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Association between *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* in subgingival plaque and clinical parameters, in Argentine patients with aggressive periodontitis

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ABSTRACT

Background: *Aggregatibacter actinomycetemcomitans* (Aa) and *Porphyromonas gingivalis* (Pg) have been associated with aggressive (AgP) and chronic periodontitis.
Objective: The aim of this study was to evaluate the levels of Aa and Pg in gingival crevicular fluid (GCF) of patients with AgP and its relation with clinical parameters.
Design: Sixteen females and fourteen males with clinical diagnosis of AgP aged 17–23 years and their match's controls, were included in this study. Clinical recording concerning probing pocket depth, clinical attachment level, plaque index and gingival bleeding index were performed at baseline, 30 and 60 days after baseline. After clinical examination GCF samples were analyzed for Aa and Pg with a real-time polymerase chain reaction technique. Patients group was treated with a combined of mechanical and oral antibiotic therapy (doxycycline 100 mg/day, during 21 days). A multivariate analysis was used to determine the relationship between Aa and Pg counts with clinical parameters.
Results: GCF from all subjects was positive for Aa and PG. In controls Pg concentration was higher than Aa (Pg: 42,420 ± 3,034 copies/ml; Aa: 66.6 ± 5.4 copies/ml $p < 0.001$) while in patients both microbes showed the same concentration (Aa: 559,878 ± 39,698 Pg: 572,321 ± 58,752). A significant and positive correlation was observed between counts of Aa and Pg (R square: 0.7965, $p < 0.0001$). Female showed more counts/ml. Aa might be closely associated with clinical parameters while Pg did not. At 30 and 60 days Aa counts in patients were similar to controls while Pg counts were equal to baseline. However, in spite of Pg presence a clinical improvement was observed in all patients.
Conclusions: In our population the presence of Aa may be associated with AgP while Pg may be in GCF as an opportunistic pathogen which might caused disease when the ecological balance was favorable.

1. Introduction

Aggressive periodontitis (AgP) is a specific form of periodontal disease with a higher rate of progression and patterns of tissue destruction, mostly affecting younger individuals. Approximately 400 bacterial species are colonizing periodontal pockets and a further 300 can be found in the rest of the oral cavity [1,2]. *Aggregatibacter actinomycetemcomitans* [3], *Porphyromonas gingivalis*, *Tannerella forsythia* (Tf) and *Treponema denticola* [4,5] are considered periodontal pathogens and frequently associated with periodontal destruction.

A. actinomycetemcomitans (Aa) is a Gram-negative, nonmotile, facultative anaerobic cocobacillus bacterium that colonizes the human oral cavity, associated with the etiology of AgP [6,7] and can also be detected in the oral cavity of chronic periodontitis (CP) patients and periodontally healthy subjects [8,9]. *P. gingivalis* (Pg), a Gram-negative black-pigmented anaerobic rod residing in subgingival biofilms, is widely recognized as a contributor to development of periodontal infections together with other oral pathogens [10]. It is considered the major pathogen that causes

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severe CP but can also be found in patients with AgP and is also found in healthy periodontal tissues without inflammation [11]. Aa produces a variety of virulence factors such as lipopolysaccharide, cytolethal distending toxin and leukotoxin which exerts its effect on the immune defence cells in different ways [12,13]. On the other hand, Pg harbors an arsenal of virulence factors including fimbriae, cysteine proteinases, hemagglutinins and lipopolysaccharide which, along with its many interactions with the host immune system, strongly support its potency as a pathogen [14].

Numerous reports aimed to characterize the microbiota associated with AgP. Some studies have associated the species Aa with Agp in different populations [3,15]. On the contrary, lack of association has also been reported and several studies found this pathogen to be prevalent in individuals with CP [7,16,17]. In addition, Tomita et al. (2013) showed no significant difference in the prevalence or levels of Aa, Pg or Tf between CP and AgP. Moreover, in most periodontitis patients these pathogens were found in combination with one another showing that certain bacterial complexes are observed together more frequently than others in subgingival plaque [18]. The aim of this study was to evaluate the prevalence and levels of Aa and Pg in subgingival plaque samples of argentine patients with AgP using a real-time polymerase chain reaction (PCR) technique, and its relation with gender, age, and clinical parameters.

2. Materials and methods

2.1. Study population

Study subjects were recruited from the patient's population of a private dental clinic, from January through December 2014. The protocol was approved by the Ethics Committee of the School of Dentistry, University of Buenos Aires, and the study was conducted in accordance with the Declaration of Helsinki (version 2008). Completed medical and dental histories were obtained from all subjects. Patients were clinically diagnosed to having AgP based on the World Workshop in Periodontology criteria [19]. Subjects of similar ethnic, race, income levels, age and gender were recruited from the students of the School of Dentistry, Buenos Aires University, as controls. All of the subjects gave their informed consent and exclusion criteria included: smokers, cardiovascular or respiratory diseases, systemic inflammatory conditions or non-plaque induced oral inflammatory conditions, immunodeficiency, current pregnancy or lactation and medicine use.

2.2. Clinical examination

All periodontal measurements were performed in four quadrants using a first-generation probe (Hu-Friedy Mfg. Co., Chicago, IL, USA). Probing pocket depth (PPD, measurements were rounded off to the nearest millimetre marking) and clinical attachment level (CAL, measuring the distance from the cemento-enemal junction to the bottom of the probable pocket) were assessed at six sites per tooth, and bleeding on probing (BOP, scored as: −, no bleeding or +, bleeding within 30 s after probing) at four sites per tooth. Plaque index (PI) was determined according to Silness and Löe [20]. Serial dental radiographs were taken of the incisor/canine/pre-molar/molar regions using a standardized periapical projection technique. On the radiographs, the alveolar bone level (ABL) was assessed by measuring the distance in mm from the cement–enamel junction (CEJ) to the alveolar bone crest, i.e. the point at which the periodontal ligament space was considered to have a normal width [21]. The measurements were made by the use of a magnifying lens (7×) to the nearest 0.5 mm at all mesial/distal tooth surfaces reproduced in the radiographs. The intra-examiner reproducibility of ABL measurements was determined by repeated assessments of 10 randomly selected subjects. Of the measurements 96% were reproduced within a difference of ±0.5 mm. The error of the method corresponded to 6% of the variance for the mean ABL in the population sample.

2.3. Gingival crevicular fluid sampling

The participants were asked to avoid oral hygiene measures, eating and drinking for 2 h before collection of subgingival plaque samples. Gingival crevicular fluid (GCF) samples were obtained from buccal aspects of two interproximal sited in single rooted teeth in each individual participating in the study. Prior to GCF sampling, plaque recording was performed and supragingival plaque was then removed carefully by sterile curretes and the surfaces were dried and isolated by cotton rolls. Filter paper strips (Periopaper, Proflow Inc., Amytyville, NY, USA) were placed in the orifices of gingival sulcus/pocket for 30 s. Care was taken to avoid mechanical trauma and strips visually contaminated with blood were discarded. Then, the strips from each patient were placed into one polypropilene tube before freezing at −20 °C. The absorbed GCF volume was estimated by a calibrated instrument (Periotron 8000, Oraflow Inc., Plainviwe, NY, USA) and converted to an actual volume (μl) by reference to the standard curve. All samples were stored at −20 °C until the laboratory analysis.

2.4. Real-time PCR

The samples were evaluated by PCR with specific primers for the presence of Aa: ′ACGCAGACGATTGACTGAATTTAA-3′; 5′GATCTTCACAGCTATATGGCAGCTA-3′, and.

5′-CCTACGTGTACGGACAGAGCTATA-3′; 5′-AGGATCGCTCAGCGTAGCATT-3′ for the presence of Pg. TaqMan probes were labeled with the reporter dye 6-carboxyfluorescein (FAM) at the 5′ end and with non-fluorescent quencher (NFQ) at the 3′ end. Amplification and detection of bacterial DNA by real-time PCR were performed using StepOne plus Real-time PCR System (Applied Biosystems, USA). Samples were assayed in a 20 ml reaction mixture containing 2 ml of template DNA, 10 ml of 2X TaqMan Universal PCR Master Mix (Applied Biosystem), 1 ml of 20× assay mix containing probe, forward and reverse primer (Applied Biosystem). The cycling conditions used were as follows: 50 °C for 2 min, 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min each. All data were analyzed using the StepOne software version 2.1.

2.5. Periodontal therapy

Patients group received non-surgical periodontal therapy under local anaesthesia follow by systemic antibiotic therapy consisting in oral doxycycline, 100 mg/day during 21 days.

2.6. Re-examination

A new examination 30 and 60 days after diagnosis and treatment was carried out. Clinical parameters and samples of GCF were taken in each subject at the same sites that had been selected prior to periodontal treatment.

2.7. Statistical analysis

Statistical significance of differences was determined by unpaired and paired (before and after treatment) Student's t test. Pearson correlations were done using GRAPHPAD Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). Categorical multivariate analysis was used to establish the relation

Table 1
Clinical attachment level (CAL), probing pocket depth (PPD), bleeding on probing (BOP), plaque index (PI) and alveolar bone level (ABL) in periodontal healthy subjects (control) and patients with aggressive periodontitis (AgP).

Group	CAL (mm)	PPD (mm)	BOP (sites)	PI	ABL (mm)
Control	0.25 ± 0.05	2.45 ± 0.05	0	0.34 ± 0.03	0.91 ± 0.04
AgP	5.95 ± 0.17***	5.54 ± 0.05***	2.6 ± 0.10	0.31 ± 0.02	2.44 ± 0.08***

The data are expressed as the mean ± SEM. *** Significantly different from control group ($p < 0.001$).

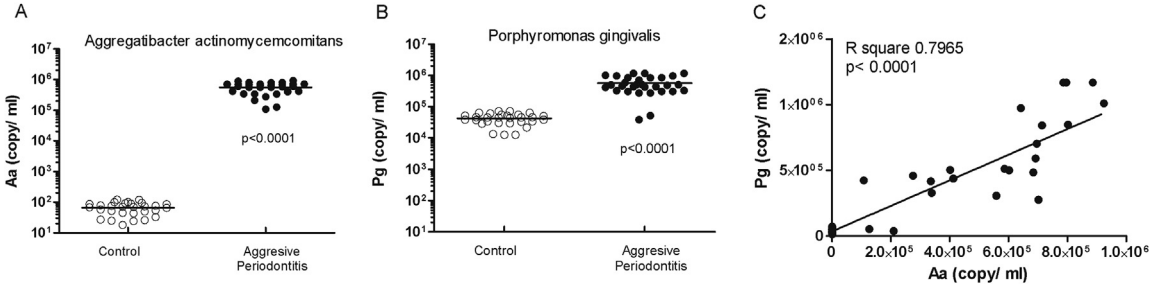

Fig. 1. Number of copies/ml of *Aggregatibacter actinomycetemcomitans* (A) and *Porphyromonas gingivalis* (B) DNA in gingival crevicular fluid of patients with aggressive periodontitis and controls, by real-time PCR. C: Pearson's correlation analysis between counts of *Aggregatibacter actynomicemcomitans* and *Porphyromonas gingivalis* in gingival crevicular fluid from all the subjects studied.

Table 2
Number of copies/ml of *Aggregatibacter actinomycemcomitans* found in subgingival plaque samples from females and males, controls and in patients with aggressive periodontitis.

Gender	Control (%)		Aggressive periodontitis (%)				
	1	2	1	2	3	4	5
Female	15 (100)	0	0	6 (37.5)	2 (12.5)	4 (25)	4 (25)
Male	10 (71.4)	4 (28.6)	4 (28.6)	4 (28.6)	4 (28.6)	2 (14.3)	0

For controls, copies/ml: **1**: $<1 \times 10^2$; **2**: $>1 \times 10^2$. For aggressive periodontitis copies/ml: **1**: $>1 \times 10^5$ to $<3 \times 10^5$; **2**: $>3 \times 10^5$ to $<5 \times 10^5$; **3**: $>5 \times 10^5$ to $<7 \times 10^5$; **4**: $>7 \times 10^5$ to $<10 \times 10^5$; **5**: $>10 \times 10^5$.

Table 3
Number of copies/ml of *Porphyromonas gingivalis* found in subgingival plaque samples from females and males, controls and in patients with aggressive periodontitis.

Gender	Control (%)			Aggressive periodontitis (%)			
	1	2	3	1	2	3	4
Female	1 (6.7)	10 (66.7)	4 (26.7)	0	7 (43.8)	5 (31.2)	4 (25)
Male	5 (35.7)	8 (57.1)	1 (7.1)	2 (14.3)	6 (42.8)	4 (28.6)	2 (14.3)

For control copies/ml: **1**: $>1 \times 10^4$ to $<3 \times 10^4$; **2**: $>3 \times 10^4$ to $<6 \times 10^4$; **3**: $>6 \times 10^6$. For aggressive periodontitis, copies/m: **1**: $<1 \times 10^5$; **2**: $>1 \times 10^5$ to $<5 \times 10^5$; **3**: $>5 \times 10^5$ to 9×10^5; **4**: $>9 \times 10^5$.

between Aa, Pg and the clinical parameters. The level of statistical significance is given when $p < 0.05$.

3. Results

The median age of patients and controls was 20 years (range 17–23). Sixteen females and 14 males were included in patient group and 15 females and 14 males comprised control group. The results of clinical parameters are described in Table 1.

All of control subjects were positive for both Aa and Pg, but Pg was much more abundant than Aa (Fig. 1A and B). In patients group the copies/ml of both, Aa and Pg, was higher than in control group but there was no significant difference in the concentration between the two bacterial species (Fig. 1A and B). With the aim of evaluating whether there was an interaction between Ag and Pg in GCF, a Pearson's correlation analysis was carried out. As can be seen in Fig. 1C, a positive and significant correlation was obtained, suggesting an interrelationship between the two bacterial species.

The concentration of Aa and Pg was not related with age neither in control nor in patients group (data not shown) but it was observed a significant difference between gender in the amount of Aa in patients group, being higher in female (female: 678,500 ± 78,130; male: 416,700 ± 54,760, $p < 0.0125$). This group also showed a higher CAL (female: 6.3 ± 0.14; male: 5.56 ± 0.18; $p < 0.0026$) which suggests a possible relationship between Aa and CAL. All patients showed more than 10^4 but less than 2×10^6 copies/ml of Aa and Pg, in subgingival plaque samples. In order to better compare the concentration of Aa and Pg in female and male, counts were classified in groups according to the number of counts/ml (Tables 2 and 3). As can be seen in the tables, a great number of females showed a high concentration of both microbes than males did.

The multivariate regression analysis suggested that the concentration of Aa in GCF might be closely associated with the volume of GCF, stimulated salivary flow rate, salivary pH, CAL, PPD and ABL

Table 4
Values of η^2 (effect size) obtained by multivariate analysis between copies/ml of *Aggregatibacter actinomycetemcomitans* (Aa) and *Porphyromonas gingivalis* (Pg) in gingival crevicular fluid (GCF) with GCF volume, stimulated (SFR) and unstimulated (UFR) salivary flow rate, salivary pH, clinical attachment level (CAL), probing pocket depth (PPD), alveolar bone loss (ABL) and plaque index (PI) in patients with aggressive periodontitis and control subjects.

Dependent variables	Independent variables	
	Aa	Pg
GCF volume (μl)	0.27 ($p < 0.0001$)	0.0002 ($p = 0.931$)
SFR (ml/min)	0.13 ($p = 0.004$)	0.004 ($p = 0.642$)
UFR (ml/min)	0.07 ($p = 0.06$)	0.015 ($p = 0.364$)
pH	0.39 ($p < 0.0001$)	0.08 ($p = 0.07$)
CAL (mm)	0.59 ($p < 0.0001$)	0.0005 ($p = 0.979$)
PPD (mm)	0.45 ($p < 0.0001$)	0.0004 ($p = 0.997$)
ABL (mm)	0.38 ($p = 0.002$)	0.0005 ($p = 0.980$)
PI	0.003 ($p = 0.87$)	0.011 ($p = 0.440$)

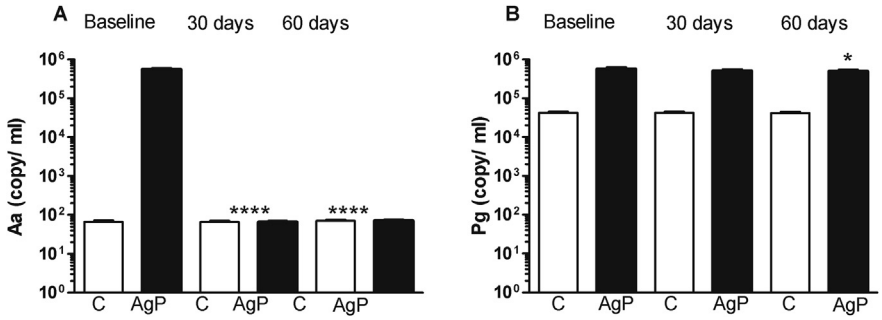

Fig. 2. Number of copies/ml of *Aggregatibacter actynomicemcomitans* and *Porphyromonas gingivalis* in control subjects and in patients with aggressive periodontitis at baseline day and after 30 and 60 days. Patients with aggressive periodontitis received local periodontal treatment and doxycycline (100 mg/day) oral therapy for 21 days. **** Significantly different from Aa in AgP at baseline ($p < 0.001$); * significantly different from Pg in AgP at baseline ($p < 0.05$).

while showed little association with unstimulated flow rate and did not show any association with PI (Table 4). Conversely, as disclosed by the multivariate regression analysis, Pg concentration in GCF might be little associated with pH but might not be associated with volume of GCF, salivary flow rate, CAL, PPD, ABL or PI (Table 4).

The result of periodontal treatment on bacterial concentration in GCF was evaluated at 30 and 60 days after diagnosis. In patients group the concentration of Aa in GCF dramatically decreased, reaching values similar to control group (Fig. 2A). Conversely, Pg copies/ml showed only a little decreased at 60 days compared with baseline (Fig. 2B).

Clinical parameters were also evaluated at 30 and 60 days after diagnosis with the aim to determine the relationship between bacterial presence and the clinical manifestation of the disease. Significant mean PPD reduction and CAL gain was observed at 30 days and went on improving at 60 days while ABL did not change (Table 5). BOP became negative and it was observed a continuous decrease in GCF volume. Stimulated and unstimulated flow rate only showed a significant increase with respect to baseline at 30 days but pH increased at 30 days and went on increasing at 60 days (Table 5).

4. Discussion

There is a growing body of evidence linking Aa and Pg with pathogenesis of periodontitis [11,16]. In this study we found the presence of both microbes in samples of GCF of patients with AgP, but also in control subjects. The positive correlation between both microbes indicates the existence of a synergistic association of Aa and Pg, as was previously described [22]. The fact that the two pathogens were present in health and disease indicates that the amount of these bacteria is more significant with regard to the disease process [23] and supports the hypothesis that periodontal disease is associated with a shift in the balance of the subgingival microbiota rather than the action of a single pathogen or a simple increase in diversity [24].

As observed in other study [25], more females showed a higher concentration of Aa and Pg in GCF samples than males did. This could be related with hormonal influences that have been described for subgingival Aa in young women [26].

Despite being considered a periodontopathogen, Aa can be frequently detected in periodontally healthy individuals, suggesting variability in its virulence [27]. There are different serotypes of Aa that are associated with periodontal health, periodontitis and non-oral infections [28]. In this study the strain of Aa has not been determined but our results indicate a closely association between the presence of Aa and the occurrence of AgP. The positive correlation between number of counts of Aa with deep pockets or serious attachment loss suggested that this microbe exhibited a great periodontal destruction potential in our population.

A number of virulence factors are known for Pg, including fimbriae, lipopolysaccharide, collagenase, and cysteine proteinases with trypsin-like activity [29,30]. It has been shown in studies using molecular typing methods that most bacterial populations consist of numerous genetic clones, and that only a small proportion of these clones cause disease. Pg is present in periodontal pockets as well as in healthy gingival margins [31,32]. Furthermore, clonal heterogeneity of subpopulation of Pg, with both high and low levels of pathogenicity, has been suggested to exist among periodontal pathogens harbored by individuals with negligible, slight, or even severe periodontal destruction. Therefore, specific virulent clones of the pathogens may be the cause of advanced and/or aggressive periodontitis [33]. We observed a lack of association between Pg copies/ml in subgingival plaque samples and severity of clinical signs of the disease. This was an unexpected finding because no significant difference was observed between copies/ml of Pg and Aa in patients with AgP. Interaction of bacteria residing in the periodontal pocket is important to sustain the infection. We found an important relationship between the concentration of Aa and Pg in GCF samples. There is a paradigm shift of the microbiome from health to disease and Aa has been identified as one of the key anaerobic species instrumental in periodontal disease progression. Because in this study Aa was the responsible of periodontal destruction, as disclosed by multivariate analysis, we speculate that it might colonized periodontal tissue inducing periodontal disease. It has been demonstrated that there was no significant difference in species diversity between the organisms harbored by individuals

Table 5
The table shows volume of gingival crevicular fluid, salivary pH, salivary flow rate and clinical parameters from patients with aggressive periodontitis at baseline and 30 and 60 days after periodontal diagnosis.

Parameter	Baseline	30 days	60 days
CAL (mm)	5.95 ± 0.13	5.85 ± 0.13***	5.82 ± 0.13##
PPD (mm)	5.54 ± 0.05	4.03 ± 0.07***	3.71 ± 0.07###
ABL (mm)	2.44 ± 0.08	2.42 ± 0.07	2.43 ± 0.08
BOP (sites)	2.63 ± 0.10	0.43 ± 0.14***	0##
PI	0.34 ± 0.03	0.32 ± 0.03	0.31 ± 0.03
GCF (μl)	0.66 ± 0.02	0.24 ± 0.01***	0.23 ± 0.01#
SFR (ml/min)	0.84 ± 0.02	0.95 ± 0.01***	0,96 ± 0.01
UFR (ml/min)	0.44 ± 0.01	0.49 ± 0.008***	0.49 ± 0.007
pH	6.87 ± 0.01	6.90 ± 0.02**	6.93 ± 0.01#

Data are expressed as the mean ± SEM. CAL: clinical attachment level; PPD: probing pocket depth; ABL: alveolar bone level; BOP: bleeding on probing; PI: plaque index; GCF: gingival crevicular fluid; SFR: stimulated flow rate; UFR: unstimulated flow rate. **Significantly different from baseline $p < 0.01$; *** significantly different from baseline $p < 0.001$; # significantly different from 30 days $p < 0.05$; ## significantly different from 30 days $p < 0.01$; ### significantly different from 30 days $p < 0.001$.

with healthy periodontal tissues and those with periodontitis [23]. In our population, a higher concentration of Pg than Aa was observed in GCF from control subjects, with healthy periodontal pocket, suggesting the presence of an avirulent strain. According to the ecological plaque hypothesis, increased gingival crevicular fluid flow in moderate gingival inflammation is responsible for early changes in the population dynamics of microorganisms at this site, causing the increment of putative periodontal pathogens [24]. Thus, we can speculate that in our population, the pathologic changes associated with infection in progressing periodontitis lesions, induced by Aa, had favored the growing of an avirulent strain of Pg, or because Pg potential to induce inflammatory response may shift according to the environment, specific virulent factors and association with other species [34] its presence in GCF did not correlate with clinical parameters.

Although root planning and surgical intervention are the foundations of periodontal therapy, adjunctive antimicrobial chemotherapy can improve the effectiveness of treatment in individuals with difficult to treat types of periodontal disease, such as AgP [35]. On the other hand, because Aa is regarded as a principal pathogen associated with AgP ant its presence is a significant factor contributing to the decision whether subgingival debridement should be performed in conjunction with systemic antibiotics [36], and doxycycline have proved to be highly effective in inhibiting this bacterium [37], patients were treated orally, during 21 days with 100 mg/day of doxycyline. Thirty days after combined mechanical-antibiotic therapy Aa and Pg were still detected in GCF samples of patients but, while Aa dramatically decreased being counts equal to controls, Pg showed no difference from baseline. Although doxycycline has been found to be active against Pg isolated from periodontal pockets in adult patients with chronic periodontitis, tested in vitro [38], systemic administration of this antibiotic failed to decrease Pg counts, present in GCF, in our population.

Both PPD and CAL should be used as clinical measures of disease severity and progression, hence our use of those measures to evaluate the result of antibiotic therapy. The data indicate that the antibiotic regimen provided a clinical benefit. Further exploratory analyses were also conducted to evaluate therapy success, as the increase in salivary flow rate and pH. The improvement of all clinical parameters, with exception of ABL, after therapy, suggests that the persistence of Pg in GCF did not lead to unfavorable clinical results.

In conclusion, in our population the presence of Aa was significantly associated with AgP. However, no significant relationship was observed between Pg and periodontal status. These results suggest that Pg, which may be an opportunistic pathogen that often exists as a member of the oral indigenous flora, causes disease only when the ecological balance is favorable [39] and provide novel insight into the apparent heterogeneity in Pg virulence properties.

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