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

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ABSTRACT

Objective: Several evidences suggested a link between human microbiome and Rheumatoid Arthritis (RA) development. *Porphyromonas gingivalis (P. gingivalis)* seems involved in RA initiation and progression, as supported by the high occurrence of periodontitis. In this case-control study, we analyzed tongue *P. gingivalis* presence and quantification in a large healthy and RA cohort.

Methods: We enrolled 143 RA patients (Male/Female 32/111, mean±SD age 57.5±19.8 years, mean±SD disease duration 155.9± 114.7 months); 36 periodontitis patients (M/F 11/25, mean±SD age56±9.9 years, mean±SD disease duration 25.5±20.9 months); 57 patients (M/F 12/45, mean ±SD age 61.4±10.9 years, mean ±SD disease duration 62.3±66.9 months) with knee osteoarthritis or fibromyalgia. All subjects underwent a standard cytologic swab to identify the rate of *P. gingivalis*/total bacteria by using quantitative real time PCR.

Results: The prevalence of *P. gingivalis* resulted similar in RA and periodontitis patients (48.9% versus 52.7%, P=NS). Moreover, the prevalence of this pathogen was significantly higher in RA and PD patients in comparison with CS (P=0.01 and P=0.003, respectively). We found a significant correlation between *P. gingivalis* rate in total bacteria genomes and DAS28(ESR) (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).

Conclusions: 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 a more active disease.

Keywords: Rheumatoid Arthritis, microbiome, P. gingivalis, disease activity.

INTRODUCTION

In the last years, several evidences 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 the 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 the bacterial species distribution have been described in autoimmune conditions including Rheumatoid Arthritis (RA), suggesting a pathogenic role of gut microbiome [6,8].

RA is a chronic, systemic, autoimmune/inflammatory disease, involving approximately 0.5-1% of the general population and affecting primarily the joints. As widely demonstrated, it is

characterized by a multifactorial aetiology, in which genetic and environmental factors interplay determining disease susceptibility and phenotype [9,10]. Beyond gut microbiome, the oral cavity commensals seem involved in RA initiation and progression, as supported by the high occurrence of periodontal inflammatory disorders in these patients [6]. Epidemiological studies showed an increased prevalence and a more severe phenotype of periodontitis in RA patients in comparison with osteoarthritis and healthy subjects [11,13]. The prevalence of periodontitis seems higher in new-onset RA: Scher and colleagues in 2012 found a moderate/severe periodontitis in 78% of early RA patients [14]. Furthermore, an 8-fold increased risk of periodontitis was observed in RA patients in comparison with healthy controls [15].

Even though periodontal disease is determined by a consortium of microorganisms, several experimental evidences have demonstrated the primary role of Gram-negative bacteria as etiological agents. Among these, *P. gingivalis* seems to be one of the prime etiological agents in development and progression of periodontal disease [16, 17]. This is the only known eubacteria expressing peptidylarginine deaminase (PAD) able to induce citrullination in human fibrinogen or α- enolase in vitro [18-20]. 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 [21, 22] 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 [23, 24].

The presence of RA-related autoantibodies, in particular ACPA, has been significantly associated with anti-*P. gingivalis* titres in RA patients and in their healthy first degree relatives [25, 26]. Distinct oral regions may present significant differences in terms of microbiome composition even in the same individual [27, 28].

Conversely, the biofilm microbiome seems to remain relatively stable in the tongue coat and unique in different pathogenic conditions. Therefore, the characterization of the different patterns of oral biofilm could provide useful insights concerning the association with human health and disease [29]. Very few studies 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 [30]. Moving from these evidences, in the present case-control study we aimed at evaluated the *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, Sapienza University of Rome [31].

As controls, we enrolled:

- 36 patients affected by periodontal disease without rheumatological comorbidity (PD), diagnosed according with Armitage and colleagues and assessed in agreement with Joint EU/USA Periodontal Epidemiology Working Group [32, 33];
- 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 American College of Rheumatology [34], while fibromyalgia according to the 1990 American College of Rheumatology classification criteria [35].

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 rheumatologic diagnosis, assessed periodontal status according to the American Academy of Periodontology guidelines [32, 33]. 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 [32, 33].

Clinical evaluation on RA patients

The same rheumatologist evaluated all RA patients. Data were collected and entered into a standardized, computerized, electronically filled-in 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 to 100 mm). Disease activity was measured according to the disease activity score in 28 joints (DAS28) [36]. The patients were asked to fill in the Health Assessment Questionnaire (HAQ).

Laboratory evaluation

For each patient, we measured the erythrocyte sedimentation rate (ESR, normal value < 20 mm/hour) by 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) by using commercially enzyme-linked immunosorbent assay kits (DELTA BIOLOGICALS, Italy). The tests were carried out in

duplicate and the results were evaluated according to the manufacturers' instructions. Values above 25 U/mL were considered positive.

Tongue biofilm specimen

The tool used to collect tongue biofilm samples was a standard cytologic swab routinely used 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 turn in two directions. After this procedure, the swab was immediately put inside an Eppendorf tube, containing 0.5 mL of Sheddler Broth with 20% glycerol (Microbiol, Uta Cagliari) 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 CTAB modified method [37]. Briefly, at 0.4 mL of the biofilm suspension in Shaedler broth previously described, were added to 0.07 mL of 10% sodium dodecyl sulphate (SDS) and 0.005 mL of proteinase K at 10 mg/ml concentration (SIGMA – Aldrich, ST. Louis, Missouri, USA). After vigorous vortexing, this mixture was incubated for 10 minutes at 65°C. Next, 0.1 mL of NaCl [5 M] and 0.1 μ /l of 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 minutes. 0.75 mL of SEVAG (Chloroform: Isoamyl Alcohol 24:1, Sigma-Aldrich) were added and the mixture was vortexed for 10 sec. After centrifuging for 5 min (at 12000 rpm) 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 12.000 rpm, the pellet was dried at room temperature for 20

min and suspended in 0.03 mLof molecular biology grade distilled water (Gibco, Invitrogen Paisley, Scotland, UK). 0.002 mL of this were used as DNA suspension for real time PCR reaction.

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 by using oligonucleotide primers from gene sequences extracted from NCBI database [37, 38]. OG 94 5'-GAATCAAATACTTCAGCCGTCT-3' and OG 95 5'-TTGCAGTTCGTATCGGATCT-3' designed on *prtC* gene, accession n. 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 rrs sequence of *Escherichia coli*, accession n. 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. The real time PCR reaction was performed with a Light Cycler instrument (Roche Diagnostics Mannheim, Germany) and a SYBR Premix Ex Taq Kit (TaKara-Clontech®) according to the manufacturer's instructions for PCR program and reagent amount. For each reaction as described before, we used 0.02 mL of DNA extract. For each analysis, three distinct biological replicas were done, and quantitative data were expressed as mean ± SD. Threshold Cycles (CT) units comprised ± 0.9 of the mean were considered significant.

The different steps of the present study were summarized in Figure 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 Mann–Whitney for pair wise comparisons within the patient groups. A chi-squared 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 ORs with their 95% CIs. In order to perform the multivariate analysis, we used a step-forward model including, progressively, those variables with $P < 0.1$ (so also those which showed a trend of an association) to have a stronger model.

RESULTS

Table 1 reports demographic data and smoking status of RA patients and CS enrolled in 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 other groups ($P<0.0001$ for both comparison). 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 the enrolment, no patient and control subject was taking antibiotic treatment.

Table 2 reports clinimetric and treatment regimen of RA patients enrolled in the present study.

By 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 higher in RA and PD patients in comparison with CS ($P=0.01$ and $P=0.003$, respectively; Figure 2).

Interestingly, the prevalence of *P. gingivalis* was similar in RA and PD patients (48.9% versus 52.7%, P=NS). As expected, we observed a significantly higher prevalence and titer of ACPA in RA patients in comparison with PD and CS (Figure 3A and B).

When considering the RA group, no significant differences in the prevalence and titers 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±SD titer 1885.0±1995.2 UI/ml) and in 60.0% of negative patients (mean±SD titer 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 patients (8.2%) than in *P. gingivalis* negative (1.7%, P=0.03).

Relationship between P. gingivalis titre on the tongue and RA

In a further step of the study, we have 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 ±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;
- 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 RA group, in which we tested about half of total cohort, no differences in terms of disease activity and prevalence of *P. gingivalis* were found.

The molecular procedure showed a sensitivity until 500 genome/μL of *P*. *gingivalis* as a meaningful level to quantify the bacterium. We showed that 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 titre of bacterial genome as absolute value (genomes/μl DNA extract), but if we evaluate the percentage of *P*. *gingivalis* among the total tongue bacteria. As reported in figure 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 microbiome significantly correlated with more severe disease activity in RA patients, evaluated by DAS28 (r=0.4, P=0.01, Figure 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 with DAS28 [39], 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, Figure 5B).

We performed the multiple logistic regression analysis, to evaluate the factors associated with disease activity in RA patients. The logistic regression confirmed the association of disease activity, evaluated by 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, for the first time we assessed 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 pro-inflammatory state in RA.

Thus, the aim of the present study was to assess the prevalence of *P. gingivalis* and its possible influence in disease features regardless the presence of periodontitis and gingivitis.

P. gingivalis is a Gram-negative anaerobic bacterium usually located in the oral cavity, as component of microbiome. Next to the established association with oral cavity diseases, such as periodontitis and halitosis, in the last years a 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 widely explored [24].

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 [24]. Nonetheless, in agreement with our, in a previous studies Scher and colleagues did not identify any correlation between *P. gingivalis* and ACPA titers when evaluating the oral microbiome [14].

Indeed, *P. gingivalis* has several virulence factors, such as LPS, fimbriae hemagglutinin, and gingipains that directly contributes to its chronic inflammation regardless of citrullination [40]. It has been suggested that the microbial persistence is a factor contributing to chronicity in inflammatory arthritides [40]. In particular, P. *gingivalis* DNA seems to be able to induce the production of pro-inflammatory cytokines such as interleukin (IL)-6, IL-1 and TNF, playing a central role in RA pathogenesis [41-43]. This production seems to occur through the signaling pathway of TLR9, highly expressed in pathologic periodontal tissue in comparison with healthy tissue [41,44,45]. Moreover, functional polymorphisms of TLR9 resulted significantly associated with alveolar bone loss in *P. gingivalis* carriers [46]. *P. gingivalis* seems also to intervene in Th-17 differentiation, potentially influencing disease

severity by IL-17 production [47]. Thus, it is plausible that *P*. *gingivalis* leads to an increased pro-inflammatory state independent from ACPA.

Another interesting aspect of our results is that, for the first time, 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 point of view, 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 [24]. From a quantitative point of view, 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 different mechanisms above-mentioned, with consequent production of pro-inflammatory cytokines. These events clinically match with an active disease, as assessed by DAS28. We may hypothesize that the presence of *P*. *gingivalis* could chronically stimulate immune system, regardless the presence of periodontitis, leading to a state of chronic inflammation.

Very few studies directly assessed the presence of this bacterium in the oral cavity, relying instead on the use of serologic 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 [14]. *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 [14].

The present study shows some limits. Firstly, 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 biologics, potentially influencing the microbiome composition. Certainly, it could be interesting to replicate our evaluation in a cohort of early free-drugs RA patients.

In conclusion, in our study, we analyzed for the first time the percentage of *P. gingivalis* on the total tongue biofilm; by 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 a more active disease.

Finally, we hypothesize 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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TABLES

Table 1: Demographic data and smoking status of RA patients and CS enrolled in the present study.

	RA (N=143)	**PD (N=36)**	**OA+FM (N=57)**	**P**
M/F	32/111	11/25	12/45	NS
mean±SD age (years)	57.5±19.8	56.0±9.9	61.4±10.9	NS
mean±SD disease duration (months)	155.9± 114.7	25.5±20.9	62.3±66.9	AR *vs* PD/OA P<0.0001
Smoking status				
Never (N/%)	86 (60.1)	12 (33.4)	34 (59.6)	RA/OA *vs* PD P=0.002
Past (N/%)	22 (15.4)	8 (22.2)	9 (15.8)	NS
Current (N/%)	35 (24.5)	16 (44.4)	14 (24.6)	RA/OA *vs* PD P=0.004

Legend: RA: Rheumatoid Arthritis; PD: periodontal disease; OA: osteoarthritis; FM (fibromyalgia); SD: standard deviation; vs: versus.

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

Clinimetric assessment at the time of enrollment	
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 if enrollment)	
N/%	48/33.6%
Mean±SD weekly dosage (mg)	37.1±37.0
Synthetic DMARDs (during disease course)	
Methotrexate (N/%)	63/44.0
Sulfasalazine (N/%)	6/4.2
Leflunomide (N/%)	11/7.7
Hydroxychloroquine (N/%)	18/12.6
Cyclosporine 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

Legend: IQR: interquartile range; VAS: visual analogue scale; SD: standard deviation.

FIGURES LEGENDS

Figure 1. Flow chart of study. First phase study conducted on 143 Rheumatoid Arthritis (RA), 36 periodontitis (PD), 57 Osteoarthritis (OA)+ Fibromyalgia (FM) to assess the presence of *P*. *gingivalis*. Second phase study conducted on 71 RA, 28 PD, 57 OA+FM to assess the percentage of *P*. *gingivalis* on total tongue biofilm.

Figure 2. Percentage of RA, PD and CS patients positive for of *P gingivalis* as evaluated by quantitative real time PCR, represented as histograms. Absolute number and percentage of positive subjects were reported.

Figure 3. (**A)** Percentage of RA, PD and CS patients positive for ACPA(A), represented as histograms. Absolute number and percentage of positive subjects were reported.

(B) ACPA titer (UI/ml) in RA, PD, CS patients, represented as box whisker plot.

*RA versus PD; RA versus CS.

Figure 4. Percentage of *P*. *gingivalis* on total tongue microbiome (%PG), evaluated by quantitative real-time PCR in the three groups evaluated (RA, PD, CS patients), represented as box whisker plot.

Figure 5. **(A)** Correlation between the prevalence of *P*. *gingivalis* on tongue microbiome (%PG) and disease activity, evaluated by DAS28 (R=0.4; P=0.01); **(B)** Prevalence of *P*. *gingivalis* on tongue microbiome (%PG) in patients with and without remission according to DAS28 values (<2.6), represented as box whisker plot. *P*. *gingivalis* was evaluated by quantitative real-time PCR.