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

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### Abstract

**Objective**—To profile the subgingival oral microbiota abundance and diversity in never-treated, new-onset rheumatoid arthritis (NORA) patients.

**Methods**—Periodontal disease (PD) status, clinical activity and sociodemographic factors were determined in patients with NORA, chronic RA (CRA) and healthy subjects. Massively parallel pyrosequencing was used to compare the composition of subgingival microbiota and establish correlations between presence/abundance of bacteria and disease phenotypes. Anti-*P. gingivalis* antibodies were tested to assess prior exposure.

**Results**—The more advanced forms of periodontitis are already present at disease onset in NORA patients. The subgingival microbiota of NORA is distinct from controls. In most cases, however, these differences can be attributed to PD severity and are not inherent to RA. The presence and abundance of *P. gingivalis* is directly associated with PD severity as well, is not unique to RA, and does not correlate with anti-citrullinated peptide antibody (ACPA) titers. Overall exposure to *P. gingivalis* is similar in RA and controls, observed in 78.4% and 83.3%, respectively. *Anaeroglobus geminatus* correlated with ACPA/RF presence. *Prevotella* and *Leptotrichia species* are the only characteristic taxa in the NORA group irrespective of PD status.

**Conclusions**—NORA patients exhibit a high prevalence of PD at disease onset, despite their young age and paucity of smoking history. The subgingival microbiota of NORA patients is similar to CRA and healthy subjects of comparable PD severity. Although colonization with *P.*

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**Study conception and design.** Scher, Ubeda, Pillinger, Weissmann, Littman, Pamer, Bretz, Abramson.

**Acquisition of data.** Scher, Ubeda, Equinda, Khanin, Buischi, Viale, Lipuma, Attur, Pamer, Bretz.

**Analysis and interpretation of data.** Scher, Ubeda, Khanin, Pillinger, Weissmann, Littman, Pamer, Bretz, Abramson.

*gingivalis* correlates with PD severity, overall exposure is similar among groups. The role of *A. geminatus* and *Prevotella/Leptotrichia* species in this process merits further study.

## INTRODUCTION

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 biological 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 we would be otherwise unable to obtain. In 2008, the NIH 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 pathological events (3). Despite recent advances in molecular pathogenesis its etiology is almost completely unknown. Although genes certainly contribute to RA susceptibility (4), genetic effects appear to require environmental factors (i.e., smoking, hormones, and infection) in order to explain differences in incidence of the disease (5).

Among the more intriguing environmental covariates modulating autoimmunity is the bidirectional crosstalk between the human host and the oral and intestinal microbiomes. Multiple lines of investigation have suggested a link between oral microbes, periodontal diseases (PD) and RA (6;7). However, most clinical studies implicating specific oral microorganisms as triggers for RA have relied only on serological methods. Data describing the subgingival microbiota in patients with RA is virtually non-existent.

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 subgingival microbiota with RA status utilizing 16S rRNA pyrosequencing. Because we wanted to understand whether specific oral microbiota is associated with the initiation of RA, we focused our attention on patients with new-onset RA (NORA) who were steroid- and DMARD-naïve at the time of enrollment.

## PATIENTS AND METHODS

### Study participants

Consecutive patients from the NYU Rheumatology clinics and offices were screened for the presence of RA. After informed consent was signed, past medical history (chart review and interview/questionnaire), diet and medications were determined. A screening musculoskeletal exam and laboratory assessments were also performed or reviewed; all RA patients who met study criteria were offered enrollment. *Inclusion criteria* involved RA patients meeting 2010 ACR/EULAR criteria for RA, including presence of rheumatoid factor (RF) and/or anti-citrullinated peptide antibodies (ACPA; Anti-CCP ELISA, EUROIMMUN), and age 18 years or older. New-onset rheumatoid arthritis (NORA) was defined as disease duration of >6 weeks and absence of any treatment with disease-modifying anti-rheumatic drug (DMARD) or steroids (ever). Chronic-established RA (CRA) was defined as any patient meeting criteria for RA with minimum disease duration of 6 months. Most CRA subjects 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. *Exclusion criteria for*

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

### Classification of periodontal diseases

All periodontal examinations were performed at the NYU College of Dentistry. Periodontal status was assessed by three calibrated examiners (blinded for RA status) and defined according to the American Academy of Periodontology (AAP) (8). The following parameters were recorded: probing depth (Prob), clinical attachment level (AL), and bleeding upon probing (BoP). Patients and controls were classified as: 1) Healthy, no bleeding upon probing; 2) Gingivitis with bleeding upon probing; 3) Slight chronic periodontitis (at least one periodontal site with 1–2 mm AL and < 4 mm Prob); 4) Moderate chronic periodontitis (at least two teeth with 3–4 mm AL or at least 2 teeth with < 4 mm Prob); 5) Severe chronic periodontitis (at least two teeth with > 5 mm AL and one tooth with > 5 mm Prob).

In all, 31 NORA patients, 34 CRA patients, and 18 healthy controls were available for the analyses presented here.

### Sample collection and DNA extraction

Oral samples were obtained by collection of subgingival biofilm from the six most periodontally diseased sites of 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 (MoBio). DNA was extracted within 1 hr of sample collection using a combination of the MoBio Power Soil kit (MoBio) and a mechanical disruption (bead-beater) method based on a previously described protocol (9). Samples were stored at –80°C.

### V1-V2 16S rRNA region amplification and 454/pyrosequencing

For each sample, 3 replicate PCRs were performed to amplify the V1 and V2 regions as previously described by Ubeda et. al. (10). PCR products were sequenced on a 454 GS FLX Titanium platform (454 Roche). Sequences have been deposited in the NCBI Sequence Read Archive under the accession number [SRA050292](https://www.ncbi.nlm.nih.gov/sra/SRA050292).

### Sequence analysis

Sequence data were compiled and processed using *mothur* (11) and converted to standard FASTA format. Sequences were trimmed and aligned to the V1-V2 region of the 16S gene, using as template the SILVA reference alignment (12). Potentially chimeric sequences were removed using *ChimeraSlayer* (13). To minimize the effect of pyrosequencing errors in overestimating microbial diversity (14), low abundance sequences were merged to the high abundant sequence using the *pre.cluster* option in *mothur*. Sequences were grouped into operational taxonomic units (OTUs) using the average neighbor algorithm. Sequences with 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 and the Simpson diversity indexes (15) and richness was estimated using the Chao index. Yue and Clayton diversity measure and Principal Coordinate of Analysis were performed using *Mothur*. Phylogenetic classification was performed using the Bayesian classifier algorithm with the bootstrap cutoff 60% (16).

## Serum ELISA for anti-HtpG P18y peptide antibodies

*P. gingivalis* HtpG peptides were prepared in the laboratories of Drs. Sweier and Shelbourne (University of Michigan School of Dentistry, Ann Arbor) as previously described (17) and loaded into the wells of microtiter plates. 25  $\mu$ l of each serum sample was added, incubated with goat anti-human IgG ( $\gamma$ -chain specific) and analyzed according to protocol.

## Statistical analyses

To determine statistically significant differences between samples from disease and healthy individuals, bacteria with <5 mean count in both conditions were removed, and t-test was applied to log<sub>2</sub> transformed scaled count-data, and rescaled using DESeq R package (18). To adjust for multiple hypothesis testing, we employed the False Discovery Rate (FDR) approach (19), and used *fdr.R* package. The final results were filtered for p value < 0.05 and a FDR = 0.1.

For cross-sectional analyses of baseline characteristics, differences were evaluated using Student's t test, Mann–Whitney U test or chi-squared tests, when appropriate. SPSS V.16.0 software (SPSS, Chicago, Illinois, USA) was used for the analysis, two-tailed significance testing was employed and significance was set as  $p < 0.05$ .

## RESULTS

### New-onset rheumatoid arthritis patients present with advanced periodontal disease

Of 31 NORA subjects included in this study, 68% were females with a mean age of 42.2 years (Table 1). Mean disease duration was 3.4 months (median 2 months) and no patient had ever received steroids, oral DMARDs or biologics. Mean disease activity score 28 (DAS28) was 5.8. In concordance with inclusion criteria all patients were “seropositive”: 96% of NORA subjects were ACPA seropositive and 92% were RF positive; more than two thirds had never smoked tobacco (16% of participants were current smokers) (Table 1). Healthy controls were age-, sex- and ethnicity-matched. The CRA group had proportionally more female participants; mean age for this group was 47.7 years ( $p = NS$  compared to NORA) and 88% were ACPA positive. Mean disease duration was 62.9 months (median 34) and the mean DAS28 was 4.4, reflecting moderate disease presumably altered by DMARD intervention; 70% of CRA subjects had no history of smoking.

As shown in Table 1, more than 75% of NORA and CRA patients were found to have moderate to severe forms of PD, a significantly higher proportion when compared to healthy controls. The prevalence of periodontal disease in our healthy controls was consistent with the expected prevalence (30–40%) of extensive PD in the general population (20). An interesting finding was the presence of PD in several of our NORA subjects younger than 30 years of age without PD risk factors, such as smoking, when periodontitis is typically absent.

### The oral microbiota is equally rich and diverse in NORA, CRA and control groups

Overall, 83 oral samples were obtained from all participants yielding a total of 206,378 16S RNA high-quality sequences (average 2037 sequences/ sample; range 443–5008 reads;  $p = NS$ ). Using a distance-based similarity of >97% for species-level operational taxonomic unit (OTU) assignment, a total of 2136 OTUs were identified. (supplemental Figure 1A).

We first studied the impact of RA status in microbial diversity by using the Inverse Simpson and Shannon indexes. Both take into account, when calculating the diversity of a sample, not only the number of OTUs (~species) present but also the relative frequency of the different OTUs within that sample. A high index reflects a more diverse microbiota. Utilizing both calculations, no significant differences in microbial diversity were observed between RA

groups and controls (supplemental Figures 1C and 1D). We then analyzed if RA status had an impact on microbial richness. When applying the Chao index (which estimates how many OTUs constitute the microbiota of a specific sample), no significant differences were found among groups (supplemental Figure 1D).

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 was able to discriminate between NORA, CRA or healthy groups. Rather, differences were evident when groups were combined by periodontal disease severity. As previously described by others (21;22), our assessment showed that the healthy periodontal microbiota is 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). The 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 if NORA microbiota was distinct from that of healthy controls, we applied the Yue and Clayton diversity measure (which compares the relative abundance of OTUs present in different samples). We then applied PCoA to the quantified similarity distances between samples and clustered them along orthogonal axes of maximal variance. Two principal coordinates (PC1 and PC2) explain most of the variation observed between the samples. No clustering due to RA status could be observed (Figure 1C). However, as shown in Figure 1D, PC1 did cluster a group of samples (circle) obtained from patients with severe and moderate periodontitis. This result suggests that differences at the OTU level characterize the more advanced forms of PD and do not represent a specific signature for RA oral microbiota.

### **Prevotella and Leptotrichia species are characteristic of the NORA oral microbiota**

We next sought to identify a bacterium or groups of bacteria responsible for the clustering that identifies patients with advanced PD. Applying multivariate statistical analyses taking into consideration patient groups, we also looked for bacterial taxa that were significantly different in the NORA group (either increased or decreased) compared to the others. At most taxonomic levels the oral microbiota of NORA patients is not significantly different from that of other groups (Table 2 and Supplementary Table). However, the genera *Corynebacterium* and *Streptococcus* are underrepresented in RA subjects, which reflects the lack of a healthy microbiota, and is therefore consistent with PD, per se. Interestingly, OTU 60 (*Prevotella spp.*) and OTU 87 (*Leptotrichia spp.*) are the only characteristic taxa in the NORA group irrespective of PD status (present in 32.2% and 25.8% of patients, respectively), and are completely absent in the oral microbiota of controls.

### **Abundance of periodontopathic bacteria is high in NORA but diminished in the oral microbiota of CRA**

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 (23) (a triad of the most virulent periodontopathic bacteria including *Porphyromonas*, *Tannerella* and *Treponema*) are more prevalent in NORA microbiota compared to CRA patients (Figure 2).

### The genus *Porphyromonas* and *P. gingivalis*-related OTUs are significantly associated with PD severity and are not specific of the RA microbiota

Although with variability in level of abundance, the genus *Porphyromonas* was present almost universally in all participants (Figures 3A and 3C).

We analyzed the 2136 different OTUs among all patient groups (including 59 OTUs within *Porphyromonas*), and found that OTU 1, with 100% 16S rRNA sequence homology to *P. gingivalis*, was significantly more prevalent and abundant in patients with PD (more than 60-fold increase in the severe forms compared to healthy gums) and had no direct correlation to RA (Figures 3B and 3D). *P. gingivalis* was present in 55% of NORA and 47% of CRA patients, while the prevalence in healthy controls was 27% ( $p=0.18$ , ANOVA). We further stratified NORA patients into two categories according to presence or absence of PD and found that *P. gingivalis* was also more prevalent (62.5% vs 28.5%;  $p<0.05$ ) and abundant (mean 6.2% vs 0.78%;  $p<0.05$ ) in the advanced PD group (supplemental Figure 2). Many other OTUs known to be associated to PD showed similar elevations, although less pronounced (data not shown). Taken together, these data suggest that although colonization of *P. gingivalis* is twice as common in RA patients compared to controls, the difference can be explained by the higher prevalence of PD in the RA population.

### Exposure to *P. gingivalis* is not significantly different among groups

Because the absence of *P. gingivalis* in the oral microbiota did not exclude prior exposure to the organism, we tested a previously validated antibody assay against the highly specific *P. gingivalis* chaperone HtpG (IgG class anti-P18 $\gamma$ ). We found that 63.3% of NORA patients, 50% of CRA patients and 72.2% of healthy controls tested positive for anti-P18 $\gamma$  ( $p=0.45$ , ANOVA; Figure 4A). We next analyzed overall exposure to *P. gingivalis* based upon either *P. gingivalis* colonization by 16s and/or a positive antibody response. Interestingly, we found that while the NORA and CRA groups had a rate of exposure of 84% and 71%, respectively, healthy individuals also revealed an exposure prevalence of 83% (N.S., ANOVA; Figure 4B), largely due to an increased serological response.

### Presence of circulating RA-related autoantibodies correlates with *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 generation of neoantigens and subsequent production of ACPA (24;25). We therefore examined the different taxonomic levels to look for phylotypes associated with circulating autoantibodies. There was no association between RF or ACPA with the higher taxonomic levels (phylum, class, order or family). In particular, there was no association between RF or ACPA and the presence of neither the genus *Porphyromonas* in the oral microbiota nor OTUs related to *P. gingivalis*. Indeed, several patients with ACPA 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 OTU99 (closely related to *Anaeroglobus geminatus*) significantly correlated with both circulating RF and ACPA ( $P<0.05$ ). Moreover, OTU99 was associated with PD, and found in 77.5% and 50% of NORA and CRA patients, respectively ( $p=NS$ ), and only 16.7% of healthy controls ( $P<0.005$  vs. NORA or CRA). OTU130, with 94% similarity to *Porphyromonas catoniae*, was also significantly associated with circulating ACPA.

## DISCUSSION

An accumulating body of epidemiological data suggests a role of clinical periodontal diseases in the development of RA. In concordance with our findings, periodontitis was more common and severe in patients with RA compared to patients with OA in a cohort of U.S. veterans (26). In another study (27), RA patients had an 8-fold increased likelihood of periodontitis compared to controls. Multiple recent studies have also implicated *P. gingivalis* as a possible triggering factor. Interestingly, however, none of these reports have directly looked at the presence of oral microorganisms. Rather, they relied upon serological methods (28;29) or limited, low-throughput PCR-based techniques (30;31). To our knowledge, no prior report has specifically assessed the presence of *P. gingivalis* (or other periodontopathic bacteria) in subgingival biofilms in RA patients.

Our study is the first utilizing multiplexed-454 pyrosequencing to compare the bacterial composition of the subgingival microbiota in RA (early and chronic) and 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 early RA patients present with incident PD (32). Our data are striking in that a high prevalence of moderate to severe periodontitis is observed in a steroid- and DMARD-naïve population, a finding reported here for the first time in this unique untreated cohort. Moreover, our NORA cohort is mostly composed of young non-smokers, whereas smoking is otherwise a significant risk indicator of periodontal diseases (33;34) and has been proposed as a central driver of gene-environment interaction in seropositive RA (35). These results are consistent with the notion that PD, present at the time of diagnosis in the majority of NORA patients, may represent a risk factor for RA development independent from smoking status. Intriguingly, some periodontopathic OTUs (e.g., *Tannerella*/OTU13, *Treponema*/OTU32) were significantly higher in NORA microbiota and tended to diminish with established better-controlled disease (CRA). We speculate that this difference could result from 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 (36;37)- have an impact on the ecological adaptation of the oral microbial niche.

Second, we found that the subgingival microbial communities of NORA patients generally do not have a unique fingerprinting compared to controls. However, two OTUs, OTU 60/*Prevotella* spp. and OTU 87/*Leptotrichia* spp., were detected only in the NORA population. Although the genus *Porphyromonas* is present in virtually all subjects, its relative abundance is directly correlated with PD severity, regardless of RA status. We corroborated prior findings using low-throughput techniques (23;38;39) that the advanced forms of PD are overrepresented by genera such as *Porphyromonas*, *Tannerella* and *Treponema*, all of which have been implicated in PD pathogenesis (23). Our ability to go beyond the genus taxonomy has 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 was similar to *P. gingivalis* were significantly overrepresented in the subgingival oral microbiota of NORA patients. Our data demonstrate that while most individuals carry *Porphyromonas* in their subgingival domain, a particular OTU (OTU1) is mostly found in advanced PD, whether in RA patients or otherwise healthy controls. However, and given the low number of non-RA PD subjects 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. A large replication cohort should help elucidate this question in the future.

Third, an unanticipated finding was that OTU99/*Anaeroglobus geminatus* significantly correlated with serum titers of RF and ACPA. *A. geminatus* is the only described species of the genus belonging to the family Veillonellaceae. A strictly anaerobic gram-negative cocci, this bacteria was originally isolated from a post-operative fluid collection (40). There is scarce literature about *A. geminatus*, although two studies have described the presence of a closely related species (*Megasphaera spp.*) in the setting of PD (41). Even more intriguingly, two other organisms were found to be prevalent only in NORA, *Prevotella*/OTU60 and *Leptotrichia*/OTU87. Using publically available alignment tools, we found that both OTUs aligned to yet-uncultured microorganisms. In the case of *Leptotrichia*, the closest known 16S gene belonged to *L. wadei* (91% identity). This species has been previously recovered from patients with periodontitis (42). OTU60 aligned only to uncultured oral *Prevotella* species. The role of the genus *Prevotella* (i.e., *P. intermedia*) in PD is well established (23). Although not fully sequenced and poorly understood, our preliminary observations show newly described species (*Prevotella*/OTU60, *Leptotrichia*/OTU87 and *A. geminatus*/OTU99) that merit further study as candidate periodontal microbial triggers of RA.

The significance of periodontal inflammation in new-onset RA continues to be an important yet unanswered question. It is clear from these and prior studies that there is a high prevalence of PD in new onset disease that cannot be explained by immunosuppressive treatments. However, it remains undetermined whether local PD precedes RA development. This question can be addressed in the future by the study of at-risk cohorts (43;44). There are remarkable similarities in the histopathologies of PD and RA, and evidence of co-association between the two, including animal models of RA that develop periodontal inflammation (6;45). 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 extra-articular feature of RA.

Several other questions remain. First, if certain *Porphyromonas* are indeed at least partially responsible triggers for RA (as suggested by many lines of investigation) (28;29;38), how is it possible to explain disease in patients without *P. gingivalis*? Based on our findings, only 55% of new-onset RA patients were colonized with *P. gingivalis*. However, when serological testing was also considered, over 80% of NORA patients exhibited evidence of exposure to *P. gingivalis*. It is possible that in those patients without prior exposure to *P. gingivalis*, other bacterial organisms might serve as disease initiators. Noticeably, a near identical proportion of healthy subjects showed similar results, albeit mostly due to presence of antibodies. Intriguingly, 72% of subjects in our healthy control group tested positive for anti-*P. gingivalis* HtpG antibodies compared to 63.3% of NORA and 50% of CRA patients, respectively. This may either reflect the proposed protective nature of these particular antibodies (17), the inability of some RA patients to mount a serological response to the organism or a combination of both. Prior studies utilized anti-*P. gingivalis* antibodies against whole cell or bacterial LPS (28;29). Although they found a similar rate of exposure in RA patients (~60%), healthy controls had a more limited antibody response. The sensitivity, specificity and biological properties of all these antibodies (including the one utilized in our study) are yet to be refined, adding complexity and potential limitations to the use of *P. gingivalis* serology 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 with HLA-DR1, -DR4 and PTPN22; data not shown. It is possible that other *Porphyromonas* strains, in combination with overabundant bacteria from other genus such as *Anaeroglobus* or *Prevotella* (and/or lower abundance of commensal symbionts, such as Actinomycetales) may also play a role, and in this regard our data suggest that OTUs 60/*Prevotella* and 87/*Leptotrichia*, which are unique to NORA patients 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 (46;47), may also contribute as triggering factors for autoimmune arthritis. The intestinal microbiome is by far the most abundant and diverse. With about 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 looked at the effects of this antigen load in animal models of RA (48). Most recently, a single commensal bacterium was sufficient to induce inflammatory arthritis in a RA-like mouse model (49). An assessment of the role of the intestinal microbiota in human RA utilizing parallel sequencing methods is currently underway in our laboratories (47).

Our studies represent a new comprehensive approach for the study of the relationship between the role of bacteria and the initiation of RA. Indeed, this approach has identified at least three novel organisms (*Anaeroglobus*, *Prevotella* and *Leptotrichia*), that merit further study. Mechanistic insights into possible 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 periodontal diseases 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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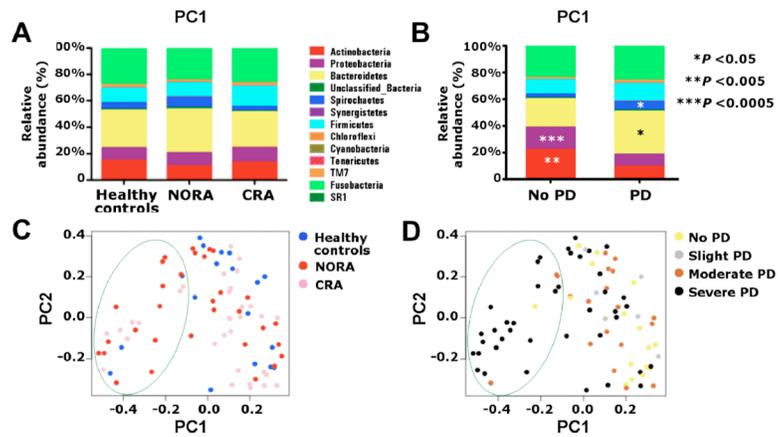
Other critical study activities: Obtained NIH funding. Littman, Abramson.

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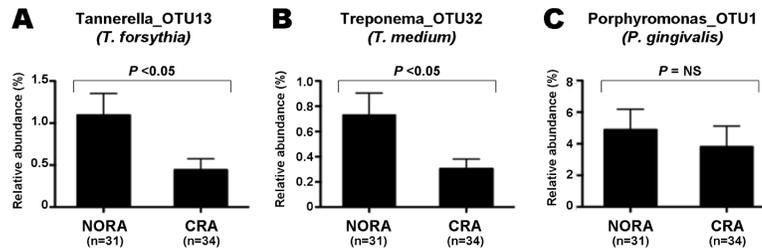
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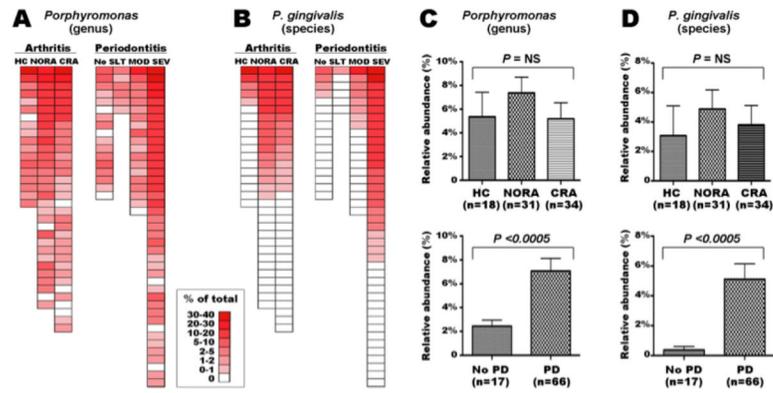
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**Figure 1.** Clustering of species-level operational taxonomic units (OTU) among groups studied. Bar graphs show relative abundance of oral microbiota (phylum level) among (A) NORA, CRA and healthy controls and (B) participants with No PD vs PD. Clustering by Yue and Clayton analysis comparing (C) patients with new-onset rheumatoid arthritis (NORA) vs patients with chronic rheumatoid arthritis (CRA) vs healthy control participants, and (D) participants with healthy gingiva or gingivitis (No PD) vs those with slight, moderate or severe periodontal disease (PD).

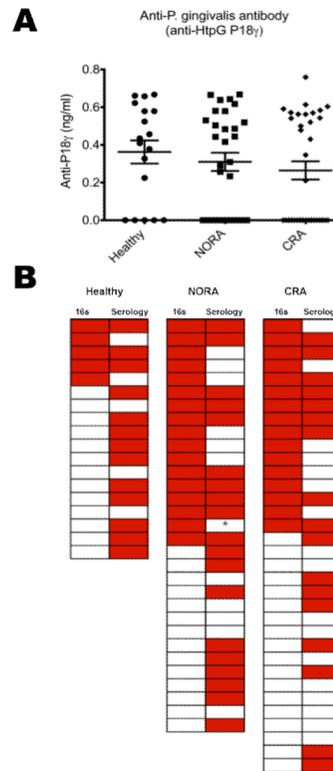


**Figure 2.** Abundance of periodontopathic bacteria (OTU-level) in patients with new-onset rheumatoid arthritis (NORA) and chronic rheumatoid arthritis (CRA). Species associated with periodontal disease, such as (A) *Tannerella forsythia* and (B) *Treponema medium*, are significantly more abundant in NORA patients compared to CRA. (C) No significant differences (NS) are observed for *Porphyromonas gingivalis*.



**Figure 3.**

Prevalence and abundance of *Porphyromonas* and *P. gingivalis* (OTU1) in 83 study participants, grouped by rheumatoid arthritis (RA) status [healthy controls (HC), new-onset RA (NORA), chronic RA (CRA)] and periodontal disease (PD) status [healthy gingiva or gingivitis (No PD); slight (SLT), moderate (MOD), and severe (SEV) PD]. Although the genus *Porphyromonas* (A, C) is present almost universally and irrespective of RA or PD status, *P. gingivalis* (B, D) is significantly associated with moderate and severe PD, and not with presence of RA. (A, B) Each square represents a single individual. The darker the intensity of the box, the greater the relative abundance of *Porphyromonas* or *P. gingivalis*. NS = not significant.



**Figure 4.**

Anti-HtpG peptide antibody (anti-P18 $\gamma$ ) serum levels and overall *P. gingivalis* exposure among different groups. **(A)** Anti-P18 $\gamma$  antibodies were found in 72% of healthy subjects, 63.3% of NORA patients, and 50% of CRA patients ( $p=0.18$ ; ANOVA); mean levels were not significantly different. **(B)** Prevalence of *P. gingivalis* in oral microbiota (16s) and anti-P18 $\gamma$  antibody (serology) in all 83 participants, grouped by healthy controls (Healthy) and RA status (NORA and CRA). Red squares denote presence of *P. gingivalis* by 16S pyrosequencing and/or detectable anti-*P. gingivalis* antibody. Each square represents a single individual and contiguous squares are representative of same participant. (\*) Serum not available in one NORA participant. HC=Healthy controls; NORA=New-onset Rheumatoid arthritis; CRA=Chronic RA; PD=Periodontal disease; No-PD=Healthy gingiva.

**Table 1**

Demographic and clinical data (top) and severity of periodontal disease (bottom) among patients with new-onset rheumatoid arthritis (NORA), patients with chronic RA (CRA), and healthy control participants.

Characteristic	NORA (n=31)	CRA (n=34)	Healthy Controls (n=18)
<b>Age, years, mean (median)</b>	42.2 (40)	47.7 (48)	42.2 (39)
<b>Female, %</b>	68%	79%	65%
<b>Disease duration, months, mean (median)</b>	3.4 (2)	62.9 (34)	n/a
<b>Disease activity parameters</b>			
ESR, mm/h, mean	37.8	31.2	14
CRP, mg/l, mean	26.7	9	0
DAS28, mean (median)	5.8 (6.01)	4.4 (4.72)	n/a
Patient VAS pain, mm, mean (median)	64.4 (55)	46.6 (50)	n/a
TJC-28, mean (median)	11.9 (12)	5.6 (4)	n/a
SJC-28, mean (median)	8.2 (8)	4 (3)	n/a
<b>Autoantibody status</b>			
IgM-RF positive, %	92%	88%	10%
ACPA positive, %	96%	88%	0
IgM-RF and/or ACPA positive, %	100%	96%	10%
IgM-RF titer, kU/l, mean (median)	377 (157)	169.6 (98)	4.4 (0)
ACPA titer, kAU/l, mean (median)	114.6 (150)	97.4 (60)	0
<b>HLA-Shared Epitope positive, %</b>	14 (45%)	11 (33%)	3 (16%)
<b>Medication use</b>			
Methotrexate, %	0	79%	0
Prednisone, %	0	45%	0
Biological agent, %	0	12%	0
<b>Smoking status</b>			
Current, %	16%	6%	6%
Former, %	16%	24%	16%
Never, %	68%	70%	78%
<b>Periodontal disease (PD) status</b>			
Healthy gingiva, %	<b>0 *</b>	<b>6% *</b>	<b>45% *</b>
Gingivitis, %	13%	3%	11%
Slight PD, %	10%	6%	5%
Moderate PD, %	16%	32%	17%
Severe PD, %	<b>62% *</b>	<b>53% *</b>	<b>22% *</b>

NORA and CRA vs healthy control participants by analysis of variance (ANOVA)

\* P < 0.01,

**Table 2**

Oral microbiota differ significantly among patients with new-onset rheumatoid arthritis (NORA), patients with chronic rheumatoid arthritis (CRA), and healthy control participants, and between individuals with healthy gingiva versus periodontal diseases (PD), at the level of both genus and species/operational taxonomic unit (OTU).

Taxonomy	NORA vs Healthy Controls	NORA vs CRA	All RA vs Healthy Controls	Healthy gingiva vs PD
<b>Genus</b>	↑Anaeroglobus*	↓Uncl. Veillonellaceae**	↑Anaeroglobus**	↑Anaeroglobus****
	↑Uncl. Prevotellaceae*	↓Mitsuokella*	↓Corynebacterium**	↑Phocaeiola****
	↑Phocaeiola*			↑Dialister***
	↓Coryne bacterium*			↑Schwartzia***
	↓Mitsuokella*			↑Uncl_Prevotellaceae****
	↓Streptococcus*			↑Prevotella*
				↑Tannerella*
				↑Treponema*
				↑Porphyromonas*
				↓Actinomyces****
				↓Corynebacterium***
				↓Neisseria***
				↓Uncl_Flavobacteriaceae****
				↓Uncl_Propionibacteriac.***
				↓Granulicatella**
				↓Streptococcus*
<b>Species (OTU)</b>	↑Anaeroglobus_OTU99****	↑Porphyromonas_OTU57*	↑Anaeroglobus_OTU99****	↑Anaeroglobus_OTU99****
	↑Leptotrichia_OTU87****	↑Selenomonas_OTU231*	↑Prevotella_OTU134****	↑Prevotella_OTU62****
	↑Prevotella_OTU60****	↑Prevotella_OTU26*	↑Prevotella_OTU60****	↑Prevotella_OTU20****
	↑Selenomonas_OTU168**	↑Treponema_OTU32*	↑Selenomonas_OTU168*	↑Treponema_OTU139****
	↑Phocaeiola_OTU92*	↑Tannerella_OTU13*	↑Prevotella_OTU31*	↑Tannerella_OTU13***
	↑Prevotella_OTU31*	↓Prevotella_OTU39****	↓Leptotrichia_OTU12****	↑Porphyromonas_OTU1***
	↑Prevotella_OTU134*		↓Corynebact_OTU4****	↑Treponema_OTU32****
	↑Neisseria_OTU16*		↓Leptotrichia_OTU86*	↑Selenomonas_OTU168****
	↑Porphyromonas_OTU1*		↓Leptotrichia_OTU9*	↑Prevotella_OTU39****
	↓Leptotrichia_OTU12**		↓Capnocytophaga_OTU74*	↓Corynebacterium_OTU4****
	↓Corynebact_OTU4*			↓Corynebacter_OTU77****
	↓Uncl.TM7_OTU58*			↓Granulicatella_OTU162**
				↓Actinomyces_OTU63**
				↓Corynebacter_OTU146**

↑ = significant increase in NORA (or PD); ↓ = significant decrease in NORA (or PD).

\*  
 $P < 0.05$ ;

\*\*  
 $P < 0.01$ ;

\*\*\*  
 $P < 0.005$ ;

\*\*\*\*  
 $P < 0.0005$ .