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Full Length Research Paper

Periodontopathogens levels and clinical response to periodontal therapy in individuals with the *Interleukin 4* haplotype of susceptibility to chronic periodontitis

Livia S. Finoti[1,2], Giovana Anovazzi[1,2], Suzane C. Pigossi[1,2], Sâmia C. T. Corbi[1,2], Silvia R. L. Teixeira[3], Guilherme V. V. Braido[2], Yeon J. Kim[4], Silvana R. P. Orrico[2], Joni A. Cirelli[1], Márcia P. A. Mayer[3], Raquel M. Scarel-Caminaga[2*]

[1]Department of Oral Diagnosis and Surgery, School of Dentistry at Araraquara, UNESP- Univ Estadual Paulista, SP, Brazil.
[2]Department of Morphology, School of Dentistry at Araraquara, UNESP- Univ Estadual Paulista, SP, Brazil.
[3]Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil;
[4]Department of Implantology, Santo Amaro University, Sao Paulo, SP, Brazil

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Periodontitis is an inflammatory disease that results from an interaction between dental biofilm agents and the host immune-inflammatory response. Periodontopathogenic organisms, such as *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola,* as well as the host's susceptibility represented by the host's genetic makeup, are the key factors that influence this complex disease. Recently, we identified haplotypes in the *IL4* gene that were associated with chronic periodontitis (CP). This study aimed to evaluate whether subjects with different *IL4* haplotypes associated with genetic susceptibility to or protection against CP would be differentially colonized by periodontopathogens and whether they would respond differently to non-surgical periodontal therapy. Thirty-nine patients carrying the *IL4* haplotype of genetic susceptibility to CP (S) or protection against CP (P) were evaluated. Those groups were further subdivided into individuals with CP [SCP or PCP] and those that were periodontally healthy (H), [SH or PH]. CP patients were submitted to non-surgical periodontal therapy. Clinical and microbiological analyses were performed considering the data at baseline, 45 and 90 days after periodontal therapy/oral hygiene instruction. Periodontopathogens levels were evaluated by absolute quantitative PCR (qPCR). Baseline data revealed that the total levels of periodontopathogens were higher in the SCP than in the PCP groups. Clinical analyses revealed that the periodontal therapy was equally effective, independent of the subject's *IL4* genetic load. The *IL4* haplotype, previously associated with genetic susceptibility to CP, was also associated with increased levels of periodontopathogenic bacteria, but this genetic background did not influence the response to non-surgical periodontal treatment.

Key words: Interleukins, genetics; polymorphism, periodontitis, disease susceptibility, periodontal therapy.

INTRODUCTION

Chronic periodontitis (CP) results from the interaction of host defense mechanisms, microbial agents, and environmental and genetic factors. Periodontopathogens, such as *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (together called the red complex) are associated with CP (Feng & Weinberg, 2006). The host's immune response to these microbiological factors triggers the expression of cytokines, which characterizes the immunopathology of periodontitis (Ferreira et al., 2008).

Cytokines are defined as regulatory proteins that are produced by immune cells and by other cells of the body (Kamma et al., 2009), including cells in the periodontium that regulate the cellular response to an inflammatory stimulus. A large number of cytokines have been found in the gingival crevicular fluid (GCF) (Birkedal-Hansen, 1993, Genco, 1992), such as interleukin-1β, interleukin-6 and interleukin-4 (Kamma et al., 2009).

*Corresponding author. E-mail: raquel@foar.unesp.br

Interleukin-4 (IL-4) is a potent down regulator of macrophage function that inhibits the secretion of proinflammatory cytokines, such as IL-1, IL-6, and tumor necrosis factor (TNF), after stimulation with the lipopolysaccharides (LPS) of periodontopathogenic bacteria. In periodontal tissues, a lack of IL-4 may cause the accumulation of macrophages, increased CD14 expression, and high production of IL-1β, TNF-α and prostaglandin E2 by human monocytes, which leads to bone resorption (Shapira et al., 1992). Variation in cytokine levels among patients with periodontitis is documented and it is associated with disease susceptibility (Trombone et al., 2009). This individual variation in the levels of interleukins can be attributed to polymorphisms in their genes.

Polymorphisms in the *IL4* gene have been largely investigated in studies regarding inflammatory and autoimmune diseases, as reviewed by Vandenbroeck & Goris (Vandenbroeck & Goris, 2003). Three polymorphisms in the *IL4* gene, -590 (C/T) rs2243250, +33(C/T) (rs2070874) and VNTR (variable number of tandem repeats, insertion/deletion of 70 bp in intron 3), which are in strong or perfect linkage disequilibrium with each other due to their physical proximity on the chromosome, form haplotypes (van der Pouw Kraan et al., 2002). In Czech and Brazilian populations, respectively, Holla et al. (2008) (Holla et al., 2008) and Anovazzi et al. (2010) (Anovazzi et al., 2010) reported that haplotypes formed by these polymorphisms are associated with susceptibility to chronic periodontitis. Individuals carrying the TCI/CCI haplotype were five times more susceptible to CP ($OR_{adjusted}$= 5.27, 95% CI = 2.28-12.18), while those carrying the TTD/CTI haplotype seemed to be genetically protected against the development of periodontitis ($OR_{adjusted}$= 0.29, 95% CI = 0.08-0.88) (Anovazzi et al., 2010).

Many studies evaluated a single factor, such as genetic polymorphisms (Anovazzi et al., 2010), periodontopathogens (Martinez-Pabon et al., 2008), and cytokine production (Duarte et al., 2010), and its relationship with periodontal disease. Some of them investigated the combined influence of more than one factor on the disease, such as genetic polymorphisms and microbiological factors. Agerbaek et al. (2006) found that individuals who were previously genotyped for *IL1A* (+4845) and *IL1B* (-3954), when identified as *IL1* gene-positive, required a lower bacterial load to develop the same level of periodontitis than *IL1* gene-negative subjects (Agerbaek et al., 2006).

Because CP is a complex disease, the interrelationship of different factors, needs to be better understood (Corbi et al., 2012). Therefore, this study aimed to evaluate whether subjects with different *IL4* haplotypes associated with genetic susceptibility to or protection against CP would be differentially colonized by periodontopathogens and whether they would respond differently to non-surgical periodontal therapy.

MATERIALS AND METHODS

Study Population

To participate in the present study, which was conducted between January and August 2011, thirty-nine individuals (15 males and 23 females) were selected among 250 genetically pre-screened individuals, considering our previous study that demonstrated the association of haplotypes in the *IL4* gene with CP (Anovazzi et al., 2010). To assess clinical differences between the genetically pre-screened groups, a sample size calculation was performed with the use of a two-tailed *t* test with an alpha of 5%, a 1-mm clinically significant difference in the probing pocket depth between groups, and a standard deviation of 0.5 mm (Gomes *et al.*, 2008, Lopes *et al.*, 2010). Then, with a total sample size of 6 subjects, the power of the study was calculated to be 95%. Therefore, the number of subjects enrolled in the present study (39 individuals) was large enough to detect clinical differences between the genetically pre-screened groups with an acceptable level of confidence.

All volunteers were informed about the aims and methods of the current study, and they gave their written consent to participate. This study was approved by the Committee of Ethical Affairs of the UNESP – Univ Estadual Paulista (Protocol number 52/08). The inclusion criteria were the following: good general health, age between 30-60 years old, presence of at least 16 teeth in the mouth, and no history of subgingival periodontal debridement or periodontal surgery in the preceding 12 months. The following additional exclusion criteria were applied: oral diseases other than caries and periodontal disease, ongoing orthodontic therapy, smoking (current and former smoker with abstinence for less than 5 years), need for antibiotic prophylaxis, a history of systemic or local disease with an influence on the immune system (cancer, cardiovascular and respiratory diseases), diabetes mellitus, hepatitis or HIV infection, immunosuppressive chemotherapy, or current pregnancy or lactation.

Clinical measurements

A single previously calibrated examiner (G.A./weighted Kappa=0.82, considering the probing pocket depth) recorded clinical measurements of the probing pocket depth (PPD – from the gingival margin to the base of the pocket), clinical attachment loss (CAL – from the cementoenamel junction to the base of the pocket), and the presence of bleeding on probing (BOP). The clinical periodontal parameters were assessed at six sites around each tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual locations) for the whole mouth, excluding third molars. All clinical measurements were performed with the use of a manual probe (Trinity-Campo Mourão, Brazil). Each

patient's clinical data were recorded at baseline, 45 and 90 days after the completion of non-surgical treatment.
The patients were considered to have CP when two or more sites in non-adjacent teeth exhibited probing pocket depths ≥ 5 mm, clinical attachment loss ≥ 3 mm and bleeding on probing. Subjects without CP exhibited no clinical signs of gingival inflammation and had probing pocket depths ≤ 3 mm.

Periodontal treatment

All individuals enrolled in the study received instructions on oral hygiene techniques and supragingival prophylaxis. The patients with CP received non-surgical periodontal treatment, which was composed of scaling and root planing over a 3- to 4-week period using manual curettes (Hu-Friedy® Manufacturing Inc., Chicago, IL, USA) and ultrasonic instrumentation (Cavitron® Ultrasonics Inc., Long Island City, NY, USA) performed by one periodontist (G.A.).

Sample Collection

In the patients with CP, subgingival samples were collected in two proximal sites of non-adjacent teeth with PPD ≥ 5 mm and BOP (diseased sites – DS) and from two proximal sites of non-adjacent teeth with PPD ≤ 2 mm(healthy sites – HS). For the periodontally healthy subjects, microbiological samples were collected only from two proximal sites of non-adjacent teeth with PPD ≤ 2 mm(control sites – CS). Each collection site was recorded at baseline, 45 and 90 days after the completion of non-surgical treatment. Thus, 324 subgingival plaque samples were analyzed and distributed into the following four groups:

- SCP: *IL4* haplotype susceptible to CP and presenting CP (6 patients; 24 sites [12 DS, 12HS]);
- PCP: *IL4* haplotype protected against CP and presenting CP (9 patients; 36 sites [18 DS, 18HS]);
- SH: *IL4* haplotype susceptible to CP, periodontally healthy (12 individuals; 24 sites [24 CS]);
- PH: *IL4* haplotype protected against CP, periodontally healthy (12 individuals; 24 sites [24CS]).

Quantitative polymerase chain reaction

Genomic DNA from bacteria and subgingival plaque samples was isolated and purified using a phenol-chloroform protocol (Ausubel, 1999) and used as the template for real-time polymerase chain reaction (q-PCR). To establish the quantitative assay, plasmids containing the target genes were used as standards. PCR amplicons were obtained for *16SrRNA* of *P. gingivalis*, *T. denticola,* and *T. forsythia*. The amplicons were individually cloned in PCR 2.1 TOPO TA (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations and transformed in *Escherichia coli DH5-α*. After the growth of transformants, plasmids were extracted using the PureLink Quick Plasmid Miniprep kit (Invitrogen) (Teixeira et al., 2009).
The standard curve with diluted plasmids that harbored a cloned copy of template DNA for *P. gingivalis*, *T. denticola*, and *T. forsythia* had a minimum R^2 value of 0.98, and the estimated amplification efficiency ranged from 97% to 102%. The seven standard dilutions (from 10^7 to 10^1 copies) were run in triplicate, the subgingival samples were run in duplicate, and the mean values of target molecule numbers were used for analysis.
Quantitative PCR was carried out using the SYBR Green Power Kit (Applied Biosystems). PCR reactions were set up in 96-well plates in a total volume of 22 µl, containing 11 µl of SYBR Green mix, 1 µl of sample DNA, and 100 nM of each of the primers for *P. gingivalis*, *T. denticola,* and *T. forsythia* (Teixeira et al., 2009, Amano et al., 1999, Shelburne et al., 2000, Mullally et al., 2000). The following thermal program was used: 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min and extension at 95°C for 15 s. Melting curve analysis was carried out in the range from 65°C to 95°C to confirm that the PCR products from samples and reference plasmids had identical melting points. Data were analyzed using SDS 7500 Applied Biosystems software. The microorganisms' levels were expressed as the number of copies of the *16SrRNA* gene.

Statistical analysis

The Shapiro-Wilk test was used to assess the normality of the quantitative data distribution. Data were categorized as "baseline", "45 days after treatment" and "90 days after treatment." Because the mean age was distributed normally, one-way analysis of variance (ANOVA) was used to assess differences between groups. The χ^2 test was used to determine whether the groups were composed of the same proportion of males and females.
Because of the absence of a normal distribution, comparisons between the SCP and PCP groups, as well as SH and PH, were accomplished by the use of unpaired Mann-Whitney U test. To assess differences between study periods (multiple comparisons), the Friedman test was utilized to evaluate clinical and microbiological data.
The unit for the analysis was either the subject or the site, and α was set at 0.05. The subject was used for demographic analysis, while the site was used for clinical and microbiological analysis. All data analyses were performed using a statistical software package (Statistica 8.0, StatSoft Inc., Tulsa, OK, USA).

Table 1. Demographic characteristics and mean (± standard deviation) of full-mouth clinical parameters of the study groups.

Characteristics (mean values ±SD)	SCP n=6	SH n=12	PCP n=9	PH n=12
Age (years) (±)	53.33(± 7.37)	38.91(± 5.79)	48.33(± 14.24)	40.75(± 10.96)
Gender (M/F)	2/4	6/6	2/7	5/7
Probing depth (mm) (±)	2.38(± 0.17)	1.52(± 0.23)	2.51(± 0.52)	1.57(± 0.31)
Attachment level (mm) (±)	2.62(± 0.21)	1.55 (± 0.20)	3.04(± 0.94)	1.77(± 0.32)
% of sites with				
Plaque	73.73(± 21.48)	18.62(± 8.29)	60.78(± 25.28)	16.65(± 10.00)
Gingival bleeding	38.31(± 20.12)	7.55(± 3.06)	26.69(± 13.11)	6.00(± 3.14)
Bleeding on probing	26.73(± 12.04)	1.08(± 1.29)	20.54(± 14.56)	0.35(± 0.90)

SCP/PCP = individuals susceptible/non-susceptible to CP by *IL8* haplotype with periodontitis
SH/PH = individuals susceptible/non-susceptible to CP by *IL8* haplotype without periodontitis

RESULTS

Clinical outcomes in evaluated periods

The clinical data of the studied population are summarized in Table 1. No statistically significant differences were observed among groups with regard to age (ANOVA; $p = 0.2587$) or gender (χ^2; $p = 0.26$). All of the full mouth clinical parameters of the individuals with similar periodontal conditions (SH *vs* PH; SCP *vs* PCP) were similar, independent of the presence of the *IL4* haplotype of susceptibility to (S) or protection (P) against CP (Mann-Whitney *U* test, $p > 0.05$).

The effects of non-surgical periodontal therapy on the clinical parameters between individuals belonging to the SCP and PCP groups in diseased sites (DS) and healthy sites (HS) were evaluated. Furthermore, the effects of the instructions of oral hygiene and supragingival prophylaxis on clinical parameters between individuals from the SH and PH groups in control sites (CS) were also evaluated (Table 2). For these analyses, data at baseline, 45 and 90 days after periodontal therapy were considered.

Table 2 shows that all clinical parameters were similar between all sites with similar periodontal conditions at baseline, independent of the subject's genetic makeup. We observed that PPD, CAL and BOP significantly decreased after non-surgical periodontal therapy in diseased sites (at 45 and 90 days) for both groups with CP (SCP and PCP) ($p \leq 0.05$). Healthy sites at baseline, either from periodontitis or periodontally healthy subjects, presented no significant changes in clinical parameters after 45 and 90 days of treatment/oral hygiene instruction and supragingival prophylaxis. Therefore, the subject's genetic background did not influence any clinical parameters, as no significant differences were found between the groups with CP (SCP *vs.* PCP) and with healthy periodontium (SH *vs.* PH) at the studied periods.

Periodontopathogens versus *IL4* haplotypes of susceptibility to/protection against chronic periodontitis

To investigate the relationship between the *IL4* genetic susceptibility to/protection against CP with the colonization of the oral cavity by periodontopathogens, the quantitative data of *P. gingivalis*, *T. forsythia,* and *T. denticola* were obtained from 30 diseased sites, 30 healthy sites (12/18 from susceptible/protected patients, respectively, with CP), and 48 control sites (24/24 from susceptible/protected subjects, respectively, without CP) at three periods of evaluation. Significantly higher levels of *P. gingivalis*, *T. denticola* and *T. forsythia* were found in the SCP group in comparison to the PCP group, mainly at baseline, for both diseased and healthy sites (Mann-Whitney U test, $p<0.05$, Table 3). Counts of *T. denticola* were significantly higher in the diseased sites of the SCP than in the PCP groups, but not in healthy sites. Each intra-group comparison (among the study periods) revealed that the non-surgical periodontal therapy efficiently reduced the counts of all the evaluated periodontopathogens in diseased sites for both the SCP and PCP groups (Friedman's test, $p<0.05$, Table 3). In the control sites, the levels of the three investigated microorganisms were similar between the groups (SH *vs.* PH) for all periods (Mann-Whitney U test, $p>0.05$, Table

Table 2. Clinical Parameters (median; min-max) in both diseased groups (at baseline, 45 and 90 days after treatment) at sample collected sites.

Clinical Parameters		Groups		*p*-value*
		SCP	**PCP**	
Disease Sites				
PPD (mm)				
Baseline		5.0 (4.0-7.0)	5.0 (4.0-7.0)	*NS*
45 days		3.0 (2.0-4.0)[#]	3.0 (2.0-5.0)[#]	*NS*
90 days		2.5(2.0-3.0)[#]	2.0 (2.0-2.0)[#]	*NS*
	p*-value*	*<0.0001*	*<0.0001*	
CAL (mm)				
Baseline		5.0 (4.0-10.0)	5.0 (4.0-6.0)	*NS*
45 days		3.0 (2.0-8.0)[#]	2.5 (2.0-4.0)[#]	*NS*
90 days		3.0 (2.0-5.0)[#]	2.0 (2.0-4.0)[#]	*NS*
	p*-value*	*<0.0001*	*<0.0001*	
BOP (Y/N)				
Baseline		12/0	18/0	*NS*
45 days		8/4[#]	5/13[#]	*NS*
90 days		1/11[#]	0/18[#]	*NS*
	p*-value*	*<0.0001*	*<0.0001*	
Healthy Sites				
PPD (mm)				
Baseline		1.0 (1.0-2.0)	2.0 (1.0-2.0)	*NS*
45 days		1.0 (1.0-2.0)	1.0 (1.0-1.0)	*NS*
90 days		1.0 (1.0-1.0)	1.0 (1.0-2.0)	*NS*
	p*-value*	*NS*	*NS*	
CAL (mm)				
Baseline		2.0 (1.0-3.0)	2.0 (2.0-5.0)	*NS*
45 days		1.5 (1.0-3.0)	1.0 (1.0-4.0)	*NS*
90 days		1.0 (1.0-3.0)	1.0 (1.0-4.0)	*NS*
	p*-value*	*NS*	*NS*	
BOP (Y/N)				
Baseline		0/12	0/18	*NS*
45 days		0/12	0/18	*NS*

Table 2 cont.

90 days		0/12	0/18	*NS*
	p*-value*	*NS*	*NS*	
Control Sites				
PPD (mm)				
Baseline		2.0 (1.0-2.0)	1.0 (1.0-2.0)	*NS*
45 days		1.5 (1.0-2.0)	1.0 (1.0-2.0)	*NS*
90 days		1.0 (1.0-2.0)	1.0 (1.0-2.0)	*NS*
	p*-value*	*NS*	*NS*	
CAL (mm)				
Baseline		2.0 (1.0-2.0)	1.0 (1.0-2.0)	*NS*
45 days		2.0 (1.0-2.0)	1.0 (1.0-2.0)	*NS*
90 days		2.0 (1.0-2.0)	1.0 (1.0-2.0)	*NS*
	p*-value*	*NS*	*NS*	
BOP (Y/N)				
Baseline		0/24	0/24	*NS*
45 days		0/24	0/24	*NS*
90 days		0/24	0/24	*NS*
	p*-value*	*NS*	*NS*	

PPD= Probing Pocket Depth; CAL=Clinical Attachment Loss; BOP=Bleeding on Probing (Y=yes/N=no)
p-value* = Mann-Whitney U for evaluation between the SCP and PCP in each period
p-value**= Friedman's test for multiple evaluation between the different periods
= Significant difference compared to baseline period
NS: no significance

3). However, *P. gingivalis* levels were significantly higher at baseline in sites of both groups than at other evaluated periods (Friedman's test, Table 3).

Effect of non-surgical periodontal therapy or oral hygiene instruction and supragingival prophylaxis on the levels of *P. gingivalis*, *T. denticola* and *T. forsythia*

In general, there was a statistically significant decrease in the counts of each of the three investigated bacterial species in diseased and healthy sites at 45 and 90 days after non-surgical periodontal treatment for both groups with CP (Table 3). In control sites, *P. gingivalis* counts reduced significantly at 90 days after oral hygiene instruction and supragingival prophylaxis, but no significant decrease was found in the counts of *T. forsythia* and *T. denticola* at the two studied periods after the treatment for both the SH and the PH groups.

DISCUSSION

Because CP is a complex disease, the mere presence of putative pathogens does not imply that disease is or will be present, but high numbers of organisms are most likely required (Socransky & Haffajee, 1992). Moreover, the increased content of some bacteria in dental plaque of patients with periodontitis is associated with increased levels of proinflammatory cytokines, such as TNF-α, which was associated with the enhanced dental plaque loaded with *P. gingivalis* (Andrukhov et al., 2011). Despite the complexity of the etiology of periodontitis, few studies have investigated more than one risk factor (e.g., microbiota, genetics, smoking habits, systemic diseases) in relation to the CP outcome.

The present study showed that in diseased and healthy sites at baseline, individuals carrying the *IL4* haplotype of susceptibility to CP (SCP) were infected by the studied periodontopathogens in a higher level than those individuals who carried the *IL4* haplotype of protection against CP (PCP) (Table 3). Interestingly, the clinical parameters shown in Table 2 demonstrated that the SCP and PCP groups had similar periodontal clinical conditions, suggesting that the differences in the bacterial counts seemed to be associated with the genetic load of the subjects. Similarly, Nibali et al. (2007) (Nibali et al., 2007) found that variants in *IL6* and *Fcγ* receptor genes were associated with an increased likelihood of detecting

Table 3. Clinical Parameters (median; min-max) in both diseased groups (at baseline, 45 and 90 days after treatment) at sample collected sites.

Counts of 16S rRNA		Groups		
		SCP	**PCP**	***p*-value***
Diseased Sites				
P. gingivalis				
Baseline		1240.5 (2.2-18810.2)	35.5 (1.0-5884.5)	*0.0397*
45 days		2.8 (0.0-7.4)[#]	2.6 (0.0-28.4)[#]	*NS*
90 days		0.5 (0.0-10.0)[#]	1.6 (0.0-14.0)[#]	*NS*
	p*-value*	*0.0002*	*<0.0001*	
T. denticola				
Baseline		6555.8 (3.4-182635.6)	129.4 (3.0-20054.9)	*0.0227*
45 days		84.1 (0.7-13332.7)[#]	41.0 (0.0-18784.6)[#]	*NS*
90 days		12.9 (0.0-597.6)[#]	0.4 (0.0-292.8)[#]	*0.0199*
	p*-value*	*<0.0001*	*<0.0001*	
T.forsytia				
Baseline		1449.8 (6.0-10793.7)	16.8 (1.6-8837.7)	*0.0257*
45 days		21.5 (2.3-5402.9)[#]	5.2 (0.9-2899.0)[#]	*NS*
90 days		5.1 (0.0-16.0)[#]	3.4 (1.4-12.2)[#]	*NS*
	p*-value*	*<0.0001*	*<0.0015*	
Healthy Sites				
P. gingivalis				
Baseline		8.5 (0.0-12.2)	1.8 (0.0-9.9)	*0.0087*
45 days		0.4 (0.0-7.2)[#]	0.9 (0.0-8.5)[#]	*NS*
90 days		0.0 (0.0-6.7) [#]	0.0 (0.0-4.4)[#]	*NS*
	p*-value*	*0.0015*	*0.0018*	
T. denticola				
Baseline		31.4 (0.0-117.2)	9.4 (0.0-119.2)	*NS*
45 days		13.3 (0.0-40.2)[#]	4.1 (0.0-47.1)[#]	*NS*
90 days		1.1 (0.0-36.8)[#]	0.0 (0.0-24.8)[#]	*NS*
	p*-value*	*0.0183*	*0.0015*	
T.forsytia				
Baseline		6.0 (0.9-26.5)	2.4 (0.0-295.1)	*0.0472*
45 days		1.9 (0.0-19.7)[#]	2.1 (0.0-59.5)	*NS*
90 days		1.8 (0.0-14.4)[#]	0.0 (0.0-2.9)[#]	*0.0158*
	p*-value*	*0.0052*	*0.0029*	
Control Sites				
P. gingivalis				
Baseline		3.4 (0.28-34.7)	3.6 (0.0-13.8)	*NS*
45 days		3.0 (0.0-17.5)	1.9 (0.0-16.7)	*NS*
90 days		1.7 (0.0-12.8)[#]	1.4 (0.0-7.1)[#]	*NS*
	p*-value*	*0.0011*	*0.0063*	
T. denticola				
Baseline		2.8 (0.0-464.5)	4.0 (0.0-121.3)	*NS*
45 days		2.9 (0.0-365.4)	2.4 (0.0-111.8)	*NS*
90 days		0.8 (0.0-61.2)	0.9 (0.0-14.6)	*NS*
	p*-value*	*NS*	*NS*	
T.forsytia				
Baseline		2.1 (0.0-5.9)	4.0 (0.0-121.3)	*NS*
45 days		2.4 (0.0-3568.2)	2.4 (0.0-111.8)	*NS*
90 days		0.5 (0.0-26.6)	0.9 (0.0-14.6)	*NS*
	p*-value*	*NS*	*NS*	

Table 3 Legend
p-value* = Mann-Whitney for evaluation between the SCP and PCP in each period
p-value** = Friedman test for multiple evaluation between the different periods
[#] = Significant difference comparing to the baseline period
NS: no significance.

Aggregatibacter actinomycetemcomitans, P. gingivalis, and *T. forsythia* after adjustment for covariates. Sequentially, haplotype analysis of five *IL6* polymorphisms confirmed an association with the detection of *A. actinomycetemcomitans*. (Nibali et al., 2008, Nibali et al., 2007). It is possible to note that the present study, as well as previous studies (Agerbaek et al., 2006, Nibali et al., 2008, Nibali et al., 2007), showed that differences in the host genotype influence the composition of the subgingival microbiota, in agreement with to the concept of infectogenomics (Nibali *et al.*, 2009). This term defines the study of the interaction between host variations and colonization by pathogenic microbes.

In the present study, it seems that the individuals who carried the *IL4* haplotype of susceptibility to CP are also affected by high levels of infection by periodontopathogens that could, in a synergistic way, contribute to the development of CP in these patients. The response to periodontal non-surgical therapy was similar for both groups, independent of the genetic background (Table 2). A decrease in the PPD and in the CAL measurements in the diseased sites were observed for both the SCP and PCP groups after 45 and 90 days of non-surgical periodontal treatment. These data indicated that non-surgical periodontal therapy was efficient for all the studied subjects. In agreement with our study, Pirhan et al. (2009) (Pirhan et al., 2009), investigating the *MMP13* gene, did not find a significant association between gene polymorphisms and the outcome of periodontal therapy.

As expected, the improvement in clinical parameters promoted by the non-surgical periodontal treatment was followed by a significant reduction in the periodontopathogens levels (Table 3). These results are in agreement with those of other studies that investigated the levels of the same microorganisms after non-surgical periodontal treatment (Doungudomdacha *et al.*, 2001, Mineoka *et al.*, 2008). Additionally, Teles et al. 2010 (Teles *et al.*, 2010) found positive correlations among the mean clinical parameters and the proportions of red complex species.

The main limitation of our study was the number of the enrolled patients, characterizing an exploratory study. However, it should be considered, that the motivation to investigate the clinical and microbiological aspects of the patients was originated from the previous results of the genetic association (Anovazzi, Kim et al. 2010) that investigated a large study population. The patients enrolled in the present study was screened from that population because the importance to find subjects carrying the specific *IL4* gene haplotypes in order to fulfill the inclusion criteria of patients. Despite the number of collected periodontal sites investigated here for the microbiological analysis seemed to be quite limited, we explored the literature for articles focusing microbiological analysis in association with genetic carriage. The few studies found examined a number of sites similar to the investigated in our study (Nibali, Ready et al. 2007; Nibali, Tonetti et al. 2008; Nibali, Donos et al. 2010).

In conclusion, the susceptibility to CP conferred by the haplotype in the *IL4* gene was associated with higher levels of periodontopathogenic bacteria. The non-surgical treatment was equally effective in improving clinical parameters independent of the *IL4* genetic background.

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