ORIGINAL ARTICLE

Prevalence of periodontal pathogens as predictor of the evolution of periodontal status

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Abstract The aim of this study was to determine the relationship between the prevalence of *Porphyromonas gingivalis*, its *fimA* genotypes, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, and *Treponema denticola* and the evolution of periodontal health. In a longitudinal prospective study, samples of subgingival plaque were taken from 114 patients (37 with chronic periodontitis, 17 with gingivitis, and 60 periodontally healthy) in the course of a full periodontal examination. PCR was employed to determine the presence of the periodontopathogenic bacteria. Four years later, a second examination and sample collection were performed in 90 of these patients (20 with chronic periodontitis, 12 with gingivitis, and 58 periodontally healthy). *T. forsythia, P. gingivalis,* and *T. denticola* are the most prevalent bacteria in patients with chronic periodontitis (78.4%, 62.2 y 56.8%, respectively). The *P. gingivalis* bacterium and its *fimA* genotypes I, II, and IV showed the highest correlation between the baseline and follow-up assessments. *P. gingivalis fimA* genotype II and *T. forsythia* were associated to a significant degree with unfavourable periodontal evolution. Of the variables studied, *P. gingivalis fimA* genotype II and *T. forsythia* increase the risk of an unfavourable evolution of periodontal status.

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Introduction

Chronic periodontitis is an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment loss, and bone loss [1]. The composition of subgingival plaque is complex and has been the subject of numerous studies, as the presence of certain bacteria is associated with worse periodontal status, greater pocket depth, and higher bleeding indices [2]. The oral cavity is colonized by a wide range of microorganisms [3]. Some are commensal bacteria that live in harmony with their hosts and do them no harm. Others, known as periodontopathogenic bacteria, are associated with periodontitis. *Aggregatibacter actinomycetemcomitans* has been associated with aggressive periodontitis, while *Porphyromonas gingivalis, A. Actinomycetemcomitans, Tannerella forsythia, Treponema denticola,* and *Eikenella corrodens* have been associated with chronic periodontitis [4].

In 1998, Socransky et al. described a 'red complex', formed by a combination of *Bacteroides forsythus* (now known as *T. forsythia*), *P. gingivalis,* and *T. denticola*, which exhibit a clearer relationship with the clinical signs of chronic periodontitis, specifically pocket depth and bleeding on probing. Subsequently, many studies have been carried out to detect the presence of these three bacteria in different populations, the association between them, and the synergic mechanisms that increase their pathogenicity [5–7].

The virulence of *P. gingivalis*, a gram-negative anaerobic bacterium, is attributed to its various surface components, such as fimbriae, lipopolysaccharides, and proteases. This surface makes it possible for the bacterium to interact with the external medium and facilitates its growth, nutrient acquisition, colonization, and formation of a biofilm that protects it against the host's defences [8, 9]. Amano et al. concluded that *P. gingivalis* can be classified into five genotypes according to genomic differences in the *fimA* gene which codes fimbrillin, a protein of the major fimbriae [10, 11]. Subsequently, Nakagawa et al. discovered a new variant of the fimA gene, which they named Ib, because it bore a great resemblance to genotype I [12]. *P. gingivalis* is frequently found in patients with chronic periodontitis, but has also been observed, although to a lesser extent, in periodontally healthy patients [13]. In recent years, studies have been conducted to evaluate the relationship between the different *P. gingivalis* genotypes and periodontal pathogenesis. Genotype II has been observed to be more prevalent in periodontal patients and to be associated with more aggressive forms of the disease. Some authors attribute this relationship to its possessing greater adhesiveness and invasiveness [14].

The aetiopathogeny of periodontitis is the result of a complex interaction between the oral biofilm and the host's immune response. New laboratory techniques have given a better understanding of this biofilm and its role in the progress of chronic periodontitis. The aim of this study was to assess the relationship between the presence of periodontal pathogenic bacteria (*P. gingivalis* and its six *fimA* genotypes, *A. actinomycetemcomitans, T. denticola, T. forsythia,* and the 'red complex') and periodontal status, whether periodontal bacteria prevalence differed significantly between adult patients in different periodontal status groups and the evolution of periodontal status 4 years later.

Materials and methods

In this longitudinal prospective cohort study, two oral examinations and determinations of periodontal pathogenic bacteria were conducted 4 years apart. The baseline assessment was made in 2009 and the follow-up assessment in 2013.

Baseline assessment

Inclusion and exclusion criteria and study size

The inclusion criteria were patients aged between 25 and 50 years with at least 20 teeth present in the mouth, excluding third molars.

Pregnant women and any with drug-induced gingival hyperplasia, or who had taken antibiotics in the previous six months, or who were taking anti-inflammatory medication to treat a chronic condition were excluded from the study, as were patients with HIV infection, Type I or II diabetes mellitus, coronary heart disease, rheumatoid conditions, lupus erythematosus, Behçet's syndrome, Crohn's disease, herpetic gingivostomatitis, pemphigous, or oral pemphigoid [15].

A consecutive sample of 140 patients of the Dentistry Clinic at the University of Valencia (Spain) who met the inclusion and exclusion criteria was selected for the study. The patients were given the necessary information and took part voluntarily after giving their informed consent. The study design and protocol were both approved by the Research in Humans Ethics Committee of the University of Valencia's Experimental Research Ethics Committee.

The sample size was calculated on the basis of data from the study by Zhao et al. [13], which estimated the presence of *P. gingivalis* as around 82% in patients with chronic periodontitis and 22% in periodontally healthy patients. With a confidence level of $1-\alpha = 95\%$ for a statistical power of 85%, a two-tailed test, and an expected loss rate of 25%, the estimated sample size is a minimum of 14 individuals in each study group.

Clinical examinations and sample collection

A full examination of the entire mouth of each patient was conducted, employing a WHO periodontal probe (PCP11.5B, Hu Friedy, Chicago, IL, USA), and the following were recorded at 6 sites on each tooth [16]: bacterial plaque, bleeding, pocket depth, and loss of periodontal attachment. The plaque index was calculated by dividing the number of surfaces with plaque by the total number of surfaces and multiplying the result by 100 to express the result as a percentage [17]. The bleeding index was calculated in the same way.

The patients were classified into three groups: Group I was periodontally healthy patients, nowhere presenting a pocket depth greater than 3 mm or loss of periodontal attachment greater than 1 mm [13], Group II was patients with gingivitis, who met the conditions for Group I but presented a Gingival Bleeding Index of over 30% [18], and Group III was patients with chronic periodontitis, presenting at least 4 areas with a probing depth of 5 mm or greater and loss of periodontal attachment of 2 mm or more [16]. Of the 140 patients selected for the study and examined, a total of 114 were included in the study: 60 were assigned to the no disease group, 17 to the gingivitis group, and 37 to the chronic periodontitis group. The other 26 were excluded, as they did not meet the requirements for classification into one of these three groups.

The patients were also classified as 'non-smokers', 'ex-smokers', or 'current smokers'. Those who had stopped smoking at least 6 months before the study began were considered 'ex-smokers'. Those who had stopped smoking less than 6 months previously were included in the 'current smokers' group. In the case of patients who smoked, the number of cigarettes per day was also recorded.

The subgingival plaque samples were obtained from the deepest pocket at Ramfjord teeth in the patients in the chronic periodontitis group and from the mesiolabial area of a Ramfjord molar in the no disease group and the gingivitis group. The supragingival plaque was first removed with a sterile Gracey curette, employed with care to avoid bleeding. Three sterile paper points (size 25, Dentsply Maillefer, Ballaigues, Switzerland) were inserted as deeply as possible into the gingival groove, left for 15 s, removed, and placed in a sterile Eppendorf tube [13]. The bacterial DNA was extracted immediately using the Wizard SV DNA Purification System (Promega Cat. # A2360) in accordance with the manufacturer's instructions. Following extraction, the DNA samples were stored at −20 °C until determination took place.

Polymerase chain reaction (PCR)

The PCR method was used to detect the four bacteria: *P. gingivalis, A. actinomycetemcomitans, T. denticola,* and *T. forsythia* [19, 20]. The method described by Zhao et al. [13] was used to analyse the *fimA* genotypes of *P. gingivalis*. To genotype the *fimA* gene, the specific primers for each subtype described by Amano et al. [11] and Nakagawa et al. [12] were used. Table 1 shows the species-specific primers used for PCR in this study.

The PCR reaction was performed with 100 ng of the bacterial DNA extracted in the previous step, 200 μM of each of the dNTPs, 3 mM MgCl2, 50 pmol of each primer, and 0.5 U of AmpliTaq Gold (9800 Fast ThermalCycler of Applied Biosystems®). The PCR conditions were an initial taq polymerase activation step at 95 °C for 10 min, followed by 40 denaturing cycles at 94 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 30 s, and finally, a single final elongation step at 72 °C for 7 min.

The PCR products were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide, then viewed under ultraviolet light. All the samples were analysed in duplicate using two different PCR reactions for each primer pair. A no-template control (PCR reactions without a DNA template) for each primer pair was included in each group of PCR reactions to check for foreign DNA contamination and for non-specific amplification (primer-dimer formation). Samples in which types I and II were both found were amplified further using specific primers for type Ib. The resulting amplicons were digested with RsaI, and cast in 1.5% agarose gels stained with ethidium bromide and viewed under ultraviolet light. Samples in which 2 fragments appeared were considered type Ib [12].

Negative controls were included for each primer set in each round of PCR reactions. Positive controls were included to verify the negative amplification results. For *Porphyromonas*, DNA isolated from cell lines (*P. gingivalis* ATCC33277 and *P. gingivalis* W83) was used. For the other bacterial species, DNA isolated from the patients' samples was used and those samples that yielded PCR amplification products were included in each round of amplification.

Follow-up assessment

Of the initial 114 patients included in the study, 90 could be re-examined at the follow-up assessment 4 years later. Two patients were not re-examined, because they did not meet the inclusion criteria established for the chronic conditions at the baseline assessment. Informed consent was again obtained, and a new examination record was filled in registering whether the patient had received any periodontal treatment since the initial examination.

The patients were again classified into one of the three groups (no disease, gingivitis, or chronic periodontitis) according to the clinical signs observed, as in the baseline assessment, to determine whether their periodontal evolution had been favourable or unfavourable. The subgingival plaque samples were obtained at the same point of the gingival sulcus in the no disease and gingivitis groups and in the same periodontal pocket in the chronic periodontitis group. The classification criteria and sample collection and processing followed the same protocol, as described above.

Study variables

- Bacteria$_0$/Genotype$_0$/Red Complex$_0$: The presence of bacteria (*A. actinomycetemcomitans, T. denticola, P. gingivalis,* or *T. forsythia*), a *P. gingivalis fimA* genotype (I–V or Ib), or Socransky's 'red complex' in the baseline assessment.
- Bacteria$_1$/Genotype$_1$/Red Complex$_1$: The presence of bacteria (*A. actinomycetemcomitans, T. denticola, P. gingivalis,* or *T. forsythia*), a *P. gingivalis fimA* genotype (I–V or Ib), or Socransky's 'red complex' in the follow-up assessment 4 years later.
- Periodontal treatment during the follow-up period (none/sporadic scaling/constant periodontal maintenance).
- Stable or favourable periodontal evolution: patients whose periodontal condition in the follow-up assessment 4 years later was the same or better than in the baseline assessment.

Table 1 Species-specific primers used for PCR

Species-specific primers	Sequence (5′–3′)	Size (bp)
Porphyromonas gingivalis	AGG CAG CTT GCC ATA CTG CG	404
	ACT GTT AGC AAC TAC CGA TGT	
FimA genotype I	CTG TGC GTT TAT GGC AAA CTT C	392
	AAC CCC GCT CCC TGT ATT CCG A	
FimA genotype II	ACA ACT ATA CTT ATG ACA ATG G	257
	AAC CCC GCT CCC TGT ATT CCG A	
FimA genotype III	ATT ACA CCT ACA CAG GTG AGG C	247
	AAC CCC GCT CCC TGT ATT CCG A	
FimA genotype IV	CTA TTC AGG TGC TAT TAC CCA A	251
	AAC CCC GCT CCC TGT ATT CCG A	
FimA genotype V	AAC AAC AGT CTC CTT GAC AGT G	462
	TAT TGG GGG TCG AAC GTT ACT GTC	
FimA genotype Ib	CAG CAG AGC CAA AAA CAA GTC	271
	TGT CAG ATA ATT AGC GTC TGC	
A. actinomycetemcomitans	AAA CCC ATC TCT GAG TTC TTC TTC	557
	ATG CCA ACT TGA CGT TAA AT	
T. forsythia	GCG TAT GTA ACC TGC CCG CA	641
	TGC TTC AGT GTC AGT TAT ACC T	
T. denticola	TAA TAC ATG TGC TCA TTT ACA	316
	TCA AAG CAT TCC CTC TTC TTC TTA	

- Unfavourable periodontal evolution: patients who had passed from the no disease to the gingivitis or periodontitis group between the baseline and follow-up assessments, or who were already affected initially by chronic periodontitis and whose pocket depth had increased during the follow-up period.

Statistical analyses

The mean values of the quantitative variables and the prevalence rates for the dichotomous or ordinal variables were obtained. The 95% confidence intervals were calculated. In the two-tailed statistical analysis, the relationships between the dichotomous variables were evaluated by the Chi-square test and the linear trend test. Student's *t* test and ANOVA were used to compare means and the Pearson coefficient to assess correlations. The significance level was set at $p < 0.05$ or $p < 0.01$. A multivariate binary logistic regression analysis was performed. Computations were carried out by the SPSS 19.0 statistical analysis software (SPSS Inc., Chicago, IL USA).

Results

The distribution of the sample by mean age, gender, or smoking did not differ significantly between the no disease, gingivitis, and chronic periodontitis groups. The data and the inter-group statistical differences found among the variables—number of cigarettes per day, plaque index, bleeding index, probing depth, and level of attachment loss—are shown in Table 2.

Percentage of individuals with periodontal bacteria at the beginning of the study, by group (baseline assessment)

Table 3 shows the prevalence of the different bacteria found in the subgingival plaque. The three periodontal status groups presented significant differences in rates of *P. gingivalis, A. actinomycetemcomitans, T. denticola, T. forsythia,* and the 'red complex', which increased significantly as periodontal condition worsened and reached their highest point in the chronic periodontitis patients. The percentages of individuals with *T. forsythia* (78.4%), *P. gingivalis* (62.2%), and *T. denticola* (56.8%) in the subgingival plaque samples were higher than those with *A. actinomycetemcomitans* (27%) in the chronic periodontitis group. The 'red complex' appeared in 35.1% of the individuals taking part in the study.

On studying the distribution of the four bacteria under study and the 'red complex' by non-smoker, ex-smoker or 'current smoker', regardless of periodontal status, the only bacterium showing statistically significant differences between smokers and non-smokers or ex-smokers was *T. denticola* (data not shown).

Table 2 Sociodemographic and clinical characteristics of the initial sample ($n = 114$) by groups in the baseline assessment

	No disease ($n = 60$)	Gingivitis ($n = 17$)	Chronic periodontitis ($n = 37$)	Statistical test for inter-group comparison
Age (mean years)	39.3 ± 8.1	35.1 ± 13.1	41.4 ± 9.6	ANOVA $p = 0.083$
Gender				
Male	25	5	21	Chi^2
$n = 51$	41.7%	29.4%	56.8%	$p = 0.135$
Female	35	12	16	
$n = 63$	58.3%	70.6%	43.2%	
Tobacco				
Non-smoker	23	6	7	Chi^2
$n = 36$	38.3%	35.3%	18.9%	$p = 0.239$
Ex-smoker	20	5	12	
$n = 37$	33.3%	29.4%	32.4%	
Smoker	17	6	18	
$n = 41$	28.3%	35.3%	48.6%	
Cigarettes per day (mean)	2.78 ± 6.2	4.59 ± 7.6	9.54 ± 11.2	ANOVA $p = 0.268$
Plaque index** (mean %)	12.4 ± 11.3	30.6 ± 22.8	26.7 ± 17.8	ANOVA $p = 0.003$
Bleeding index** (mean %)	12.1 ± 8.2	47.8 ± 15.3	33.9 ± 21.9	ANOVA $p = 0.000$
Probing depth** (mean)	0.72 ± 0.31	1.14 ± 0.34	2.01 ± 0.45	ANOVA $p = 0.000$
Loss attachment level** (mean)	0.17 ± 0.11	0.18 ± 0.12	1.07 ± 0.33	ANOVA $p = 0.000$

Significant statistical differences between groups * $p < 0.05$; ** $p < 0.01$

Changes in periodontal status and in the percentage of individuals with periodontal bacteria 4 years later, at the end of this study (follow-up assessment)

Of the 114 persons initially examined, 90 remained for the follow-up assessment 4 years later, signifying a loss of 21.1% of the sample. At follow-up, most of the patients remained in their initial groups. On the Landis and Koch scale, the agreement between the baseline status and follow-up status was considered high (kappa statistic = 0.722). The clinical status of 10 patients from the initial no disease group had worsened (8 had developed gingivitis and 2 periodontitis). Of the patients initially placed in the gingivitis group, 2 were moved to the no disease group and another 2 to the periodontitis group. All the patients initially placed in the periodontitis group remained there. Of the 90 individuals examined at follow-up, only 6 had received constant periodontal maintenance (periodical scaling and root planning with chlorhexidine mouthrinses before treatment), 40 had received sporadic scaling, and the remaining 44 had not received any type of treatment.

Variations in the composition of the patients' subgingival plaque were recorded. Table 4 shows the correlations for each bacterium, the 'red complex', and *P. gingivalis fimA* genotypes at the beginning (baseline assessment) and end of the study (follow-up assessment 4 years later). Those with the greatest correlation between baseline and follow-up were genotypes I, II, and IV, the bacterium *P. gingivalis,* and the 'red complex'.

Bacteria associated with unfavourable periodontal evolution

Table 5 shows the prevalence of *P. gingivalis* bacteria and *fimA* genotypes in the baseline assessment of the patients with a stable or favourable periodontal evolution and those with an unfavourable periodontal evolution. The bacteria with statistically significant differences were *P. gingivalis* and its *fimA* genotype II, *T. forsythia,* and the 'red complex'. This table also shows the incidence ratios of unfavourable periodontal evolution due to the presence of the different bacteria. The highest are those associated with the

Table 3. Prevalence of periodontal pathogenic bacteria and *fimA* genotypes of *P. gingivalis* by periodontal status at baseline and at 4-year follow-up

	Baseline			4-Year follow-up		
	No disease (*n* = 60) *n* % CI 95%	Gingivitis (*n* = 17) *n* % CI 95%	Chronic periodontitis (*n* = 37) *n* % CI 95%	No disease (*n* = 50) *n* % CI 95%	Gingivitis (*n* = 16) *n* % CI 95%	Chronic periodontitis (*n* = 24) *n* % CI 95%
P. gingivalis[a,b,c,d]	14	6	23	12	10	16
	23.3%	35.3%	62.2%	24%	62.5%	66.7%
	(14.4–35.4)	(17.3–58.7)	(46.1–75.9)	(14.3–37.4)	(38.6–81.5)	(46.7–82)
FimA genotype I	1	0	4	4	4	4
	1.7%	0%	10.8%	8%	25%	16.7%
	(0.3–8.9)	(0–18.4)	(4.3–24.7)	(3.2–18.8)	(10.2–49.5)	(6.7–35.9)
FimA genotype II[a]	11	1	13	6	4	8
	18.3%	5.9%	35.1%	12%	25%	33.3%
	(10.6–29.9)	(1–26.9)	(21.8–51.2)	(5.6–23.8)	(10.2–49.5)	(18–53.3)
FimA genotype III[a,b]	0	0	4	4	2	2
	0%	0%	10.8%	8%	12.5%	8.3%
	(0–6)	(0–18.4)	(4.3–24.7)	(3.2–18.8)	(3.5–36)	(3.2–25.8)
FimA genotype IV	6	5	6	4	6	4
	10%	29.4%	16.2%	8%	37.5%	16.7%
	(4.7–20.1)	(13.3–53.1)	(7.7–31.1)	(3.2–18.8)	(18.5–61.4)	(6.7–35.9)
FimA genotype Ib[a,b,d]	0	0	3	0	0	4
	0%	0%	8.1%	0%	0%	16.7%
	(0–6)	(0–18.4)	(2.8–21.3)	(0–7.1)	(0–19.4)	(6.7–35.9)
A. actinomycetemcomitans[a,b]	5	2	10	4	0	0
	8.3%	11.8%	27%	8%	0%	0%
	(3.6–18)	(3.3–34.3)	(15.4–43)	(3.2–18.8)	(0–19.4)	(0–13.8)
T. forsythia[a,b]	13	10	29	30	10	16
	21.7%	58.8%	78.4%	60%	62.5%	66.7%
	(13.2–33.6)	(36–78.4)	(62.8–88.6)	(46.2–72.4)	(38.6–81.5)	(46.7–82)
T. denticola[a,b,d]	8	3	21	12	6	14
	13.3%	17.6%	56.8%	24%	37.5%	58.3%
	(6.9–24.2)	(6.2–41)	(40.9–71.3)	(14.3–37.4)	(18.5–61.4)	(38.8–75.5)
Red complex[a,b,d]	3	1	13	4	4	10
	5%	5.9%	35.1%	8%	25%	41.7%
	(1.7–13.7)	(1–26.9)	(21.8–51.2)	(3.2–18,8)	(10.2–49.5)	(42.5–61.2)

FimA genotype V was not found in any of the patients. Red complex: combination of *P. gingivalis. T. forsythia* and *T. denticola*

[a] Significant differences in Chi-squared test *p* < 0.05 at baseline

[b] Significant differences in linear trend test *p* < 0.05 at baseline

[c] Significant differences in Chi-squared test *p* < 0.05 at 4-year follow-up

[d] Significant differences in linear trend test *p* < 0.05 at 4-year follow-up

presence of *A. actinomycetemcomitans* and the 'red complex'.

A multivariate binary logistic regression analysis was performed with 'unfavourable periodontal evolution' as the dependent variable and all the measurements of bacteria or *P. gingivalis fimA* genotype presence in the baseline assessment as the independent variables, together with age, gender, the number of cigarettes smoked by the patients who were smokers, and any periodontal treatment received during the 4-year follow-up period. The final model contained significant independent variables: *P. gingivalis fimA* genotype II (*p* = 0.017; Expβ = 3.84) and *T. forsythia* (*p* = 0.008; Expβ = 4.16). The other variables entered did not prove significant. The omnibus test of the model was

Table 4. Correlation of bacteria and *P. gingivalis fimA* genotypes in baseline and follow-up assessments

	Pearson correlation coefficient	*p* value
FimA genotype I_0 − *fimA* genotype I_1	0.384*	<0.01**
FimA genotype II_0 − *fimA* genotype II_1	0.362*	<0.01**
FimA genotype III_0 − *fimA* genotype III_1	−0.047	0.659
FimA genotype IV_0 − *fimA* genotype IV_1	0.442*	<0.01**
P. gingivalis$_0$ − *P. gingivalis$_1$*	0.312*	<0.01**
T. denticola$_0$ − *T. denticola$_1$*	0.287	<0.01**
T. forsythia$_0$ − *T. forsythia$_1$*	0.134	0.206
A. actinomycetemcomitans$_0$ − *A. actinomycetemcomitans$_1$*	−0.076	0.475
Red complex$_0$ − red complex$_1$	0.469*	<0.01**

$_0$ Bacteria and genotypes in baseline assessment plaque samples, $_1$ Bacteria and genotypes in follow-up assessment plaque samples

* Pearson correlation coefficient >0.3; ** $p < 0.01$. It was not possible to find a correlation for *fimA* genotypes V and Ib

significant, with $p = 0.001$ and a Nagelkerke's R^2 value of 0.212. The Hosmer and Lemeshow goodness of fit test found $p = 0.901$.

Discussion

The composition of the subgingival plaque and, more specifically, the presence of particular periodontal pathogenic bacteria are related to clinical signs which define the patient's periodontal status and its evolution. The three study groups presented significant differences in *T. forsythia*, *P. gingivalis,* and *T. denticola* prevalence. These differences also existed in the case of *A. actinomycetemcomitans*, but this bacterium was only found in 27% of the chronic periodontitis patients. The same differences were found in *P. gingivalis fimA* genotypes II, III, and Ib. *FimA* genotype V was not found in any of the patients. On studying 90 of the same patients 4 years later, collecting the subgingival plaque sample from the same area as before, genotypes I, II, and IV, the *P. gingivalis* bacterium, and the 'red complex' were the pathogens with the highest correlation between the baseline and follow-up assessments. The bacteria with significant differences between patients whose periodontal status had remained steady or improved and those in whom it had worsened were *P. gingivalis* and its *fimA* genotype II, *T. forsythia,* and the 'red complex'. The results showed that *P. gingivalis* and *T. forsythia* were the most prevalent bacteria in patients with an unfavourable evolution. However, the incidence ratio shows that *A. actinomycetemcomitans* presents the highest relative risk (4.63) despite being the least prevalent of the bacteria studied.

The aim of most longitudinal studies has been to follow the evolution of cases who had been given a particular treatment compared to a control group, or to compare two different treatments [21, 22]. In the present case, the treatment received during the follow-up interval did not influence the results ($p = 0.237$). It should be noted that this study had a lengthy follow-up interval, 4 years, in keeping with the evolution of chronic periodontitis. One salient result is that 2 patients who were included in group II at the beginning of the study were placed in group I in the follow-up assessment, because their gingival bleeding index had dropped below 30%. This confirms that gingivitis is a reversible condition. The main limitation in this type of study is the loss of a proportion of the sample for a variety of reasons, such as a change of address or of the contact telephone number. It must be acknowledged that the loss rate of 21.1% in this case constitutes a limitation for the evaluation of the results.

It has already been reported by Simonson et al. that *T. denticola* can serve as a prognostic marker for periodontal disease recurrence [23]. In 2012, Nomura et al. published a longitudinal study with the aim of ascertaining which salivary biomarkers and which bacteria could be used to predict the progression of chronic periodontitis. The bacteria they studied were *P. gingivalis, Prevotella intermedia,* and *T. forsythia,* and their follow-up period was 18 months. The bacteria that proved to be significant predictors of the progression of chronic periodontitis were *P. gingivalis and P. intermedia* [24]. As regard longitudinal studies of the presence of *P. gingivalis fimA* genotypes, Fujise et al. [25] assessed the influence of the different genotypes in patients with chronic periodontitis who received scaling and root planing treatment. They found that genotype I was a predictor of bleeding on treatment.

The *fimA* genotypes of *P. gingivalis* and the bacteria *A. actinomycetemcomitans, T. forsythia,* and *T. denticola* have all been widely investigated, with numerous cross-sectional studies in different age groups and ethnic groups, and have been related to the presence of systemic pathologies, such as diabetes mellitus or tobacco use. These studies show that

Table 5 Prevalence of periodontal pathogenic bacteria and *P. gingivalis fimA* genotypes at the beginning of the study (baseline assessment) in the samples taken from the 90 patients who remained in the study, classified by periodontal evolution at the end of the follow-up time (follow-up assessment) and the incidence ratio of unfavourable periodontal evolution

	Periodontal evolution stable or favourable (n = 68) n % CI 95%	Periodontal evolution unfavourable (n = 22) n % CI 95%	p value	Incidence ratio (IR)
*P. gingivalis***	18 26.5% (17.4–38.0)	18 81.8% (61.4–92.7)	0.000	3.09 (1.61–5.94)
FimA genotype I	0 0% (0–5.3)	2 9.1% (2.5–27.8)	0.058	–
FimA genotype II**	12 17.6% (10.3–28.4)	10 45.5% (26.9–65.3)	0.008	2.57 (1.11–5.96)
FimA genotype III	0 0% (0–5.3)	2 9.1% (2.5–27.8)	0.058	–
FimA genotype IV	12 17.6% (10.3–28.4)	4 18.2% (7.3–38.5)	0.955	1.03 (0.33–3.19)
*A. actinomycetemcomitans**	4 5.9% (2.3–14.2)	6 27.3% (13.1–48.2)	0.012	4.63 (1.30–16.4)
*T. forsythia***	20 29.4% (19.9–41.1)	14 63.6% (42.9–80.3)	0.004	2.16 (1.09–4.28)
T. denticola	14 20.6% (12.6–31.6)	10 45.5% (26.9–65.3)	0.022	2.21 (0.98–4.97)
Red complex**	6 8.8% (4.1–17.9)	8 36.4% (19.7–57.0)	0.005	4.12 (1.43–11.8)

Red complex combination of *P. gingivalis, T. forsythia,* and *T. denticola*

Significant differences in Chi-squared test or Fisher's test: * $p < 0.05$; ** $p < 0.01$

P. gingivalis very frequently appears in the subgingival plaque of patients with chronic periodontitis, ranging from 29.6 to 97.5% prevalence [10, 13, 18, 26–29]. While *P. gingivalis* is considered one of the bacteria most closely associated with chronic periodontitis, it is also present in the subgingival plaque of periodontally healthy patients, although in a smaller percentage, ranging from 1.5 to 57.8% depending on the study [11, 13, 18, 27–30]. The present study detected *P. gingivalis* in 62.2% of the patients with chronic periodontitis and 23.3% of the healthy individuals.

As regard the distribution of *P. gingivalis fimA* genotypes, one fact that every study repeats is the greater prevalence of *fimA* genotype II in patients with chronic periodontitis. In the first studies in which the 6 genotypes were known, it was already being said that type II had the strongest association with periodontitis, while type Ib, the last to be identified, was also shown to be associated with progression of the disease, although to a lesser degree [12]. Nevertheless, variations in the distribution of the genotypes have been found, attributed to ethnic and geographical differences [13, 18]. Indeed, certain studies show marked variations in the composition of the subgingival plaque of chronic periodontitis patients from different countries with the same age, pocket depth, gender, and exposure to tobacco use [17, 31, 32].

In the same way as *P. gingivalis* is present in both healthy subjects and in patients with chronic periodontitis, so are *T forsythia, T denticola,* and *A. actinomycetemcomitans*. In all cases, the prevalence of these bacteria is lower in healthy individuals than in patients with chronic periodontitis. It should be mentioned that of the four bacteria studied, *A. actinomycetemcomitans* is the one that appears least frequently in both healthy and periodontal patients (around 30%), even in studies conducted in patients with aggressive periodontitis [4]. Wara-aswapati et al. [7] found a high frequency of the three 'red complex' bacteria in the moderate to severe periodontitis patients group, whereas *A. actinomycetemcomitans* was only present in 35% of the patients.

On studying the influence of tobacco on the composition of subgingival bacterial plaque, the present study found no differences in the distribution of *P. gingivalis fimA* genotypes when current smokers were compared with ex-smokers and non-smokers. Some previous studies have found no differences in the quantities of *P. gingivalis* obtained from the subgingival plaque samples of smokers and non-smokers [7]. As regard tobacco use and the bacteria studied, the present study did find significant differences in *T. denticola* prevalence between smokers, on the one hand, and ex-smokers and non-smokers, on the other hand. Haffajee and Socransky [31] found that certain bacteria, such as *P. gingivalis* and *T. denticola*, were more prevalent in smokers than in ex-smokers or non-smokers.

Of all the variables studied, those that proved significant in the multivariate logistic regression analysis were *P. gingivalis fimA* genotype II and *T. forsythia*, although owing to the complexity of the aetiopathogeny of chronic periodontitis, further longitudinal studies are needed to determine which variables influence the evolution of periodontitis for the worse to develop different patient follow-up protocols depending on the risk profile.

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