**Concise Report**

# *Prevotella copri* in individuals at risk for rheumatoid arthritis

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**Abstract**

**Objectives:** Rheumatoid arthritis (RA) has been associated with a relative expansion of faecal Prevotellaceae. To determine the microbiome composition and prevalence of *Prevotella* spp. in a group of individuals at increased risk for RA, but prior to the development of the disease.

**Methods:** In an ongoing cohort study of first degree relatives (FDR) of RA patients, we identified 'FDR controls', asymptomatic and without autoantibodies, and individuals in pre-clinical RA stages, who had either developed anti-citrullinated peptide antibodies or rheumatoid factor positivity and/or symptoms and signs associated with possible RA. Stool sampling and culture-independent microbiota analyses were performed followed by descriptive statistics and statistical analyses of community structures.

**Results:** A total of 133 participants were included, of which 50 were categorized as 'FDR controls' and 83 in 'pre-clinical RA stages'. The microbiota of individuals in 'pre-clinical RA stages' was significantly altered compared to FDR controls. We found a significant enrichment of the bacterial family Prevotellaceae*,* particularly *Prevotella* spp., in the 'pre-clinical RA' group ($p = 0.04$).

**Conclusions:** *Prevotella* spp. enrichment in individuals in pre-clinical stages of RA, before the onset of RA, suggest a role of intestinal dysbiosis in the development of RA.

**‘Key messages’**

What is already known about this subject?

- A high relative abundance of *Prevotella copri* has been identified in patients newly diagnosed with RA, suggesting a role of gut microbiota dysbiosis in the etiopathogenesis of the disease.

What does this study add?

- This is the first study to describe a significantly altered microbiota, particularly a *Prevotella spp*. enrichment, already in individuals in pre-clinical stages of RA, compared to controls.

How might this impact on clinical practice or future developments?

- Our results, together with previous studies in early RA patients and recent mechanistic studies, support the mucosal origins hypothesis and the role of intestinal dysbiosis in the development of RA.
- Intestinal dysbiosis could act as an early environmental modulator and may be a target of future preventive interventions in individuals at risk of RA, before the onset of the disease.

## INTRODUCTION

The etiopathogenesis of rheumatoid arthritis (RA) is thought to result from a multi-step process, whereby environmental factors induce a pathological activation of the immune system in susceptible individuals.[1] Recent studies have suggested that the initial steps of the pathological autoimmune response originate in mucosal sites, rather than in the joints.[2] Intestinal dysbiosis has been suggested to have a causal role in the pathogenesis of RA and has been shown to trigger arthritis development in genetically susceptible mice.[3-6] *Prevotella copri* has been identified as highly enriched in the gut microbiota of patients newly diagnosed with RA and an increased humoral and cellular immune response to this organism has been demonstrated in RA patients suggesting a role of *Prevotella copri* in the disease onset.[7-9] Sequence homology between RA-specific autoantigens and epitopes from proteins of *Prevotella* spp. have been reported, supporting the molecular mimicry hypothesis, although exact mechanisms remain uncertain.[8] Considering these observations, intestinal dysbiosis involving *Prevotella* spp. may be a risk factor for RA and a potential therapeutic target. However, to formally establish a causal role of intestinal dysbiosis in RA development, longitudinal studies prior to the onset of RA are required, to demonstrate that the presence of *Prevotella* spp. precedes the development of RA. The aim of this study was thus to characterize the microbiota and determine the prevalence of *Prevotella* spp. in individuals during the pre-clinical phases of RA, before the development of clinically apparent RA.

## MATERIAL AND METHODS

### Study design and study population

First degree relatives of patients with RA (RA-FDRs) have an increased risk of developing RA compared to the general population.[10 11] The SCREEN-RA study is an ongoing cohort study of RA-FDRs, comprising subjects without a diagnosis of RA at enrolment, described in detail elsewhere (online supplementary text).[12]

We performed a nested case-control study within SCREEN-RA cohort to analyse the intestinal microbiota in individuals in pre-clinical phases of the disease. We identified participants in 'pre-clinical RA' stages based on the EULAR terminology for pre-clinical phases of RA.[13] Operationally, we combined two pre-clinical RA stages for statistical power reasons: (i) individuals with 'systemic autoimmunity associated with RA' defined by anticitrullinated protein autoantibodies (ACPA) positivity and/or rheumatoid factor (RF) positivity,[14] and/or (ii)

'individuals with symptoms and signs associated with possible RA' as defined by the Connective Tissue Disease Screening Questionnaire (CSQ) with or without undifferentiated arthritis (UA) (see online supplementary text for details).[15-17] We included a control group, namely 'FDR controls', namely RA-FDRs without any autoantibodies or symptoms associated with possible RA.

Participants were contacted by telephone to explain the objectives of the study and invited to provide stool samples for microbiome analysis. We included individuals with complete clinical information at the time of the stool sampling. We excluded participants who had undergone antibiotic therapy within the last 3 months, with a known history of inflammatory bowel disease and/or gastrointestinal tract surgery. The protocol was approved by the ethics committee and all participants signed an informed consent before providing a stool sample.

**Sampling, DNA extraction and amplicon sequencing analysis to analyse the faecal microbiota**

The DNA Genotek OMNIgene·Gut Stool Microbiome Kit was used to collect, store and ship the stool samples.[18] Stool samples processing and culture-independent analyses were performed. After DNA extraction from stool samples, the variable region 4 (V4) region of the 16S rRNA gene was amplified using barcoded primers (F515/R806) and sequencing was performed on an Illumina MiSeq as previously described.[19] (Details in the online supplementary text).

**Statistical analysis**

Controls and individuals in pre-clinical stages of RA were matched by sex, age and tobacco at the sampling stage. We used descriptive statistics to compare demographic data and various putative environmental factors.

Based on our *a priori* hypothesis, the primary outcome of the study was the prevalence of bacteria from the family of Prevotellaceae, particularly *Prevotella* spp. Based on the mucosal origins hypothesis of RA,[2] we postulated that the relative prevalence of Prevotellaceae in the stool of individuals in pre-clinical stages of RA would be increased compared to FDR controls. Statistical analyses of community structures were performed. We used *linear discriminant analysis (LDA) effect size (LEfSe)*, an algorithm to compare the relative abundance of the different features between groups, as previously described.[19 20] We performed subgroup analyses, dividing the group of

participants in 'pre-clinical stages of RA' into 'systemic autoimmunity associated with RA' and 'individuals with symptoms and signs associated with possible RA'. We further explored the general characteristics association with Prevotellaceae abundance.

## RESULTS

### Study population

Among the 1067 RA-FDRs participants in the SCREEN RA cohort, 183 (17%) were invited to provide stool samples, based on *a priori* inclusion criteria and the matching algorithm. A total of 133 RA-FDRs sent stool samples and could be analysed. General characteristics were balanced between the two groups (Table 1).

Table 1. General characteristics at stool collection (133 participants)

| Characteristics | FDR controls n=50 | Pre-clinical RA stages [a] n=83 |
|---|---|---|
| Age [years], median (IQR) | 55 (47-62) | 58 (50-66) |
| Female sex, n (%) | 39 (78) | 74 (89) |
| Current Smoking, n (%) | 11 (22) | 16 (19) |
| Past Smoking, n(%) | 26 (55) | 29 (41) |
| Pack years smoked, median (IQR) | 0.4 (0.4-0.7) | 0.4 (0.4-0.7) |
| Current Alcohol, n (%) | 22 (47) | 29 (41) |
| Body mass index, median(IQR) | 24 (22-27) | 24 (22-27) |
| Swollen joints on examination, median (IQR)• | 0 (0-1) | 1 (0-3) * |
| Tender joints at examination, median (IQR) | 0 (0-1) | 1 (0-2) * |
| ACPA positivity, n (%) | 0 (0) | 38 (46) * |
| RF positivity, n (%) | 0 (0) | 28 (34) * |
| Shared epitope (1 or 2 copies), n (%) | 32 (65) | 42 (53) |
| *p-value <0.05, Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables. [a]Pre-RA group includes individuals with 'Systemic autoimmunity associated with RA' and individuals with 'symptoms and signs | | |

associated with possible RA'. An isolated asymptomatic swollen joint was not sufficient to be classified as being in a 'pre-clinical stage of RA'.

**Microbiota analysis**

Our initial comparison of microbial diversity in the faecal microbiota within individuals and between individuals, i.e. alpha and beta diversity respectively, of the FDR control and the pre-clinical RA groups did not reveal significant differences (see online supplementary figure S1-S3). We utilized the LEfSe method to analyse potentially more specific differences in microbiota composition between FDR controls and individuals in the 'pre-clinical stages of RA'.[20] Indeed, we found statistically significant differences in the relative abundances of bacterial taxonomic groups between the participants in pre-clinical stages of RA development and FDR controls (Figure 1, LDA score >2, p-value <0.05). The family Prevotellaceae was the group of bacteria with the highest LDA score and was significantly enriched in individuals in 'pre-clinical stages of RA' (LEfSe p-value=0.041).

In a subgroup analysis, we split the 'pre-clinical RA group' into the 'systemic autoimmunity associated with RA' and the 'individuals with symptoms and signs associated with possible RA' groups. The family Prevotellaceae was enriched particularly in participants with 'systemic autoimmunity associated with RA' compared to 'FDR controls' (online supplementary figure S4, LEFSe p-value=0.019), and no significant difference was found between individuals in the two groups of pre-clinical stages of RA (online supplementary figure S5), which allowed us to analyse them together.

We then specifically analysed the relative abundance of the family Prevotellaceae and associated taxa to evaluate whether all individuals of the pre-clinical phases display an enrichment of Prevotellaceae or whether an enrichment is observed only in some (Figure 2). This analysis confirmed that a larger proportion of individuals within the pre-clinical RA group compared to controls (53 % vs 30 %) had significant levels of Prevotellaceae (>1%), but Prevotellaceae are not present in all individuals. The general characteristics of individuals with high relative abundance (>1%) of Prevotellaceae were not different compared to individuals with no Prevotellaceae or lower relative abundance, but for a higher prevalence of RF positivity (Online supplementary Table S2). Furthermore, besides *P. copri* other *Prevotella* spp. in other operational taxonomic units (OTUs) contribute to the Prevotellaceae enrichment in 'pre-clinical RA' (online supplementary figure S6).

## DISCUSSION

The present study focused on the prevalence of *Prevotella* spp. in the stool of individuals at risk for RA during pre-clinical phases of the disease. The microbiota of individuals in pre-clinical RA stages was significantly altered compared to FDR controls. In particular, the relative abundance of bacteria of the Prevotellaceae family and associated taxa were enriched among individuals in pre-clinical stages of RA and differed significantly from controls, in particular in individuals with 'systemic autoimmunity associated with RA', which is consistent with the mucosal origins hypothesis of RA development.[2]

A previous study analysed the microbiome of faecal samples of American patients with new-onset untreated RA and detected high abundance (>5%) of *P. copri* in 75% (33 of 44) compared to only 21.4% (6 of 28) of healthy individuals.[9] This finding was not replicated in a study involving Chinese RA patients.[21] Cross-sectional studies in RA patients with established disease do not allow making causal inferences, as this association could be due to differences in behaviours between RA patients and controls. Our study describes an increased relative abundance in *Prevotella* spp. in individuals in 'pre-clinical RA stages', using participants enrolled in a FDR-RA cohort. While this is still not a longitudinal study, the demonstration of a larger proportion of individuals in pre-clinical stages of RA with a significant abundance of Prevotellaceae strengthens the case for an involvement of *Prevotella* spp. in the RA ethiopathogenesis. However, longitudinal studies are needed to determine the specific role of intestinal dysbiosis and whether *P. copri* or other *Prevotella* spp. trigger systemic autoimmunity or drives the development of signs and symptoms associated with RA.

Our study had limitations. The demonstration of a specific immune response against *P. copri* during pre-clinical stages would have strengthened our findings. In RA patients, an increased humoral and Th1 cellular immune response against *P. copri* has been demonstrated and a sequence homology between RA-specific autoantigens and epitopes from proteins of *P. copri* has been found.[78] The microbiome study of the family members with RA and a replication of our results in a new-onset RA population would have further reinforced internal consistency.. Our results, together with previous studies in established RA patients and recent mechanistic studies, support the mucosal origins hypothesis and the role of *Prevotella* spp. dysbiosis in RA development.

In conclusion, we demonstrated that individuals at risk for RA with systemic autoimmunity and/or symptoms associated with RA have an enrichment of *Prevotella* spp. compared to FDR controls. Our findings support the mucosal origins hypothesis in the development of RA. Intestinal dysbiosis could act as an early environmental modulator, and may be the target of future preventive interventions.

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**COMPETING INTERESTS.** The authors declare no competing interest.

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**Figure 1: Linear discriminant analysis (LDA) effect size (LEfSe) evaluates the different relative abundance of bacteria.** The fecal microbiota composition of a subset of participants of the SCREEN-RA cohort was compared using 16S rRNA gene sequencing. (A) Bacterial families identified using LEfSe (LDA > 2, $p < 0.05$). Red bars: Bacterial taxa enriched in the preclinical RA group. (B) Relative abundance (range 0 to 1) of the bacterial families Prevotellaceae (left panel) and Lactobacillaceae (right panel) in individuals samples of the two groups. The thick horizontal dashed line in each graph shows median relative abundance and the solid line indicates mean relative abundance.

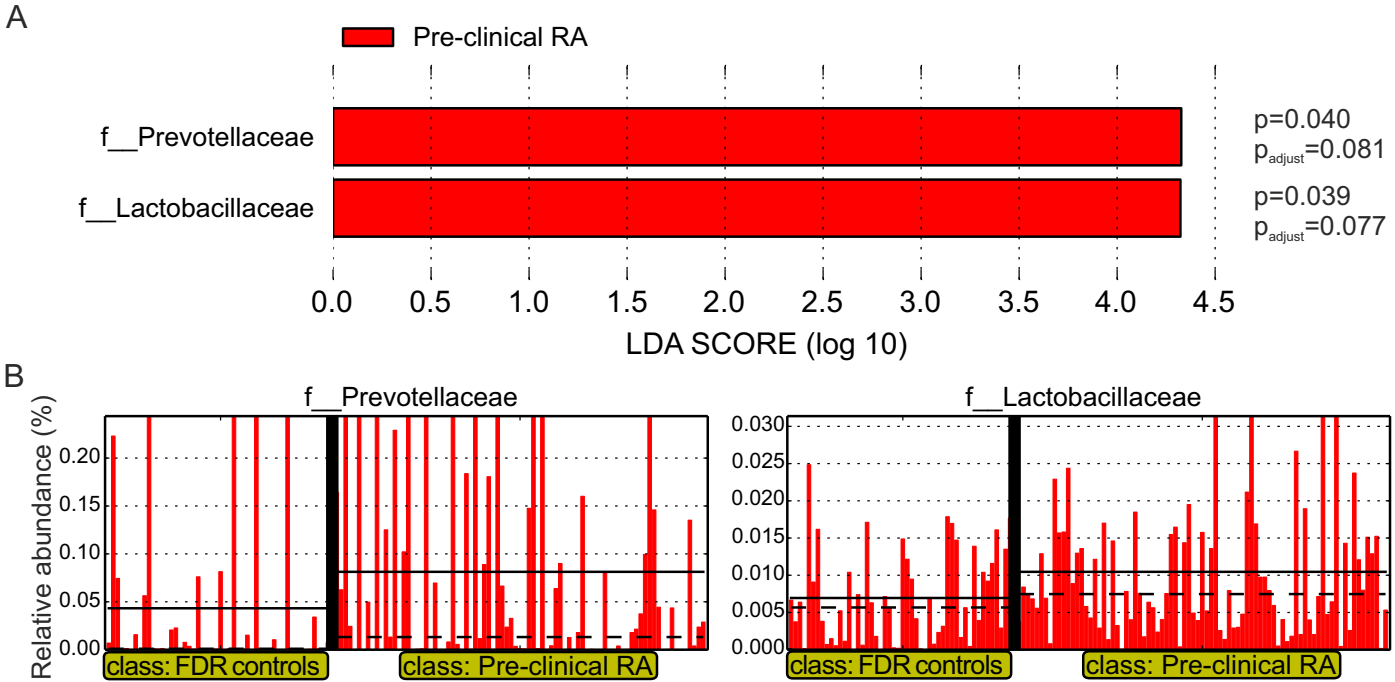
A
Pre-clinical RA
f__Prevotellaceae
f__Lactobacillaceae
p=0.040
p_adjust=0.081
p=0.039
p_adjust=0.077
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
LDA SCORE (log 10)
B
f__Prevotellaceae
f__Lactobacillaceae
Relative abundance (%)
0.00
0.05
0.10
0.15
0.20
0.000
0.005
0.010
0.015
0.020
0.025
0.030
class: FDR controls
class: Pre-clinical RA
class: FDR controls
class: Pre-clinical RA

**Figure 2. Relative abundance of species belonging to the Prevotellaceae family in individual samples.** The samples are ordered by decreasing cumulative relative abundance of OTUs assigned to the taxonomic level of Prevotella species. OTUs assigned only to the level of family or genus are not displayed. For each listed OTU the closest related taxonomically described species is listed and the "D=" brackets indicates the sequence similarity between OTU and this species.

Or: For each listed OTU the closest related taxonomically described species is listed. The "D=" brackets indicates the sequence similarity between them.

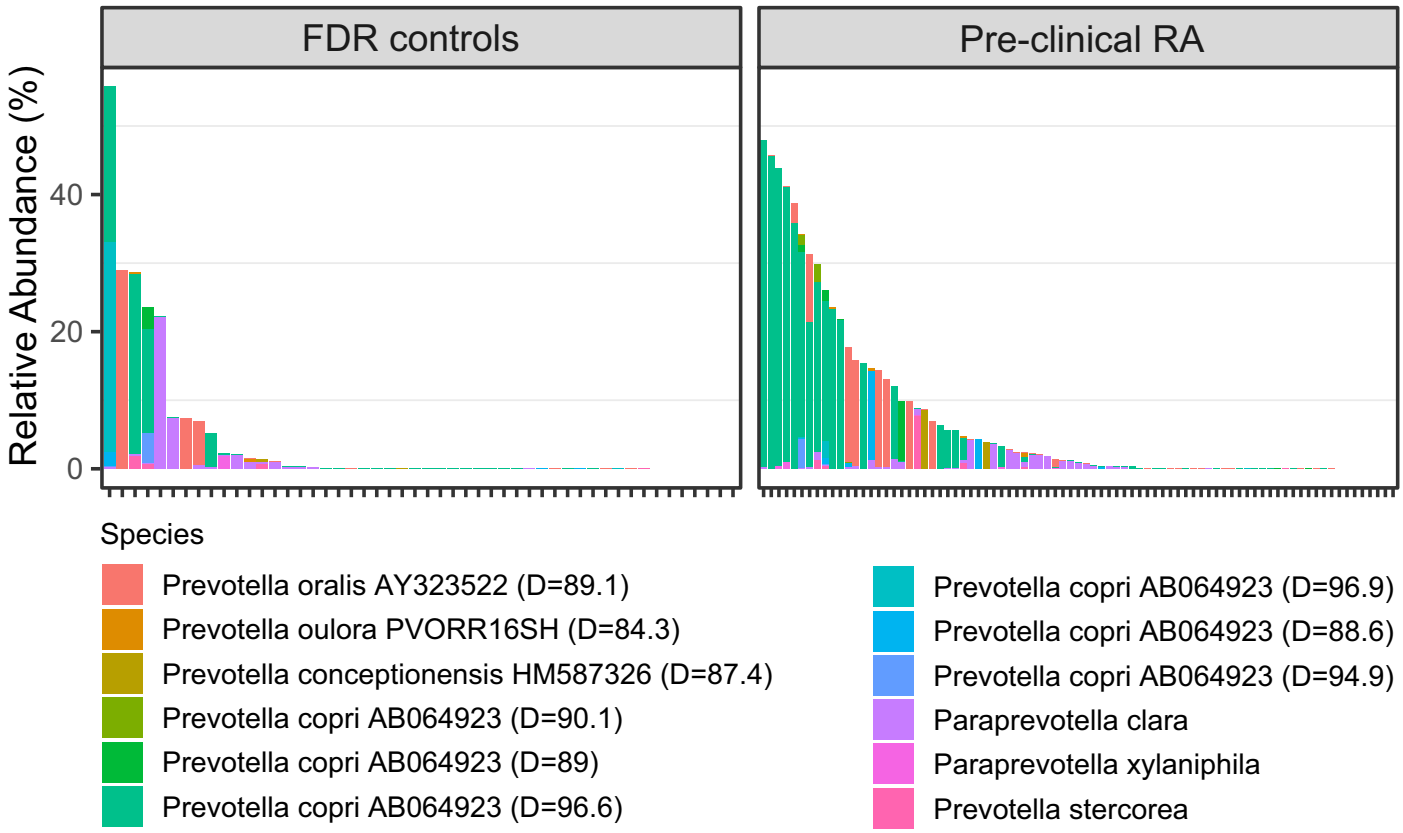
FDR controls
Pre-clinical RA
Relative Abundance (%)
0
20
40
Species
Prevotella oralis AY323522 (D=89.1)
Prevotella oulora PVORR16SH (D=84.3)
Prevotella conceptionensis HM587326 (D=87.4)
Prevotella copri AB064923 (D=90.1)
Prevotella copri AB064923 (D=89)
Prevotella copri AB064923 (D=96.6)
Prevotella copri AB064923 (D=96.9)
Prevotella copri AB064923 (D=88.6)
Prevotella copri AB064923 (D=94.9)
Paraprevotella clara
Paraprevotella xylaniphila
Prevotella stercorea

Figure 2