Porphyromonas gingivalis in the tongue biofilm is associated with clinical outcome in rheumatoid arthritis patients

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Summary

Several studies have suggested a link between human microbiome and rheumatoid arthritis (RA) development. *Porphyromonas gingivalis* seems involved in RA initiation and progression, as supported by the high occurrence of periodontitis. In this case–control study, we analysed tongue *P. gingivalis* presence and quantification in a large healthy and RA cohort. We enrolled 143 RA patients [male/female (M/F) 32/111, mean ± standard deviation (s.d.), age 57·5 ± 19·8 years, mean ± s.d. disease duration 155·9 ± 114·7 months); 36 periodontitis patients (M/F 11/25, mean ± s.d., age 56 ± 9·9 years, mean ± s.d. disease duration 25·5 ± 20·9 months); and 57 patients (M/F 12/45, mean ± s.d., age 61·4 ± 10·9 years, mean ± s.d. disease duration 62·3 ± 66·9 months) with knee osteoarthritis or fibromyalgia. All subjects underwent a standard cytological swab to identify the rate of *P. gingivalis*/total bacteria by using quantitative real-time polymerase chain reaction. The prevalence of *P. gingivalis* resulted similarly in RA and periodontitis patients (48·9 *versus* 52·7%, *P* = not significant). Moreover, the prevalence of this pathogen was significantly higher in RA and periodontitis patients in comparison with control subjects (*P* = 0·01 and *P* = 0·003, respectively). We found a significant correlation between *P. gingivalis* rate in total bacteria genomes and disease activity score in 28 joints (DAS28) (erythrocyte sedimentation rate) (*r* = 0·4, *P* = 0·01). RA patients in remission showed a significantly lower prevalence of *P. gingivalis* in comparison with non-remission (*P* = 0·02). We demonstrated a significant association between the percentage of *P. gingivalis* on the total tongue biofilm and RA disease activity (DAS28), suggesting that the oral cavity microbiological status could play a role in the pathogenic mechanisms of inflammation, leading to more active disease.

Keywords: disease activity, microbiome, *Porphyromonas gingivalis*, rheumatoid arthritis

Introduction

In recent years, several studies have suggested a link between human microbiome and the development of a number of pathological conditions, including autoimmune diseases [1,2]. In particular, the advent of new technologies allowed the characterization of functional properties and composition of microbial communities [3]. Of note, dysbiosis, defined as an imbalance of microbiome, has been associated with the development of several pathological disorders [4,5]. In particular, changes in bacterial species distribution have been described in autoimmune conditions, including rheumatoid arthritis (RA), suggesting a pathogenic role of gut microbiome [6,7].

RA is a chronic, systemic, autoimmune/inflammatory disease, involving approximately 0·5–1% of the general population and primarily affecting the joints. As demonstrated widely, it is characterized by a multi-factorial aetiology in which genetic and environmental factors interplay, determining disease susceptibility and phenotype [8,9]. Beyond gut microbiome, the oral cavity commensals

seem to be involved in RA initiation and progression, as supported by the high occurrence of periodontal inflammatory disorders in these patients [6]. Epidemiological studies have shown an increased prevalence and a more severe phenotype of periodontitis in RA patients in comparison with osteoarthritis and healthy subjects [10,11]. The prevalence of periodontitis seems higher in new-onset RA: Scher and colleagues in 2012 found moderate/severe periodontitis in 78% of early RA patients [12]. Furthermore, an eightfold increased risk of periodontitis was observed in RA patients in comparison with healthy controls [13].

Even though periodontal disease is determined by a consortium of microorganisms, several experimental studies have demonstrated the primary role of Gram-negative bacteria as aetiological agents. Among these, *Porphyromomas gingivalis* seems to be one of the prime aetiological agents in the development and progression of periodontal disease [14,15]. This is the only known eubacteria expressing peptidylarginine deaminase (PAD), able to induce citrullination in human fibrinogen or α- enolase *in vitro* [16-18]. Citrullination, a post-translational protein modification, leads to loss of tolerance to self-proteins in genetically susceptible individuals, inducing an immune response driving RA onset [19,20] Therefore, it is postulated that *P. gingivalis* infection, by generating citrullinated peptides, could induce the production of anti-citrullinated protein antibodies (ACPA) and subsequent RA development, especially in shared epitope-carrying subjects [21,22].

The presence of RA-related autoantibodies, in particular ACPA, has been associated significantly with anti-*P. gingivalis* titres in RA patients and in their healthy first-degree relatives [23,24]. Distinct oral regions may present significant differences in terms of microbiome composition even in the same individual [25,26].

Conversely, the biofilm microbiome seems to remain relatively stable in the tongue coat and is unique in different pathogenic conditions. Therefore, characterization of the different patterns of oral biofilm could provide useful insights concerning the association with human health and disease [27]. Very few studies have evaluated the tongue microbiome: the overall results suggest that significant differences exist in its composition when comparing healthy controls with patients affected by gastrointestinal diseases [28]. Moving from these findings, in the present case–control study we aimed at evaluating *P. gingivalis* infection on the tongue in a large cohort of healthy and RA patients. We applied a new evaluation method to assess this pathogen by evaluating the amount of *P. gingivalis* on total tongue biofilm.

Methods

Study subjects

In order to perform a case–control study, 143 patients affected by RA diagnosed according to the 1987 American College of Rheumatology (ACR) criteria were enrolled at the Rheumatology Unit, La Sapienza University of Rome [29].

As controls, we enrolled:

- 36 patients affected by periodontal disease without rheumatological comorbidity (PD), diagnosed according to Armitage and colleagues and assessed in agreement with the joint European Union/United States (EU/USA) Periodontal Epidemiology Working Group [30,31]; and
- 57 patients affected by knee osteoarthritis or fibromyalgia (control subjects: CS). Specifically, knee osteoarthritis was diagnosed according to the clinical and radiographic criteria of the ACR [32], while fibromyalgia was diagnosed according according to the 1990 ACR classification criteria [33].

Concerning PD patients, periodontal assessment was performed at the Dipartimento di Scienze Odontostomatologiche e Maxillo Facciali of Sapienza University of Rome. One examiner, blinded with regard to the rheumatological diagnosis, assessed periodontal status according to the American Academy of Periodontology guidelines [30,31]. Periodontitis was defined as the presence of at least one periodontal site with an attachment level of 1–2 mm and probing depth ≥ 4 mm. Moreover, the following parameters were recorded: probing depth, clinical attachment level and bleeding on probing [30,31].

Clinical evaluation of RA patients

The same rheumatologist evaluated all RA patients. Data were collected and entered into a standardized, computerized, electronically completed form. Data included patient demographics, date of diagnosis, comorbidities and previous and concomitant medications.

The clinical evaluation included the count of swollen and tender joints and the patient's and physician's global disease assessment based on a visual analogue scale (VAS; range = 0–100 mm). Disease activity was measured according to the disease activity score in 28 joints (DAS28) [34]. The patients were asked to complete the Health Assessment Questionnaire (HAQ).

Laboratory evaluation

For each patient, we measured the erythrocyte sedimentation rate (ESR, normal value < 20 mm/h) using the

Westergren method, as well as the C-reactive protein level (CRP, normal value < 5 mg/dl).

Furthermore, blood serum samples were obtained from all subjects and stored at −20°C until use. We evaluated the presence of ACPA (anti-CCP2) using commercially enzyme-linked immunosorbent assay kits (Delta Biologicals, Rome, Italy). The tests were carried out in duplicate and the results were evaluated according to the manufacturer's instructions. Values above 25 U/ml were considered positive.

Tongue biofilm specimen

The tool used to collect tongue biofilm samples was a standard cytological swab used routinely for mucosal sampling (DOC cytobrush; Gardening Spa, Genova, Italy). For each subject, the swab was inserted into the oral cavity, positioned on the tongue posterior dorsum surface and slowly rotated 1/4 or 1/2 turns in two directions. After this procedure, the swab was immediately put inside an Eppendorf tube, containing 0·5 ml Sheddler Broth with 20% glycerol (Microbiol, Uta Cagliari, Italy) and maintained at −20°C until DNA extraction.

DNA extraction

DNA extraction was carried out in the Molecular Biology Service at the University of Cagliari. Genomic DNA from tongue biofilm samples was obtained by the previously described cetyl trimethylammonium bromide (CTAB) modified method [35]. Briefly, 0·4 ml of the biofilm suspension in Shaedler broth, described previously, was added to 0·07 ml of 10% sodium dodecyl sulphate (SDS) and 0·005 ml proteinase K at 10 mg/ml concentration (Sigma-Aldrich, St Louis, MO, USA). After vigorous vortexing, this mixture was incubated for 10 min at 65°C. Next, 0·1 ml NaCl (5 M) and 0·1 μ/l CTAB/NaCl (0.274 M CTAB, hexadecyl trimetylammonium bromide and 0·877 M NaCl; Sigma-Aldrich) were added to the tube, which was vortexed briefly and incubated at 65°C for 10 min; 0·75 ml of Sevag (chloroform : isoamyl alcohol 24 : 1; Sigma-Aldrich) was added and the mixture was vortexed for 10 s. After centrifuging for 5 min (at 24 000 g), 0·6 volumes of 90% ethanol (Sigma-Aldrich) were added to the supernatant. After 30 min at −20°C and after being centrifuged for 30 min at 24 000 g, the pellet was dried at room temperature for 20 min and suspended in 0·03 ml of molecular biology-grade distilled water (Gibco, Invitrogen, Paisley, Scotland, UK); 0·002 ml were used as DNA suspension for real-time polymerase chain reaction (PCR).

Microbiological analysis

At the Molecular Biology Service at the University of Cagliari, the detection and enumeration of *P. gingivalis*

and the total microbiome amount was evaluated by real-time PCR procedure using oligonucleotide (OG) primers from gene sequences extracted from the NCBI database [35,36]. OG 94 5′-GAATCAAATACTTCAGCCGTCT-3′ and OG 95 5′-TTGCAGTTCGTATCGGATCT-3′, designed on the *prtC* gene, Accession no. AB00697, were used as PCR oligos for *P. gingivalis* recognition on tongue biofilm, while the total microbiome was evaluated by a set of universal primers designed on the rrs sequence of *Escherichia coli*, Accession no. X80724, with the oligos: OG 33 5′-AGCAGCCGCGGTAATA-3′ and OG 123 5′-GACTACCAGGGTATCTAATC-3′. The amount of *P. gingivalis* was expressed as a percentage of the co-respective total bacteria (biofilm) in the sample. Real-time PCR was performed with a Light Cycler instrument (Roche Diagnostics, Mannheim, Germany) and a SYBR Premix Ex Taq Kit (TaKara-Clontech®, Kusatsu, Shiga, Japan), according to the manufacturer's instructions for the PCR programme and reagent amount. We used 0·02 ml of DNA extract for each reaction, as described previously. Three distinct biological replicas were performed for each analysis, and quantitative data were expressed as mean ± standard deviation (s.d.). Threshold cycle (CT) units comprising ± 0·9 of the mean were considered significant. The different steps of the present study are summarized in Fig. 1.

Statistical analysis

All statistical analyses were performed by spss program version 13 (IBM Corp, Armonk, NY, USA) and version 5.0 of the GraphPad statistical package (La Jolla, CA, USA). The Kruskall–Wallis test was used to compare the patient groups and the Mann–Whitney test for pairwise comparisons between the patient groups. A χ^2 or Fisher's exact test was used to compare non-continuous data. For correlation analyses, a Spearman's test was used. Two-tailed *P*-values less than 0·05 were considered statistically significant. Multivariate analysis was performed using binary logistic regression. The results are presented as odds ratios (ORs) with 95% confidence intervals (CIs). In order to perform the multivariate analysis, we used a step-forward model including, progressively, those variables with $P < 0.1$ (also those which showed a trend of an association) to achieve a stronger model.

Results

Table 1 reports the demographic data and smoking status of RA patients and CS enrolled into the present study. No differences were found in terms of gender and mean age among the three groups. RA patients showed a significantly higher disease duration in comparison with
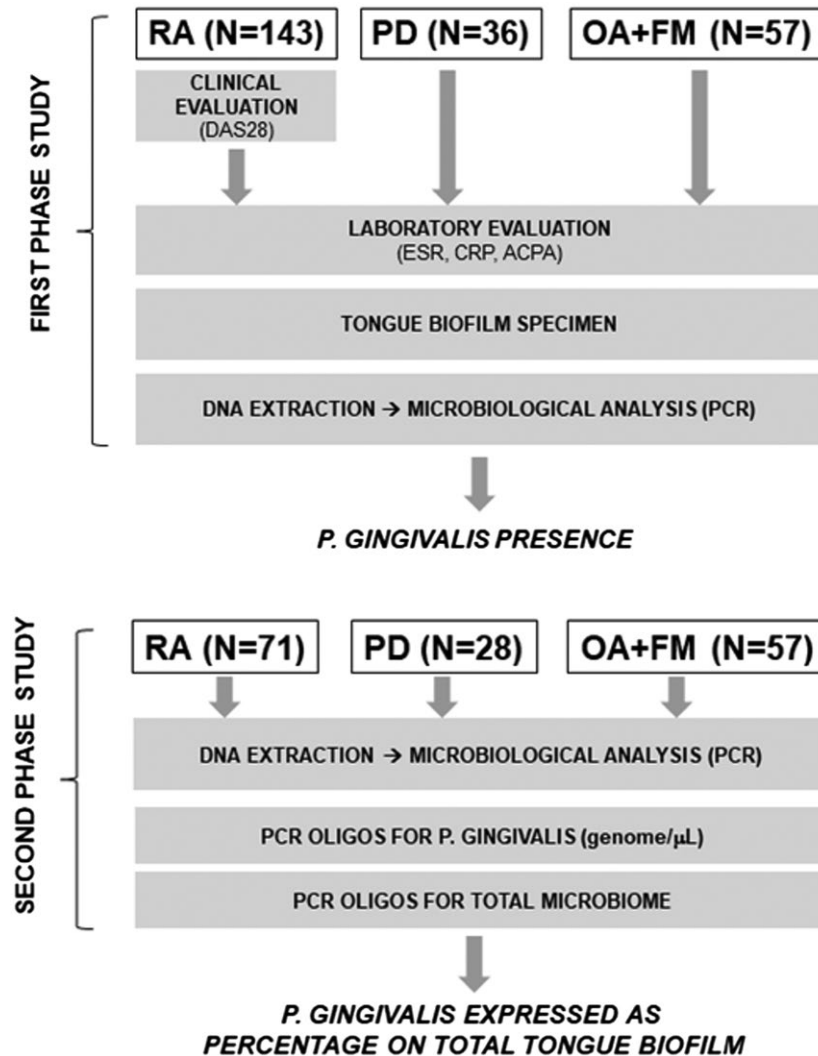
Fig. 1. Flow-chart of the study. The first phase of the study wasconducted on 143 rheumatoid arthritis (RA), 36 periodontitis (PD) and 57 osteoarthritis (OA) + fibromyalgia (FM) to assess the presence of *Porphyromonas gingivalis*. The second-phase study was conducted on 71 RA, 28 PD and 57 OA + FM to assess the percentage of *P. gingivalis* on total tongue biofilm.

other groups ($P < 0.0001$ for both comparisons). A significantly higher percentage of PD patients were current smokers in comparison with RA and OA+FM patients ($P = 0.004$).

At the time of enrolment, no patient or control subject was taking antibiotic treatment. Table 2 reports the clinimetric and treatment regimen of RA patients enrolled into the present study. Using the real-time PCR procedure we evaluated the rate of *P. gingivalis* on total bacteria at tongue biofilm level. The prevalence of this pathogen resulted significantly more highly in RA and PD patients in comparison with CS ($P = 0.01$ and $P = 0.003$, respectively; Fig. 2). Interestingly, the prevalence of *P. gingivalis* was similar in RA and PD patients [48·9 *versus* 52·7%, P = not significant (n.s.)]. As expected, we observed a

significantly higher prevalence and titre of ACPA in RA patients in comparison with PD and CS (Fig. 3).

When considering the RA group, no significant differences in the prevalence and titres of ACPA were identified comparing patients according with the presence of *P. gingivalis*. Specifically, ACPA were detected in 69·2% of patients positive for *P. gingivalis* (mean ± s.d., titre 1885·0 ± 1995·2 UI/ml) and in 60·0% of negative patients (mean ± s.d., titre 1545·9 ± 1905·1 UI/ml). The evaluation of disease activity according with DAS28 values demonstrated an association between disease activity and the presence of *P. gingivalis* at the tongue level;: higher disease activity was observed more frequently in *P. gingivalis*-positive (8·2%) than in *P. gingivalis*-negative (1·7%, P = 0·03) patients.

Table 1. Demographic data and smoking status of rheumatoid arthritis (RA) patients and control subjects (CS) enrolled into the present study

	RA (*n* = 143)	PD (*n* = 36)	OA+FM (*n* = 57)	*P*
M/F	32/111	11/25	12/45	n.s.
Mean ± s.d. age (years)	57·5 ± 19·8	56·0 ± 9·9	61·4 ± 10·9	n.s.
Mean ± s.d disease duration (months)	155·9 ± 114·7	25·5 ± 20·9	62·3 ± 66·9	AR *versus* PD/OA *P* < 0·0001
Smoking status				
Never (*n*/%)	86 (60·1)	12 (33·4)	34 (59·6)	RA/OA *versus* PD *P* = 0·002
Past (*n*/%)	22 (15·4)	8 (22·2)	9 (15·8)	n.s.
Current (*n*/%)	35 (24·5)	16 (44·4)	14 (24·6)	RA/OA *versus* PD *P* = 0·004

RA = rheumatoid arthritis; PD = periodontal disease; OA = osteoarthritis; FM = fibromyalgia; s.d. = standard deviation.

Table 2. Clinimetric and treatment regimen of RA patients (*n* = 143) enrolled in the present study

Clinimetric assessment at the time of enrolment	
DAS28, median (IQR)	2·3 (1·4–3·8)
Number of tender joints, median (IQR)	1 (0–3)
Number of swollen joints, median (IQR)	0 (0–2)
VAS for pain, median (IQR)	20 (10–30)
VAS for global health assessment by the patient, median (IQR)	20 (10–30)
VAS for global health assessment by the physician, median (IQR)	10 (0–20)
Glucocorticoid (at the time of enrolment)	
n/%	48/33·6%
Mean ± s.d. weekly dosage (mg)	37·1 ± 37·0
Synthetic DMARDs (during disease course)	
Methotrexate (*n*/%)	63/44·0
Sulphasalazine (*n*/%)	6/4·2
Leflunomide (*n*/%)	11/7·7
Hydroxychloroquine (*n*/%)	18/12·6
Cyclosporin A (*n*/%)	5/3·5
Biological DMARDs (during disease course)	
Etanercept (*n*/%)	21/14·7
Adalimumab (*n*/%)	13/9·1
Infliximab (*n*/%)	6/4·2
Tocilizumab (*n*/%)	25/17·5
Rituximab (*n*/%)	8/5·6
Abatacept (*n*/%)	9/6·3

IQR = interquartile range; VAS = visual analogue scale; s.d. = standard deviation; DMARDs = disease-modifying anti-rheumatic drugs.

Relationship between **P. gingivalis** *titres on the tongue and RA*

In a further step in the study, we considered microbiological analysis in order to quantify the *P. gingivalis* genomes in the tongue.

For this analysis, we tested:

- 71 RA patients [M/F 17/54, mean ± disease duration (DS) age 60·9 ± 11·9 years, mean ±DS disease duration 153·2 ± 114·0 months] matched with the overall RA patients in terms of age, disease duration and disease activity;
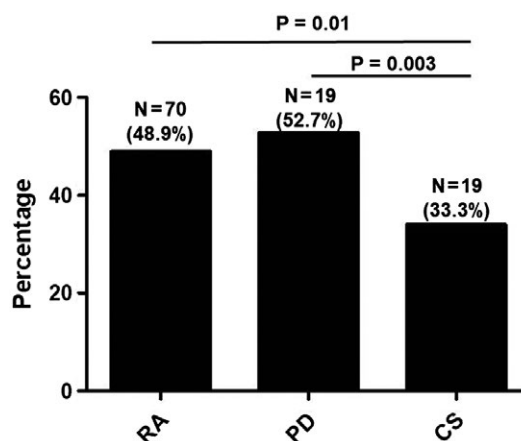

Fig. 2. The percentage of rheumatoid arthritis (RA) and periodontitis (PD) patients and control subjects (CS) positive for of *Porphyromonas gingivalis* as evaluated by quantitative real-time polymerase chain reaction (PCR), represented as histograms. The absolute number and percentage of positive subjects are reported.

- 28 PD (M/F 7/21, mean ± DS age 56·0 ± 10·9 years, mean ± DS disease duration 28·1 ± 22·5 months);
- all the 57 CS.

In the RA group, in which we tested approximately half of the total cohort; no differences in terms of disease activity and prevalence of *P. gingivalis* were found.

The molecular procedure showed sensitivity until 500 genomes/μl of *P. gingivalis* was reached as a meaningful level to quantify the bacterium. PD patients had a significantly higher prevalence of *P. gingivalis* amounts above the cut-off than RA patients (60·7 *versus* 28·1%, *P* = 0·000004) and CS (31·5%, *P* = 0·00003). No significant differences were observed among the three groups in terms of mean titres of bacterial genome as absolute value (genomes/μl DNA extract). Moreover, we evaluated the percentage of *P. gingivalis* among the total bacteria. As reported in Fig. 4, RA patients showed a higher mean percentage of *P. gingivalis* (0·2 ± 0·5) compared with PD patients (0·02 ± 0·04) and CS (0·07 ± 0·02). Interestingly, a higher percentage of *P. gingivalis* on total tongue
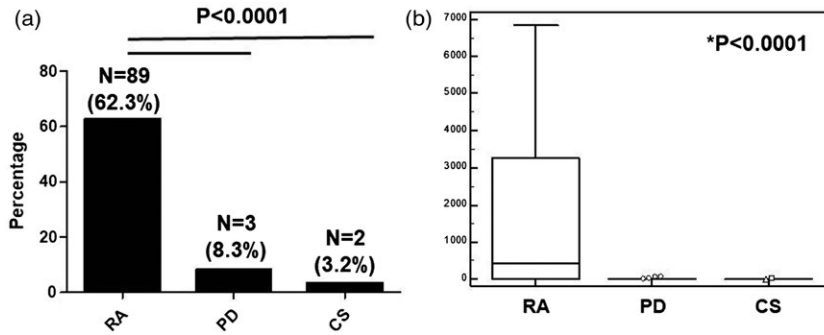
Fig. 3. (a) The percentage of rheumatoid arthritis (RA) and periodontitis (PD) patients and control subjects (CS) positive for anti-citrullinated protein antibodies (ACPA) (a), represented as histograms. The absolute number and percentage of positive subjects are reported. (b) ACPA titre (UI/ml) in RA, PD and CS patients, are represented as a box-and-whisker plot. *RA *versus* PD; RA *versus* CS.
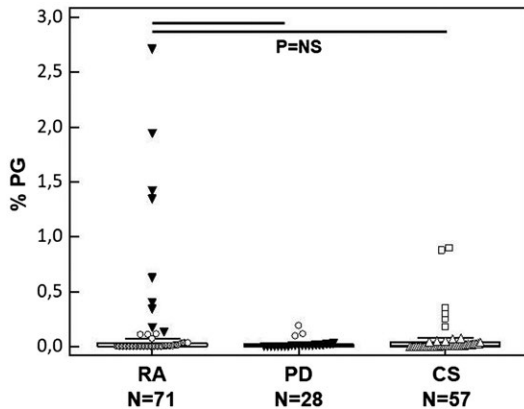

Fig. 4. The percentage of *Porphyromonas gingivalis* on total tongue microbiome (%PG), evaluated by quantitative real-time polymerase chain reaction (PCR) in the three groups evaluated [rheumatoid arthritis (RA), periodontitis (PD) and control subjects (CS) patients], represented as box-and-whisker plot.

microbiome correlated significantly with more severe disease activity in RA patients, evaluated by DAS28 ($r = 0.4$, $P = 0.01$, Fig. 5a) and with the number of tender joints ($r = 0.3$, $P = 0.03$). When we compared

RA patients with and without a remission status according to DAS28 [37], a higher prevalence of *P. gingivalis* among the total bacterial titres in the tongue was identified in non-remission patients (0.4 ± 0.9) than in those in remission (0.02 ± 0.04, $P = 0.02$, Fig. 5b).

We performed multiple logistic regression analyses to evaluate the factors associated with disease activity in RA patients. The logistic regression confirmed the association of disease activity, evaluated using DAS28, and the presence of *P. gingivalis* in tongue biofilm ($P = 0.04$, OR = 6.6, 95% CI = 1.01–43.6).

Discussion

In the present study, we assessed for the first time the prevalence of *P. gingivalis*, i.e. its percentage on the total tongue biofilm, in a large cohort of RA patients. A significant correlation between the amount of *P. gingivalis* on total tongue biofilm and disease activity was observed. There was no association with ACPA, suggesting that this bacterium, beyond citrullination and antibody production, could be implicated in triggering a proinflammatory state in RA. Thus, the aim of the present study was to assess
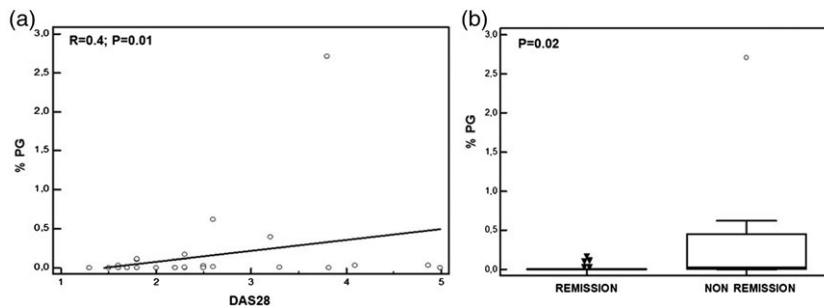

Fig. 5. (a) Correlation between the prevalence of *Porphyromonas gingivalis* on tongue microbiome (%PG) and disease activity, evaluated by DAS28 ($r = 0.4$; $P = 0.01$); (b) the prevalence of *P. gingivalis* on tongue microbiome (%PG) in patients with and without remission according to disease activity score in 28 joints (DAS28) values (< 2·6), represented as a box-and-whisker plot. *P. gingivalis* was evaluated by quantitative real-time polymerase chain reaction (PCR).

the prevalence of *P. gingivalis* and its possible influence in disease features regardless of the presence of periodontitis and gingivitis.

P. gingivalis is a Gram-negative anaerobic bacterium located usually in the oral cavity, as a component of microbiome. Next to the established association with oral cavity diseases, such as periodontitis and halitosis, in recent years growing interest has been addressed to the implication of *P. gingivalis* in the development of autoimmune diseases; in particular, its role in RA has been explored widely [22].

The evidence that this bacterium expresses the PAD enzyme leading to citrullination is the most relevant link between *P. gingivalis* and RA. Indeed, it has been proposed that the presence of this microorganism in genetically prone subjects could induce an autoimmune response against citrullinated peptides, leading to disease development [22]. Nonetheless, in agreement with our work, in a previous study Scher and colleagues did not identify any correlation between *P. gingivalis* and ACPA titres when evaluating the oral microbiome [12].

P. gingivalis has several virulence factors, such as lipopolysaccharide (LPS), fimbriae haemagglutinin and gingipains, that contribute directly to chronic inflammation regardless of citrullination [38]. It has been suggested that the microbial persistence is a factor contributing to chronicity in inflammatory arthritides [38]. In particular, *P. gingivalis* DNA seems to be able to induce the production of proinflammatory cytokines such as interleukin (IL)-6, IL-1 and tumour necrosis factor (TNF), playing a central role in RA pathogenesis [39–41]. This production seems to occur through the signalling pathway of Toll-like receptor (TLR)-9, expressed highly in pathological periodontal tissue in comparison with healthy tissue [39,42,43]. Moreover, functional polymorphisms of TLR-9 were associated significantly with alveolar bone loss in *P. gingivalis* carriers [44]. *P. gingivalis* seems also to intervene in T helper type 17 (Th)-17 differentiation, potentially influencing disease severity by IL-17 production [45]. Thus, it is plausible that *P. gingivalis* leads to an increased proinflammatory state independent from ACPA.

Another interesting aspect of our results is that, for the first time, to our knowledge, that the presence of the bacterium in relation with total tongue microbiome was assessed. We evaluated the presence of *P. gingivalis* in the context of tongue microbiome providing a qualitative and quantitative assessment. From a qualitative viewpoint, we found a similar prevalence of *P. gingivalis* in RA and PD patients. This is in agreement with previous data from the literature, confirming an increased presence of the bacterium in RA [22]. From a quantitative viewpoint, we assessed the rate of *P. gingivalis*, expressed as its percentage on the total biofilm. By using this molecular approach, a significant correlation between the *P. gingivalis* rate and DAS28 values was observed. This result is strengthened by the observation that patients not in remission status showed a significantly higher rate in comparison with patients in remission. We could speculate that *P. gingivalis* is able to induce the activation of the above-mentioned different mechanisms, with consequent production of proinflammatory cytokines. These events match clinically with active disease, as assessed by DAS28. We may hypothesize that the presence of *P. gingivalis* could chronically stimulate the immune system, regardless of the presence of periodontitis, leading to a state of chronic inflammation.

Very few studies have assessed the presence of this bacterium directly in the oral cavity, relying instead on the use of serological methods or low-throughput PCR-based techniques. Scher and colleagues compared RA and healthy controls in terms of subgingival microbiota composition by using multiplexed-454 16S rRNA pyrosequencing [12]. *P. gingivalis* was present at heterogeneous levels in the participants (55% of new-onset RA, 47% of chronic RA, 27% of healthy controls), and it was significantly more prevalent and abundant in patients with PD [12].

The present study shows some limits. First, other oral sites, such as periodontal pockets or saliva, were not evaluated in terms of microbiota. Secondly, it should be considered that RA patients enrolled in our analysis were treated by different drugs, including biologicals, potentially influencing the microbiome composition. Certainly, it could be interesting to replicate our evaluation in a cohort of early drugs-free RA patients.

In conclusion, in our study, we have analysed for the first time the percentage of *P. gingivalis* on the total tongue biofilm; using this new measurement, an association between this value and disease activity was identified, providing new information about the influence of this bacterium on RA. In particular, we suggest that the microbial persistence could play a role in the pathogenic mechanisms of inflammation, leading to more active disease.

Finally, we suggest the application of this new measurement tool for *P. gingivalis* in order to identify patients with higher disease activity, requiring a more aggressive treatment. Further studies are needed in order to confirm this suggestion.

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Disclosures

None of the authors have any conflicts of interest.

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