periodontopathic bacteria (OTU level) in patients with new-onset RA and patients with chronic RA. Species associated with periodontal disease, such as Tannerella forsythia (A) and Treponema medium (B), were significantly more abundant in patients with new-onset RA compared to patients with chronic RA, whereas between-group differences were not significant (NS) for Porphyromonas gingivalis (C). Bars show the mean ± SEM. See Figure 1 for other definitions.

Figure 3. Prevalence and abundance of Porphyromonas and P gingivalis (OTU1) in 83 study participants, grouped by RA status (healthy controls [HC], new-onset RA, chronic RA) and periodontal status (healthy gingiva or gingivitis [No PD] versus slight [SLT], moderate [MOD], or severe [SEV] PD). Whereas the genus Porphyromonas (A and C) was present almost universally and irrespective of RA or PD status, P gingivalis (B and D) was significantly associated with moderate and severe PD and was not associated with the presence of RA. In A and B, each square represents a single individual; the darker the intensity of the box, the greater the relative abundance of Porphyromonas or P gingivalis. In C and D, bars show the mean ± SEM. NS = not significant (see Figure 1 for other definitions).
the sera using a previously validated assay with an antibody against HtpG (IgG-class anti-P18γ), which is a highly specific chaperone for P. gingivalis. We found that 63.3% of patients with new-onset RA, 50% of patients with chronic RA, and 72.2% of healthy controls tested positive for anti-P18γ (P = 0.45 by analysis of variance) (Figure 4A).

We next analyzed the overall exposure to P. gingivalis on the basis of findings from 16S rRNA pyrosequencing for P. gingivalis colonization and/or a positive anti-P18γ antibody response. Interestingly, we found that patients with new-onset RA and patients with chronic RA had a rate of exposure to P. gingivalis of 84% and 71%, respectively (78% overall), which was comparable to the exposure prevalence in healthy individuals (83%) (P not significant by analysis of variance) (Figure 4B). This was largely attributable to the increased serologic response observed in all groups.

**Correlation of circulating RA-related antibodies with the presence of Anaeroglobus, an unusual bacterial taxon.** Proposed mechanisms through which P. gingivalis might promote the pathogenesis of RA include its capacity to citrullinate peptides via the enzyme peptidylarginine deiminase (PAD), theoretically promoting the generation of neoantigens and subsequent production of ACPAs (26,27). We therefore examined the different taxonomic levels to try to identify the phylotypes associated with circulating autoantibodies.

There was no association between the presence of RF or ACPAs and the presence of the higher taxonomic levels (phylum, class, order, or family). In particular, there was no association between the presence of RF or ACPAs and the genus Porphyromonas in the oral microbiota, nor was there an association of RF or ACPAs with OTUs related to P. gingivalis. Indeed, several patients who were positive for ACPAs lacked P. gingivalis–related OTUs, and the autoantibody titer was not positively associated with the genera abundance (data not shown). Unexpectedly, however, the presence of the genus Anaeroglobus and its species-level OTU (OTU99; closely related to Anaeroglobus guminattus) significantly correlated with the serum titers of both circulating RF and ACPAs (both P < 0.05). Moreover, OTU99 was associated with the presence of PD, and was found in 77.5% of patients with new-onset RA and 50% of patients with chronic RA (P not significant) compared to only 16.7% of healthy controls (both P < 0.005). OTU130 (with 94% similarity to Porphyromonas catoniae) was also significantly associated with the presence of circulating ACPAs.

![Figure 4. Serum levels of anti–HtpG peptide (anti-P18γ) antibodies and overall Porphyromonas gingivalis exposure among patients with new-onset RA, patients with chronic RA, and healthy controls. A, Anti-P18γ antibodies were found in 72% of healthy subjects, 63.3% of patients with new-onset RA, and 50% of patients with chronic RA (P = 0.18 by analysis of variance). Bars show the mean ± SEM. B, The prevalence of P. gingivalis in the oral microbiota was determined by 16S ribosomal RNA pyrosequencing and by serology (for anti-P18γ antibodies) in all 83 participants in the 3 groups. Red squares denote the presence of P. gingivalis as indicated by 16S pyrosequencing and/or detectable anti–P. gingivalis antibodies. Each square represents a single individual, and contiguous squares are representative of the same participant. * = serum not available. See Figure 1 for definitions.](image_url)
DISCUSSION

An accumulating body of epidemiologic data suggests a role of clinical PD in the development of RA. In accordance with our findings, periodontitis was more common and severe in patients with RA compared to patients with osteoarthritis in a cohort of US veterans (28). In another study (29), RA patients had an 8-fold increased likelihood of periodontitis compared to controls. Many recent studies have also implicated *P. gingivalis* as a possible triggering factor. Interestingly, however, none of these studies directly assessed the presence of oral microorganisms. Rather, they relied on serologic methods (30,31) or limited, low-throughput PCR–based techniques (32,33). To our knowledge, no prior study has specifically assessed the presence of *P. gingivalis* (or other periodontopathic bacteria) in subgingival biofilms from RA patients.

Our study is the first to utilize multiplexed-454 16S rRNA pyrosequencing to compare the bacterial composition of the subgingival microbiota between patients with RA (early and chronic) and healthy controls. This approach permitted a broad and comprehensive portrayal of the subgingival microbial communities associated with RA at different stages of the disease. Several conclusions can be drawn from our data.

First, we corroborated previous observations that patients with early RA present with incident PD (34). Our data are striking in that a high prevalence of moderate-to-severe periodontitis was observed in patients who had never been treated with steroids or DMARDs, a finding reported for the first time herein in this unique cohort of untreated patients. Moreover, our population of patients with new-onset RA was mostly composed of young subjects and nonsmokers, whereas smoking is often a significant risk indicator of PD (35,36) and has been proposed as a central driver of gene–environment interactions in seropositive RA (37). These results are consistent with the notion that PD, present at the time of diagnosis in the majority of patients with new-onset RA, may represent a risk factor for RA development independent of smoking status. Intriguingly, some periodontopathic OTUs (e.g., Tannerella/OTU13, Treponema/OTU32) showed significantly higher abundance in the microbiota of patients with new-onset RA, and tended to diminish with established, better-controlled disease. We speculate that this difference could be attributed to the effects of RA therapeutic regimens over time. It is conceivable that a variety of immunomodulatory regimens, particularly those with proposed antibacterial properties, such as methotrexate or hydroxychloroquine (38,39), have an impact on the ecologic adaptation of the oral microbial niche.

Second, we found that the subgingival microbial communities in patients with new-onset RA generally did not have a unique fingerprinting signature when compared to healthy controls. However, 2 OTUs, OTU60 (Prevotella species) and OTU87 (Leptotrichia species), were detected only in the patients with new-onset RA. Although the genus *Porphyromonas* was present in virtually all subjects, its relative abundance was directly correlated with the severity of PD regardless of RA status. We corroborated prior findings, obtained using low-throughput techniques (25,40,41), that the advanced forms of PD are overrepresented by genera such as *Porphyromonas, Tannerella, and Treponema*, all of which have been implicated in the pathogenesis of PD (25).

Our ability to go beyond the genus taxonomy also allowed us to investigate the OTUs within the genus *Porphyromonas*. The most abundant OTU within the genus was identical to *P. gingivalis*. Interestingly, none of the other OTUs whose 16S rRNA sequences were similar to those of *P. gingivalis* were significantly over-represented in the subgingival oral microbiota of patients with new-onset RA. Our data demonstrate that while most individuals carry *Porphyromonas* in their subgingival domain, a particular OTU (OTU1) is found mostly in conditions of advanced PD, regardless of whether RA is present. However, given the low number of non-RA subjects with PD in our study, we could not categorically establish whether bacterial exposure can be attributed to the presence of subgingival inflammation alone. It is quite possible that *P. gingivalis* may serve as a shared causal pathway in some cases of RA. Studies of a large replication cohort should help elucidate this question in the future.

Third, an unanticipated finding was that the presence of OTU99 (*A. geminatus*) significantly correlated with the serum titers of RF and ACPAs. *A. geminatus* is the only described species of the genus belonging to the family Veillonellaceae. A strictly anaerobic gram-negative coccii, this bacterium was originally isolated from a collection of postoperative fluid samples (42). Existing literature on *A. geminatus* is scarce, although 2 reports have described the presence of a closely related species (*Megasphaera* species) in the setting of PD (43).

Even more intriguingly, 2 other organisms were found to be prevalent only in the patients with new-onset RA, *Prevotella* (OTU60) and *Leptotrichia* (OTU87). Using publicly available alignment tools, we found that both OTUs aligned to microorganisms that have not yet
been cultured. In the case of Leptotrichia, the closest known 16S gene belonged to Leptotrichia wadei (91% identity). This species has been previously recovered from patients with periodontitis (44). OTU60 aligned only to uncultured oral Prevotella species. The role of the genus Prevotella (i.e., P. intermedia) in PD is well established (25). Although they are not yet fully sequenced and are poorly understood, our preliminary observations show that these newly described species (Prevotella [OTU60], Leptotrichia [OTU87], and A. gernatus [OTU99]) merit further study as candidate periodontal microbial triggers of RA.

The significance of periodontal inflammation in new-onset RA continues to be an important, as-yet unanswered question. It is clear from these and prior studies that there is a high prevalence of PD in new-onset RA that cannot be explained by the effects of immunosuppressive treatments. However, it remains to be determined whether local development of PD precedes the development of RA. This question can be addressed in the future by the study of at-risk cohorts (45,46). There are remarkable similarities in the histopathologic features of PD and RA, and there is evidence of an association between the 2 conditions, as has been observed in animal models of RA and periodontal inflammation (6,47). It is possible, therefore, that both the periodontal tissue and the joints are preferential targets of the same autoimmune process, thus raising an alternative concept, namely that periodontitis may be an extraarticular feature of RA.

Several other questions remain. First, if certain species of Porphyromonas are indeed at least partially responsible for the triggering of RA (as has been suggested by many lines of investigation) (30,31,40), how can the presence of RA in patients without P. gingivalis be explained? Based on our findings, the subgingival biofilm was colonized with P. gingivalis in samples from only 55% of patients with new-onset RA. However, when the results of serologic testing were also considered, >80% of the patients with new-onset RA exhibited evidence of exposure to P. gingivalis.

It is possible that in those patients who had no prior exposure to P. gingivalis, other bacterial organisms might serve as disease initiators. Notably, a near-identical proportion of healthy subjects showed similar results, albeit mostly due to the presence of antibodies. Intriguingly, 72.2% of subjects in our healthy control group tested positive for anti-P. gingivalis HtpG antibodies, compared to 63.3% of patients with new-onset RA and 50% of patients with chronic RA. This may reflect either the proposed protective nature of these particular antibodies (18), the inability of some RA patients to mount a serologic response to the organism, or a combination of both. Prior studies utilized anti-P. gingivalis antibodies against whole-cell or bacterial lipopolysaccharide (30,31). Although those investigators found a similar rate of exposure to P. gingivalis in RA patients (~60%), healthy controls had a more limited antibody response. The sensitivity, specificity, and biologic properties of all of these antibodies (including those utilized in our study) are yet to be refined, adding complexity and potential limitations to the use of P. gingivalis serologic responses as a surrogate for prior exposure.

Our data remain consistent with the prevailing speculation that P. gingivalis may serve as an environmental trigger for RA. It is reasonable to posit that a particular Porphyromonas species with defined virulent attributes (i.e., invasion properties, high PAD enzyme activity) might serve as a triggering factor for RA in susceptible individuals. We did not find any correlation between the presence of Porphyromonas and the presence of HLA–DR1, HLA–DR4, or PTPN22 (results not shown). It is possible that other Porphyromonas strains, in combination with overabundant bacteria from other genera, such as Anaeroglobus or Prevotella (and/or lower abundance of commensal symbionts, such as Actinomycetales), may also play a role; in this regard, our findings suggested that OTU60 (Prevotella) and OTU87 (Leptotrichia), which were unique to the patients with new-onset RA irrespective of PD status, should be further studied as potential pathogenic triggers.

Exposure to bacterial antigens at other body sites, such as the lung or intestine (48,49), may also contribute as a triggering factor for autoimmune arthritis. The intestinal microbiome is, by far, the most abundant and diverse. With ~3.3 million protein-coding genes (100 times more than the human genome), it outnumbers the host cells in a ratio of 10 to 1. Several studies have examined the effects of this antigen load in animal models of RA (50). Most recently, a single commensal bacterium was shown to be sufficient to induce inflammatory arthritis in a mouse RA-like disease model (51). An assessment of the role of the intestinal microbiota in human RA, utilizing parallel sequencing methods, is currently under way in our laboratories (49).

Our studies represent a new comprehensive approach for the study of the relationship between the role of bacteria and the initiation of RA. Indeed, investigations using this approach have identified at least 3 novel organisms (Anaeroglobus, Prevotella, and Leptotrichia) that merit further study. Mechanistic insights into pos-
sible causation will require analyses of microbial virulent factors, isolation of candidate microorganisms, and in vivo experiments in animal models. A prospective cohort of individuals with PD and other risk factors for the development of RA (e.g., first-degree relatives or individuals with autoantibodies and/or genetic predisposition) may help elucidate some of these questions and continue to narrow the knowledge gap in the field.

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