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Analysis of *Porphyromonas gingivalis fimA* genotypes in severe periodontitis patients

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Abstract: The aim of this study was to i) evaluate the prevalence of *P. gingivalis* and the genotypes fimA I, Ib, II, III, IV, and V in Brazilian patients with periodontitis stage III and IV, grades B and C, ii) compare periodontitis grades B and C with regard to the prevalence of *P. gingivalis* and fimA genotypes, and iii) correlate the presence of these pathogens with clinical periodontal variables. Two samples of subgingival biofilm were collected from the interproximal sites with the greatest clinical attachment loss (CAL) of each patient (grade B = 38; grade C = 54) and submitted to polymerase chain reaction (PCR) for the identification of *P. gingivalis* and fimA genotypes. The collected periodontal clinical parameters included gingival index, plaque index, probing depth (PD), bleeding on probing (BoP) and CAL. *P. gingivalis* was present in 61.96% of the samples, but more prevalent in patients with grade C periodontitis ($p = 0.048$) and higher CAL ($p < 0.001$), PD ($p < 0.001$), and BoP ($p = 0.01$) values, and at sites with high CAL values ($p = 0.01$). The fimA II genotype was more prevalent in patients with greater mean PD ($p = 0.04$) and a higher proportion of bleeding sites ($p = 0.006$). Thus, in this sample of Brazilian periodontitis patients, the presence of *P. gingivalis* was associated with grade C periodontitis and periodontal destruction, while the fimA II genotype was associated with increased PD and BoP, supporting the notion that *P. gingivalis* fimA II is an important virulence factor in periodontal tissues.

Keywords: Periodontitis; *Porphyromonas gingivalis*; Virulence Factors.

Introduction

Periodontitis is a multifactorial pathology associated with bacterial plaque dysbiosis in the subgingival environment and immunoinflammatory reactions.[1,2,3] Currently, periodontitis is classified into four stages according to severity and treatment complexity. Stages III and IV are the most severe. Periodontitis can also be classified into grades A, B, and C according to the rate of progression and presence of risk factors.[3]

Several pathogens have been associated with periodontal destruction. A periodontal pathogen of Socransky's red complex,[4] *Porphyromonas gingivalis* is highly prevalent in the periodontal pockets of periodontitis patients,[5,6,7,8,9,10] but may also be found in periodontally healthy individuals.[9,11,12] The great genotypical and phenotypical diversity of *P. gingivalis* results in

variations in virulence and in the ability to induce periodontal destruction.[11]

The fimbriae are the main structures responsible for the virulent behavior of *P. gingivalis*. They adhere to epithelial cells, fibroblasts, salivary components, and collagen and thus play an important role in the colonization and invasion of periodontal tissues.[13] The main fimbria is encoded by the *fimA* gene, allelic variations of which have been correlated with pathogenic potential and specific periodontal conditions.[9,11]

Based on the nucleotide sequence, six genotypes of the *fimA* gene have been identified (*fimA* I, Ib, II, III, IV and V).[14,15] Several studies have found associations between periodontitis and increased prevalence of *fimA* II, followed by *fimA* IV and *fimA* Ib.[9,11,16,17] Likewise, in a study on peri-implantitis, *fimA* II was the most prevalent genotype, while *fimA* Ib was associated with greater peri-implant probing depth.[18] In another study, the prevalence of *P. gingivalis* and the genotypes *fimA* II and IV was positively associated with the gingival index in adolescents three months after orthodontic appliance placement.[19] In contrast, *fimA* I has been associated with chronic gingivitis not progressing to periodontitis, suggesting this genotype is less virulent.[20]

The knowledge of periodontal pathogens and their association with clinical presentations of periodontitis can help identify individuals at risk for development and progression of periodontal disease. In addition, the distribution of periodontal pathogens and their genotypes is influenced by geographic and ethnic factors. Thus, the present study was designed to meet the following objectives: i) evaluate the prevalence of *P. gingivalis* and the genotypes *fimA* I, Ib, II, III, IV and V in Brazilian patients diagnosed with periodontitis stage III and IV, grades B and C, ii) compare periodontitis grades B and C regarding the prevalence of *P. gingivalis* and *fimA* genotypes, and iii) correlate the presence of these pathogens with clinical periodontal variables.

Methodology

This cross-sectional study included 94 patients recruited at the periodontology service of the School of Pharmacy, Dentistry and Nursing of the Federal University of Ceará (Northeastern Brazil). The study protocol was previously approved by the institutional ethics committee and filed under entry #20/08. All participants (or guardians) gave their informed written consent.

The patients were initially distributed into two groups (chronic vs. aggressive periodontitis). However, following the new periodontitis classification criteria published in 2018,[3] the patients were reclassified into stages I, II, III, or IV and grades A, B, or C, based on clinical data (clinical attachment loss, probing depth, tooth mobility, furcation involvement, number of remaining teeth, and radiographic bone loss) collected during the periodontal examination. Clinical patterns suggestive of rapid progression and/or early onset of periodontitis and the correlation between plaque deposits and periodontal destruction were used for grading (A, B, or C). In our sample, all patients were stage III or IV and grade B or C. We therefore divided the sample into 'Group B' (stage III/IV, grade B) and 'Group C' (stage III/IV, grade C). The exclusion criteria were: i) periodontal treatment within the previous 6 months, ii) use of antibiotics within the previous 3 months, iii) smoking, and iv) systemic changes capable of interfering with periodontal health.

As stated before, this study was originally designed to evaluate differences between the prevalence of *P. gingivalis fimA* genotypes in patients with aggressive periodontitis and chronic periodontitis, characteristics of the previous classification of periodontal diseases. Then, using data from a pilot study,[5] and considering a ratio of 0.16 between *fimA* II negative to positive patients, a sample size of at least 28 patients with aggressive periodontitis would be needed to provide 80% power with $\alpha = 0.05$ (Epi-Info™, CDC, Atlanta, GA, USA).

Clinical measurements

All completely erupted permanent teeth (except third molars) were evaluated using a periodontal probe (PCP-UNC 15, Trinity, São Paulo, Brazil). The clinical parameters included: plaque index (PI)[21] gingival index (GI)[21], probing depth (PD), clinical attachment loss (CAL), bleeding on probing (BoP), tooth mobility, and furcation involvement. PD and CAL were taken at six sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual).

A single examiner, a calibrated periodontist, evaluated all clinical parameters. For reproducibility analysis, duplicate measurements were performed on two occasions, one prior to initiating the study and a second during the study. Twelve individuals diagnosed with periodontitis stage III or IV were evaluated twice, with a three-day interval. Data were analyzed, and the intraclass correlation coefficient (ICC) was 0.88 and 0.83 for PD and CAL measurements, respectively.

Microbiological analysis

Supragingival plaque was removed with curettes and sterile cotton pellets, and the area was isolated with sterile cotton rolls. Subgingival plaque samples were then collected from two different sites per patient using two sterile paper points (Dentsply Maillefer 35, Dentsply, Rio de Janeiro, Brazil) per site, inserted for 20 seconds.[22] The proximal site with the greatest PD and CAL of molars or incisors was selected for each patient. Each sample was placed in a microtube containing 1 mL Ringer's solution (8.6 g NaCl, 0.3 g KCl, and 0.33g CaCl·2HO in 1000 mL HO) and stored at -80°C until the time of use.

Each sample was processed separately. The samples were thawed on ice, and bacterial cells were dispersed by vortexing at the maximum setting for 1 min, then centrifuged at 12,000 *g* for 10 min. Genomic DNA was extracted from the pellet following the manufacturer's instructions (InstaGene Matrix, Bio-Rad Laboratories, Hercules, USA). A 20-µL aliquot of the resulting supernatant was added to 30 µL reaction mixture containing 25 µM PCR buffer (Promega Corporation, Madison, USA), 25 µM $MgCl_2$ (Promega Corporation), 0.2 µM dNTP mix (Promega Corporation), 1.25 U *Taq* polymerase (Promega Corporation), and 100 ng of each primer (Invitrogen, São Paulo, Brazil), resulting in a final volume of 50 µL.[22,23] Negative and positive controls were included in each reaction (Table 1).

First, PCR was performed with universal primers[24] for 16S ribosomal DNA (16S rDNA) to confirm the presence of bacterial DNA. Subsequently, samples were evaluated by PCR with specific primers for the presence of *P. gingivalis*[25] and *fimA* genotypes I,[14] Ib,[15] II,[14] III,[14] IV,[14] and V.[26] Amplification was performed with a biocycler (Biosystems, Curitiba, Brazil), followed by analysis of the products by electrophoresis on agarose gel. The gels were stained with SYBR® Safe (Invitrogen, São Paulo, SP, Brazil) and photographed (Canon Powershot A640, Canon, USA) under ultraviolet light (LTA/LTB GE, Loccus Biotecnologia, São Paulo, Brazil). PCR was repeated three times for each sample and for *P.gingivalis* and its genotypes.

Table 1. Strains used as positive control.

Clonal type	Strain
fimA I	*P. gingivalis* ATCC 33277
fimA Ib	*P. gingivalis* HG1691
fimA II	*P. gingivalis* HW24D-1
fimA III	*P. gingivalis* 6/26
fimA IV	*P. gingivalis* HG564
fimA V	*P. gingivalis* HNA99

Statistical analysis

The normality of the data distribution was verified with the Shapiro-Wilk test. Comparisons between Group B and Group C with regard to age, clinical data, and the presence of *P. gingivalis* and its genotypes were made with the unpaired *t* test and the Mann-Whitney U test. Pearson's chi-squared test was used to compare the groups with regard to the prevalence of *P. gingivalis* and *fimA* genotypes. The level of statistical significance was set at 5% ($p < 0.05$). All analyses were performed with the software SPSS 25.0 (IBM Corp., Armonk, USA).

Results

Fifty patients were classified as stage III and 44 were classified as stage IV. As for grading, 40 subjects were graded 'B' and 54 subjects were graded 'C'. Two grade B patients yielded samples that could not be amplified, leaving 38 patients in Group B vs. 54 patients in Group C. Table 2 shows the demographic and clinical data of all 92 patients. The patients in Group C were significantly younger, had more teeth, and greater PD and BoP values than the patients in Group B.

P. gingivalis was present in 57 patients (61.96%), but was more prevalent in Group C than in Group B ($p = 0.048$). The groups did not differ significantly with regard to the prevalence of *fimA* genotypes (Table 3).

Table 2. Characteristics of the study participants.

Variables	Group B	Group C	p-value
N	38	54	
Age (years; mean ± SD)	41.82 ± 8.49	27.94 ± 6.15	< 0.001*
Male/female	16/ 22	20/ 34	0.62
White/non-white	11/27	11/43	0.85
Number of teeth (mean ± SD)	23.32 ± 3.17	25.94 ± 1.89	< 0.001*
PD (mm; mean ± SD)	2.90 ± 0.64	3.42 ± 0.68	< 0.001*
CAL (mm; mean ± SD)	3.54 ± 1.04	3.81 ± 0.89	0.17
BoP (% sites; mean ± SD)	33.46 ± 17.07	45.00 ± 16.67	0.02*
PD (sampled site 1) (mm; mean ± SD)	7.71 ± 2.09	8.42 ± 2.18	0.12
CAL (sampled site 1) (mm; mean ± SD)	8.79 ± 2.41	9.31 ± 2.48	0.31
PD (sampled site 2) (mm; mean ± SD)	6.60 ± 1.53	7.28 ± 2.06	0.09
CAL (sampled site 2) (mm; mean ± SD)	7.31 ±1.72	8.02 ± 2.54	0.14

*

Table 3. Prevalence of *P. gingivalis* and *fimA* genotypes according to group.

Pg	Group B	Group C	p-value
	19 (50.0%)	38 (70.4%)	0.048*
*fim*A I	6 (31.6%)	6 (15.8%)	0.17
*fim*A Ib	6 (31.6%)	7 (18.4%)	0.26
*fim*A II	16 (84.2%)	35 (92.1%)	0.36
*fim*A III	1 (5.3%)	1 (2.6%)	0.61
*fim*A IV	1 (5.3%)	0 (0.0%)	0.15
*fim*A V	0 (0.0%)	1 (2.6%)	0.48

*

Patients positive for *P. gingivalis* presented significantly higher mean PD, CAL, interproximal CAL, and BoP, and greater PD and CAL at selected sites, than did *P. gingivalis*-negative patients. The mean PD and BoP values were also higher among *fimA* II-positive than *fimA* II-negative patients. No significant associations were found between clinical variables and the five other *fimA* genotypes. Moreover, the proportion of selected sites with means for PD ≥7 mm, PD ≥8 mm, and CAL ≥8 mm was significantly greater for *P. gingivalis*-positive than *P. gingivalis*-negative patients (Table 4).

Discussion

Our study was originally designed to evaluate the possible correlations between aggressive vs. chronic periodontitis and *P. gingivalis fimA* genotypes. However, in 2018, the World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions concluded there is insufficient evidence to support the concept of aggressive and chronic periodontitis as two pathophysiologically distinct diseases.[3,27] A new classification system was proposed, according to which periodontitis is no longer divided into chronic and aggressive but classified into stages and grades based on clinical parameters and other elements influencing clinical management, prognosis, and, potentially, both oral and systemic health.[3,27]

Thus, all patients were reclassified as stage III or IV primarily based on the finding of interproximal CAL ≥ 5 mm and PD ≥ 6 mm. The classification also took into account dental mobility (≥ 2), furcation involvement (class II or III), the number of remaining teeth, and radiographic bone loss.[3,27]

Due to the cross-sectional design of the study, no direct data on disease progression was collected, but indirect evidence of progression was used to grade the patients as A, B, or C. Smoking habits and systemic disease were exclusion criteria and so were not included in the analysis. None of our patients presented deposits of heavy biofilm with low levels of destruction (Grade A). When destruction exceeded expectations for the level of biofilm deposits and a specific clinical pattern was observed (*e.g.*, incisive/molar pattern or early-onset disease), the patient was graded 'C'. When destruction matched expectations for the level of biofilm deposits, with no specific clinical pattern suggestive of grade A or C, the patient was graded 'B'.[3]

Table 4. Clinical variables according to the presence/absence of *P. gingivalis* and *fimA* genotypes.

Variable	*Pg* +	*Pg* -	p-value	*fimA I* +	*fimA I* -	p-value	*fimA Ib* +	*fimA Ib* -	p-value	*fimA II* +	*fimA II* -	p-value
n	57	35	-	12	45		13	44		51	6	
Age (years; mean ± SD)	33.93 ± 9.50	33.25 ± 10.70	0.67	35.92 ± 11.15	33.40 ± 9.08	0.540	35.92 ± 10.68	33.34 ± 9.18	0.424	33.50 ± 9.15	37.70 ± 12.51	0.31
PD (mm; mean ± SD)	3.40 ± 0.69	2.89 ± 0.65	< 0.001*	3.09 ± 0.50	3.47 ± 0.71	0.085	3.18 ± 0.57	3.46 ± 0.71	0.199	3.47 ± 0.67	2.72 ± 0.46	0.04*
CAL (mm; mean ± SD)	4.00 ± 0.98	3.21 ± 0.70	< 0.001*	3.88 ± 0.96	4.03 ± 0.99	0.550	3.92 ± 0.93	4.02 ± 1.00	0.711	4.04 ± 0.97	3.70 ± 0.12	0.36
Interproximal CAL (mm; mean ± SD)	4.63 ± 1.22	3.64 ± 0.87	< 0.001*	4.62 ± 1.34	4.64 = ± 1.20	0.784	4.68 ± 1.30	4.62 ± 1.21	0.985	4.62 ± 1.15	4.80 ± 1.87	0.83
PD selected sampled sites (mm; mean ± SD)	8.03 ± 1.96	6.81 ± 1.34	0.01*	8.00 ± 2.14	8.03 ± 1.93	0.962	8.12 ± 2.09	8.00 ± 1.94	0.854	8.16 ± 2.00	6.92 ± 1.16	0.14
CAL selected sampled sites (mm; mean ± SD)	8.98 ± 2.15	7.48 ± 1.65	0.01**	9.25 ± 2.32	8.91 ± 2.12	0.632	9.27 ± 2.22	8.89 ± 2.14	0.588	9.04 ± 2.21	8.50 ± 1.52	0.57
BoP (% sites; mean ± SD)	44.65 ± 16.83	32.30 ± 16.61	0.01***	40.63 ± 14.97	45.76 ± 17.31	0.655	42.24 ± 15.46	45.39 ± 17.34	0.651	46.80 ± 16.47	27.10 ± 6.40	0.006*
PD ≥7 mm (mean - sampled sites)	67.2%	44.4%	0.029***	66.7%	66.7%	1.000	69.2%	65.9%	0.823	66.7%	66.7%	1.000
CAL≥7 mm (mean - sampled sites)	77.6%	63.9%	0.149	75.0%	77.8%	0.839	76.9%	77.3%	0.979	76.5%	83.3%	0.705
PD ≥8 mm (mean - sampled sites)	50.0%	27.8%	0.034***	41.7%	51.1%	0.561	46.2%	50.0%	0.907	51.0%	33.3%	0.413
CAL ≥8 mm (mean - sampled sites)	69.0%	41.7%	0.009***	66.7%	68.9%	0.883	69.2%	68.2%	0.943	68.6%	66.7%	0.922

*Statistically significant difference between patients positive and negative for *P. gingivalis* and *fimA* II genotype (Mann-Whitney test; $p < 0.05$); **Statistically significant differences between patients positive and negative for *P. gingivalis* (unpaired t-test; $p < 0.05$); ***Statistically significant differences between patients positive and negative for *P. gingivalis* (Pearson's chi-squared test; $p < 0.05$). Due to the very low prevalence, *fimA* genotypes III, IV and V were not included. PD: probing depth; CAL: clinical attachment loss; BoP: bleeding on probing.

The patients were organized in groups according to grade, not stage. While the stage depends on the severity and extent of the disease at the time of presentation, the grade provides information on biological features of the disease such as specific clinical patterns suggestive of periods of rapid progression and/or early-onset of the disease and the relationship between plaque deposits and periodontal destruction,[3,27] as previously required for the diagnosis of aggressive periodontitis.[28,29]

The mean age was higher, and the mean number of teeth was smaller in Group B than in Group C. Despite the lower mean age in Group C, the mean BoP value was higher, suggesting greater disease severity. This was expected since Group C consisted of patients with destruction patterns suggestive of periods of rapid progression and early-onset of disease, especially those previously classified as having aggressive periodontitis.

In our sample, the high prevalence of *P. gingivalis* was associated with periodontitis stage III and IV, especially grade C. This finding matches the results of a recent metanalysis of 42 case-control studies showing a 78.7% prevalence of *P. gingivalis* in periodontal disease,[30] although the reported prevalence rates vary widely in the literature.[7,11,17,31,32] The prevalence of *P. gingivalis* was higher in Group C than in Group B. Since the classification of periodontitis into stages and grades was introduced very recently, no data was available for comparison. Nevertheless, the presence of *P. gingivalis* has been positively correlated with greater probing depth and periodontitis severity.[17,32,33] Likewise, in our study, Group C was associated with greater mean probing depth and the proportion of sampled sites with PD and CAL ≥8 mm was greater in *P. gingivalis*-positive than *P. gingivalis*-negative patients.

Among *P. gingivalis*-positive samples, the *fimA* II genotype was the most prevalent regardless of group (B = 84.2%; C = 92.1%), matching a previous Brazilian study on a sample of patients with aggressive periodontitis.[5] However, the prevalence of the *fimA* II genotype was higher in our study than in many other studies involving patients with chronic and aggressive periodontitis in a variety of populations from Japan,[9,26] Germany,[17] and even Brazil.[11] When only patients with aggressive periodontitis (equivalent to grade C) were considered, the prevalence of the *fimA* II genotype was still substantially higher than that reported for Japanese[7] and Chinese cohorts.[31] These discrepancies may be explained by differences in the severity of periodontal disease and by ethnic and geographic variability. In any case, the *fimA* II genotype very likely played an important role in the development and progression of periodontitis in our sample of Brazilian patients.

Despite the higher prevalence of *fimA* II and the lower prevalence of *fimA* I in group C, the difference between the groups was not statistically significant. This trend has been observed in several other studies, invariably associating the *fimA* II genotype with greater virulence and periodontal destruction,[5,7,9,11,17,31] whereas the *fimA* I genotype has been associated with lower virulence and healthy patients.[9,31] Since Group C consisted mainly of patients previously diagnosed with aggressive periodontitis, our findings support the results of Japanese and Chinese studies on aggressive periodontitis.[7,31] The absence of statistical significance may be due to the narrow range of disease severity in the sample: both groups consisted of patients with advanced periodontal disease (stages III and IV).

In addition to the high prevalence of *P. gingivalis* and *fimA* II, the periodontal clinical variables (such as mean PD and BoP) were also more severe in *P. gingivalis*-positive patients.

P. gingivalis of the *fimA* II genotype is related to the progression of periodontal disease,[35] adhering to and invading human epithelial cells and inducing inflammation more efficiently than any other genotype.[13,35,36,37] The presence of *fimA* II is therefore believed to be an important *P. gingivalis* virulence factor in periodontal tissues.[34,35,36,37] The detection of this genotype can help identify individuals at risk, make adequate treatment plans, and establish a more accurate prognosis. In addition, studies on the *fimA* II genotype are crucial to the development of new treatment strategies such as passive immunization with monoclonal antibodies from *P. gingivalis fimA* II strains, as demonstrated by Hijiya et al.[38] in a rat model.

Conclusions

In our sample of Brazilian patients with periodontitis stages III and IV, the prevalence of

P. gingivalis was higher in patients graded 'C' than 'B' and in patients with severe periodontal destruction as expressed by greater clinical attachment loss, probing depth, and bleeding on probing. Moreover, the prevalence of the *fimA* II genotype was higher in patients with greater mean probing depth and a greater proportion of bleeding sites.

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