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RESEARCH ARTICLE

Absolute quantification of *Aggregatibacter actinomycetemcomitans* in patients carrying haplotypes associated with susceptibility to chronic periodontitis: multifaceted evaluation with periodontitis covariants

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ABSTRACT

This study aimed to evaluate the association between haplotypes in the interleukin 8 (*IL8*) and *IL4* genes previously associated to chronic periodontitis (CP) and the levels of *Aggregatibacter actinomycetemcomitans* (*A.a.*) in subgingival sites of patients with and without CP. Moreover, multifaceted evaluations were made to search associations among patients' genetic background with the *A.a.* levels and previous clinical/immunological/microbiological findings. Subgingival sites (n = 596) of 104 patients were divided into susceptible to CP by the *IL8* haplotype ATC/TTC (*IL8*+); non-susceptible to CP by the *IL8* AGT/TTC (*IL8*–); susceptible to CP by the *IL4* TCI/CCI (*IL4*+); protection against CP by the *IL4* TTD/CTI (*IL4*–). Subgingival biofilm samples from diseased and healthy sites of CP patients and from control sites of health patients were obtained for absolute quantification of *A.a.* by quantitative real-time polymerase chain reaction. For diseased sites, samples were collected before and 45 days after periodontal treatment. The *IL4* but not the *IL8* haplotypes were associated with levels of *A.a.* (in both periods). After periodontal treatment, higher levels of *A.a.* were found in subgingival sites of (*IL4*–) patients, and higher levels of IL-4 were associated with deeper probing pockets in these same patients. Significant correlations were found among genetic (patients carrying *IL8* or *IL4* haplotypes), microbiological and immunological data showing the interrelationship of different factors in the CP.

Keywords: chronic periodontitis; interleukin-8; interleukin-4; *Aggregatibacter actinomycetemcomitans*; genetic polymorphism

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INTRODUCTION

Periodontal disease (PD), a primary reason for human adult tooth loss (Darveau 2010), is an infectious disease that affects tooth-supporting tissues, and is mainly found as Chronic Periodontitis and Aggressive Periodontitis (International Workshop for a Classification of Periodontal Diseases and Conditions, 1999). Oral biofilms play an important role in the pathogenesis of PD (Socransky and Haffajee 2005) with more than 700 species identified in the oral cavity (Aas *et al.* 2005). This biofilm presents a complex and dynamic ecosystem, whose growth is dictated by microenvironmental factors, such as changes in the physical or chemical properties of the region or changes in the host (Socransky and Haffajee 2005). Many studies have attempted to clarify the relationship between a specific bacterium and/or a group of microorganisms and the pathogenesis of periodontitis(Mombelli *et al.* 1994; Slots and Ting 1999; Tan *et al.* 2001; van Winkelhoff *et al.* 2002; Nibali *et al.* 2007, 2010; Teixeira *et al.* 2009; Finoti *et al.* 2013b,c).

Chronic periodontitis (CP) is dominated by the presence of Gram-negative anaerobic and capnophilic bacteria, especially by *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans*.(Mombelli, Gmur, Gobbi 1994; Socransky *et al.* 1998; Albandar 2002; van Winkelhoff, Loos, van der Reijden 2002; Kumar *et al.* 2003). Although *A. actinomycetemcomitans* has been mainly associated with aggressive periodontitis (AgP), it has also been frequently detected in sites and/or subjects with periodontal health and CP (Slots 1999; Colombo *et al.* 2002; Rosalem Junior, de Andrade and Colombo 2006). Likewise, the prevalence of *A. actinomycetemcomitans* in Brazilians was reported in 70% of healthy subjects and 90% of patients with CP, while *P. gingivalis* was found in 66% of healthy subjects and 78% of CP patients (Avila-Campos and Velasquez-Melendez 2002). High percentage of *A. actinotnycetemcomitans* was also found in Chinese, African and Hispanic populations with CP (Dahlen *et al.* 1989; Ali *et al.* 1997; Tan, Woo, Ong 2001).

Aggregatibacter actinomycetemcomitans is able to survive and colonize periodontal pockets, probably because of its various virulence factors that include proteolytic activity, ability to modulate the host immune response, evasion of phagocytosis, ability to escape and damage the immune system and produce periodontal destruction (Ardila, Alzate and Guzman 2012). In a biofilm environment, these functions may not only be favoring *A. actinomycetemcomitans* itself, but might also support all other species in the biofilm in escaping the host immune system. Besides, this bacterium affects other species commonly found in subgingival biofilms, including *P. gingivalis* (Periasamy and Kolenbrander 2009b) and *Fusobacterium nucleatum* (Periasamy and Kolenbrander 2009a). Slots and Ting (1999) described a synergistic association of *A. actinomycetemcomitans* and *P. gingivalis* in patients with evolutive lesions. In addition, *A. actinomycetemcomitans*, *P. gingivalis*, *Pr. intermedia* and *Tannerella forsythia* were the markers of a destructive periodontal lesion (van Winkelhoff, Loos, van der Reijden 2002; Curtis, Slaney and Aduse-Opoku 2005). However, as suggested by Hajishengallis *et al.* (Hajishengallis, Darveau and Curtis 2012; Lamont and Hajishengallis 2015), *A. actinomycetemcomitans* could be a keystone pathogen for periodontal infection which might not actually be the dominant species within the biofilm.

In general, microbiological studies have shown that, even in different populations, the same periodontal species are found in sites or individuals with periodontitis. However, the prevalence and proportion of these species in the subgingival microbiota may vary (Papapanou *et al.* 2002; Haffajee *et al.* 2004). Although the microbiota is essential for the initiation of PD, differences in the species and amount of bacteria have not been sufficient to explain the differences in disease severity that are routinely observed in adults (Kornman, Page and Tonetti 1997). The imbalance between colonizing microorganisms and the immunological potential of the host can define the state of the PD (Nibali *et al.* 2016). In addition, the host's immunological response may be influenced by several risk factors (Kurdowska, Noble and Adcock 2003), such as smoking (Haber 1994), diabetes (Mealey 2000) considered a multifactorial disease, in which genetic factors were estimated to have ~50% heritability, even after following adjustments for behavioral variables including smoking (Michalowicz *et al.* 2000).

The genetic background may influence many functions, which in turn might affect inflammation and PD expression (Shapira, Wilensky and Kinane 2005; Repeke *et al.* 2009). Genetic variants, mainly polymorphisms, which contribute to complex diseases are prevalent in any population and are often reported to differ between diseased and healthy individuals (Kinane and Hart 2003; Shapira, Wilensky and Kinane 2005). Amongst the plethora of immunological factors involved in the pathogenesis of CP (Gera 2004; Loos, John and Laine 2005; Yoshie *et al.* 2007), previous studies of our group have associated polymorphisms in the *IL8* and *IL4* genes with CP. Polymorphisms –251(T>A)(rs4073), + 396(T>G)(rs2227307) and + 781(C>T)(rs2227306) in the *IL8* gene formed the haplotype ATC/TTC, which conferred 2-fold greater susceptibility to CP than the other haplotypes (odds $\text{ratio}_{\text{adjusted}}$ = 2.24, 95% confidence interval [CI] = 1.10–4.55) (Scarel-Caminaga *et al.* 2011). Therefore, we named here this ATC/TTC haplotype as (*IL8*+) because of its association with susceptibility to CP. Regarding the *IL4* gene, individuals carrying the haplotype TCI/CCI formed by the polymorphisms –590(T>C)(rs2243250), + 33 (T>C)(rs2070874) and VNTR (I>D) were five times more susceptible to CP ($\text{OR}_{\text{adjusted}}$ = 5.3; 95% CI = 2.2–12.9), while the haplotype TTD/CTI conferred protection against the CP ($\text{OR}_{\text{adjusted}}$ = 0.18; 95% CI = 0.04–0.88) (Anovazzi *et al.* 2013). All results remained significant after adjusting for variables such as age, gender, ethnicity and smoking. Following, we verified that patients carrying the TCI/CCI haplotype in the *IL4* gene (named as *IL4*+, because of its association with susceptibility to CP) demonstrated increased levels of periodontopathogenic bacteria (Finoti *et al.* 2013a). Regarding to the *IL8* haplotype, we found that periodontal destruction occurred even in a lower microbial challenge in patients carrying the *IL8* haplotype associated with the non-susceptibility to CP (Finoti *et al.* 2013b). However, when we investigated whether the genetic background could influence the outcome of the non-surgical periodontal treatment, we did not find any relationship between the treatment results and the *IL4* haplotype (Finoti *et al.* 2013a) or the *IL8* haplotype (Finoti *et al.* 2013c).

Notably, these previous studies showed the absolute quantification of *P. gingivalis*, *T. denticola* and *Ta. forsythia*, but the *A. actinomycetemcomitans* was not investigated. Therefore, considering (i) the indubitable importance of *A. actinomycetemcomitans* in the panel of CP; (ii) the growing knowledge of the relationship among bacteria in the periodontium biofilm; (iii) and the necessity to better understand the interrelationship of different factors that influence the CP, we hypothesize that quantifying the *A. actinomycetemcomitans* in patients with known genetic carriage of *IL4* or *IL8* haplotypes, together with the levels of cytokines, *P. gingivalis*, *T. denticola* and *Ta. forsythia* could be important to better understand the multifactorial characteristic of CP. In spite of investigations are growing in this aspect, more

studies are necessary, mainly assessing different CP influence factors in the same patients. Therefore, the aim of this study was to evaluate the potential association between the genetic susceptibility to CP given by the mentioned *IL8* and *IL4* haplotypes and the levels of *A. actinomycetemcomitans* in patients' subgingival sites with and without CP, before and after the non-surgical periodontal treatment. Multifaceted evaluations were made by multiple logistic regressions and correlation analyses to search relationships among patients' genetic background with the previous clinical, immunological (IL-8 or IL-4 protein levels) and microbiological (*P. gingivalis, Ta. forsythia, T. denticola*) findings.

MATERIALS AND METHODS

Timeline of methodological procedures and analyses

Patients enrolled in this study were selected among participants who were genetically pre-screened by PCR-RFLP (polymerase chain reaction–restriction fragment length polymorphism) from two previous studies that identified patients considered genetically susceptible or not to CP. The first study investigated in 250 individuals the haplotypes in the *IL4* gene (Anovazzi *et al.* 2010), while the second study investigated in 493 individuals the haplotypes in the *IL8* gene (Scarel-Caminaga *et al.* 2011). Buccal epithelial cells from each subject were obtained by a mouthwash of 3 ml of 3% glucose for 2 min. DNA was extracted with sequential phenol/chloroform/isoamyl alcohol (25:24:1) solution and precipitated with salt ethanol solution(Ausubel 1999). Primers sequences and conditions for the PCR and RFLP reactions are described in the study by Scarel-Caminaga *et al.* (2011) in regard to the study of the *IL8* gene, and in the study by Anovazzi *et al.* (2010) for the *IL4* gene.

Further studies, enrolling part of the same patients considered susceptible (*IL8+/IL4+*) to CP or patients non-susceptible (*IL8–*) or protected (*IL4–*) against CP, investigated whether these genetic carriage were associated with periodontal clinical data before and after the non-surgical periodontal therapy and the immunological levels of IL-8 (Corbi *et al.* 2012, 2014) or IL-4 proteins (Anovazzi *et al.* 2013).

Moreover, 1 week after gingival crevicular fluid (GCF) collection, patients were asked to return for biofilm collection from the same subgingival sites. This was performed before and after the non-surgical periodontal therapy. Therefore, we also investigated the levels of *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* present in the same subgingival sites of the patients considered susceptible (*IL8+/IL4+*) to CP, or patients non-susceptible (*IL8–*) or protected (*IL4–*) against CP, before and after the non-surgical periodontal therapy (Finoti *et al.* 2013a, b,c). In the development of these studies, we utilized the same clinical procedures made for the studies that investigated the immunological IL-8 and IL-4 levels.

Clinical measurements

Clinical measurement was performed separated according to patients who participated in the *IL8* or *IL4* haplotype studies. For each of them, a single calibrated examiner blinded to the genetic carriage of the patient examined the patients. The examiner SCTC was responsible for the periodontal clinical examination of patients enrolled in the *IL8* haplotype study (weighted Kappa = 0.80; considering probing pocket depth [PPD] as a clinical outcome), while GA was responsible for the periodontal examination of patients enrolled in the *IL4* haplotype study (weighted Kappa = 0.82; considering PPD as a clinical outcome). The examiner recorded the following clinical parameters: plaque and gingival bleeding index, bleeding on probing (BOP), PPD and clinical attachment loss (CAL). The teeth were examined at six sites: mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual. The cemento-enamel junction was used as a reference point in the measurement of CAL. The PPD and CAL measurements were performed using a manual probe (Trinity-Campo Mourão, Brazil). The patients were considered to have CP when two or more sites in non-adjacent teeth exhibited PPD $\geq$ 5 mm, CAL $\geq$ 3 mm and BOP (Armitage 1999). Subjects without CP exhibited low clinical signs of BOP and had PPD and CAL $\leq$ 3 mm. Patient clinical data were recorded at baseline and 45 days after the completion of the non-surgical periodontal therapy.

Periodontal treatment

Patients with CP carrying each *IL8* or *IL4* haplotypes received non-surgical periodontal treatment, composed of oral hygiene instruction and scaling and root planing under local anesthesia. The treatment required, on average, four sessions using manual instruments (Hu-Friedy® Manufacturing Inc., Chicago, IL, USA) and ultrasonic instrumentation (Cavitron® Ultrasonics Inc., Long Island City, NY, USA). The periodontal treatment was performed by one periodontist (MHT for patients carrying *IL8* haplotypes, and GA for patients carrying *IL4* haplotypes). After the periodontal treatment, a professional plaque control program was performed twice a month for 45 days, consisting of supragingival plaque removal and reinstruction of oral hygiene procedures.

GCF sampling and immunological evaluations

In the patients with CP, GCF samples were collected from two proximal sites of non-adjacent teeth with PPD $\geq$ 5 mm, CAL $\geq$ 3 mm and BOP. In the periodontally healthy subjects, GCF samples were collected from two proximal sites of non-adjacent teeth with PPD $\leq$ 2 mm and no BOP. Before GCF sampling, supragingival plaque was removed from the interproximal surfaces with a sterile curette; these surfaces were gently dried with an air syringe and were isolated using cotton rolls. The GCF samples were collected using absorbent paper strips (Periopaper, ProFlow Inc., Amityville, NY, USA), which were placed into the sulcus/pocket until mild resistance was felt and were held in place for. Strips contaminated by saliva or blood were excluded. The volume of GCF was determined by means of a previously calibrated electronic device (Periotron 8000, ProFlow) and was converted into an actual volume (μl) by reference to a standard curve. All strips with GCF were immediately and individually placed into a sterile polypropylene tube (Giannopoulou, Cappuyns and Mombelli 2003; Kurdowska, Noble and Adcock 2003) and kept at –80°C until further analysis (Finoti *et al.* 2013a,b). Concentrations of IL-8 protein were assessed by a sandwich ELISA kit (R&D Systems Inc. Minneapolis, MN, USA) (Corbi *et al.* 2012, 2014), while the IL-4 concentrations were assayed by a highly sensitive ELISA kit (R&D Systems Inc) (Anovazzi *et al.* 2013). All of the assay procedures were conducted according to the manufacturer's instruction, and all determinations were carried out in duplicate. Immunological data were assessed at baseline and 45 days after the completion of the non-surgical periodontal therapy.

Periodontal clinical characteristics of sites selected to subgingival biofilm sampling and microbiological analyses

In the patients with CP, subgingival biofilm samples were collected from two proximal sites of non-adjacent teeth with PPD $\geq$ 5 mm, CAL $\geq$ 3 mm and BOP (diseased sites [DS]), and two

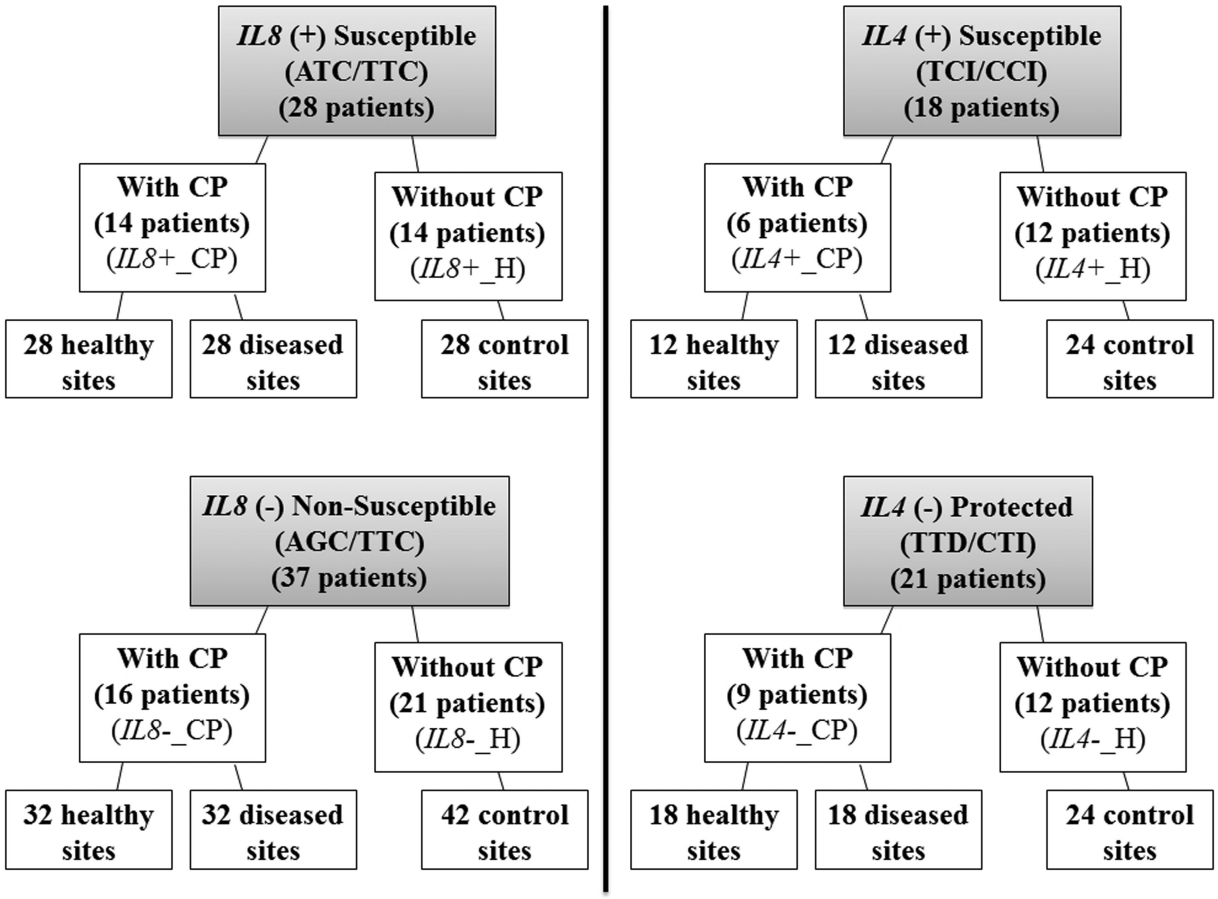

Figure 1. Flowchart showing the subgingivel samples collected from each group divided according to the genetic and periodontal statuses.

proximal sites of non-adjacent teeth with PPD ≤ 2 mm and no BOP (healthy sites [HS]). In the periodontally healthy patients, subgingival biofilm samples were collected from two proximal sites of non-adjacent teeth with PPD ≤ 2 mm and no BOP (control sites [CS]). After the removal of supragingival plaque, subgingival biofilm samples were gathered by inserting a sterile endodontic paper point for 30 s, placing the paper points in separate microtubes containing 100 μl of TE buffer (10 mM Tris-HCl, 0,1 mM EDTA, pH 7.6), which were stored at –80°C until use. Genomic DNA from these samples was extracted and purified using a phenol-chloroform protocol (Ausubel 1999), and absolute quantification of *P. gingivalis*, *Ta. forsythia* and *T. denticola* were assayed by quantitative real-time PCR (Finoti *et al.* 2013a,b,c). Microbiological levels were quantified at baseline and 45 days after the completion of the non-surgical periodontal therapy.

Procedures performed in this study

Study population

In this study, 104 patients (37 males and 67 females) were selected among genetically pre-screened participants from two previous studies focusing on haplotypes in the *IL4* gene (Anovazzi *et al.* 2010) and in the *IL8* haplotypes (Scarel-Caminaga *et al.* 2011). All of the 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 for Ethical Affairs of the UNESP- São Paulo State University (protocol number 52/08). The inclusion and exclusion criteria are described in the previous studies (Finoti *et al.* 2013a,b). To assess clinical differences between the genetically prescreened groups, sample size calculation was performed using a two-sided t-test, considering α error of 5%, with 1 mm of clinical significant difference in PPD between groups and a standard deviation (SD) of 0.5 mm (Lamster, Hartley and Vogel 1985; Lopes *et al.* 2010). With a total sample size of 39 subjects (for *IL4*) and 65 subjects (for *IL8*), the power of the study was calculated as 95%. Therefore, the number of subjects enrolled in this study was sufficiently large to detect clinical differences between the genetically prescreened groups with an acceptable level of confidence (Finoti *et al.* 2013a,b).

Subgingival sites selected for microbiological analysis

Figure 1 shows the flowchart of the biofilm sampling collected according its division into patients' groups. Subgingival biofilm was collected from the same sites selected for the previous quantification of *P. gingivalis*, *Ta. forsythia* and *T. denticola*. According to each group, the number of selected sites was as follows: IL8+_CP = 56 sites [28 DS, 28HS]; IL8+_H = 28 sites [28CS] IL8–_CP = 64 sites [32DS, 32HS]), IL8–_H = 42 sites [42CS]; IL4+_CP = 24 sites [12 DS, 12HS]; IL4+_H = 24 sites [24CS], IL4–_CP = 36 sites [18DS, 18HS]), IL4–_H = 24 sites [24CS]. Biofilm samples from 298 subgingival sites were collected at baseline and after 45 days of completion of the non-surgical treatment. Therefore, a total of 596 samples were evaluated.

***Aggregatibacter actinomycetemcomitans* absolute quantification by q-PCR**

DNA from the mentioned subgingival biofilm samples was used for absolute quantification of *A. actinomycetemcomitans* by q-PCR. Each reaction was set up in 96-well plates in a total volume of 22 μl, containing 11 μl of SYBR Green mix (Applied Biosystems), 1 μl of DNA sample and 0.69 ng/μl of specie-specific primer pair for *A. actinomycetemcomitans* (Ando-Suguimoto *et al.* 2014). To establish the quantitative assay, plasmids containing the target 16S rRNA gene of *A. actinomycetemcomitans* (JP2) were used as standard. This standard was developed obtaining PCR amplicon for 16S rRNA gene of *A. actinomycetemcomitans* that was individually cloned in PCR 2.1 TOPO TA (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations, and transformed in *Escherichia coli* DH5-a. After the growth of transformants, plasmids were extracted using the PureLink Quick Plasmid Miniprep kit (Invitrogen) (Teixeira, Mattarazo, Feres 2009). The standard curve with six dilutions (from 10^6 to 10^1 copies) was run in triplicate, and the subgingival samples were run in duplicate. 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 65°C–95°C to confirm that the PCR products from samples and reference plasmids had identical melting points. The mean values of target molecule numbers were used for the analysis using the SDS 7500 Applied Biosystems software. The levels of *A. actinomycetemcomitans* were expressed as the number of copies of the 16S rRNA gene.

Statistical analysis

The unit for the analysis was either the subject or the site. The subject was used for the demographic analysis, and the subgingival site was used for the microbiological and clinical analyses. The Shapiro–Wilk test was used to assess the distribution normality of quantitative data. Because the mean age was distributed normally, one-way analysis of variance was used to assess differences between groups. The $\chi 2$ test was used to determine whether the groups were composed of the same proportion of males and females. Both age and gender were patient-based analyses. Because of the absence of a normal distribution, the Mann–Whitney U-test was used to compare the microbiological findings (site-based analyses) in the groups composed of individuals who were genetically susceptible and non-susceptible to/protected against CP with the same clinical condition (e.g.: *IL8*+_CP vs *IL8*–_CP/*IL4*+_CP vs *IL4*–_CP; and *IL8*+_H vs *IL8*–_H/*IL4*+_H vs *IL4*–_H). Their respective sites (DS, HS and CS) were compared by the use of the Kruskal–Wallis test, followed by Dunn's post test. Differences between groups with and without CP with the same genetic condition (*IL8*+_CP vs *IL8*+_H/*IL4*+_CP vs *IL4*+_H; and *IL8*–_CP vs *IL8*–_H/*IL4*–_CP vs *IL4*–_H) were also assessed. Baseline versus after treatment comparisons for evaluations of CP patients between the *IL8*+_vs *IL8*–_groups or *IL4*+_vs *IL4*- groups were made using the Mann-Whitney U-test. The Wilcoxon test was made to determine clinical difference between data of the baseline and after treatment of each haplotype group alone or the intragroup analysis.

Spearman's correlation analyses were used to investigate the associations among the present data with previous clinical findings, IL-4 and IL-8 protein levels in the GCF and levels of *P. gingivalis*, *T. denticola* and *Ta. forsythia* (Finoti *et al.* 2013a,b) .Multilevel logistic regressions were undertaken by using the R statistical package to investigate the association between the *IL8* and *IL4* haplotypes and bacterial load and clinical parameters (at baseline and after periodontal therapy). All of the other data analyses were performed using the statistical software package Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA). Differences were considered significant when $P < 0.05$.

RESULTS

Patients

The clinical data of the studied population were summarized in the Table 1. No statistical significant differences ($P > 0.05$) were observed among groups carrying the *IL8* haplotypes with regard to age (analysis of variance; $P = 0.2587$) or gender ($\chi 2$; $P = 0.26$) (Finoti *et al.* 2013b). Considering groups carrying the *IL4* haplotypes, there were no differences regarding gender ($\chi 2$; $P = 0.99$), and with regard to age the analysis of variance demonstrated significant difference among the groups ($P = 0.0251$), in which the difference was only between the groups *IL4*+_CP and *IL4*+_H (Tukey's test, $P < 0.05$). In baseline, all of the clinical parameters of the CP patients or healthy subjects (H) were similar, independently of the carriage of the *IL8* or the *IL4* haplotype (Table 1, Mann–Whitney U-test, $P > 0.05$).

Baseline clinical data of CP patients were compared between the *IL8*+_vs *IL8*–_groups or *IL4*+_vs *IL4*– groups using the Mann-Whitney U-test. The same was made for clinical data 45 days after the periodontal treatment. In other words, after the non-surgical periodontal treatment, periodontal clinical parameters were compared between patients carrying opposite haplotypes from each gene: *IL8*+_CP group was compared to *IL8*–_CP, as well the *IL4*+_CP was compared to *IL4*–_CP. This analysis showed no statistical significant results, demonstrating that the outcome to the periodontal treatment was not influenced by the genetic carriage.

Otherwise, to determine clinical difference between data of the baseline and after the periodontal treatment of each haplotype group alone, i.e. the intragroup analysis (e.g.: *IL8*+_CP before compared to after treatment), we used the Wilcoxon test. We observed for all the PPD, CAL and BOP that the periodontal treatment was intragroup effective, i.e. considering each haplotype group (before and after treatment), the periodontal treatment was effective for all the haplotype groups.

Microbiological analysis

Table 1 shows the *Aggregatibacter actinomycetemcomitans* levels in each type of periodontal site from individuals carrying the opposite haplotypes of *IL8* or *IL4*. After the non-parametric statistical analysis, it was found that for the *IL8* haplotypes, before and after the non-surgical periodontal treatment there were no differences in the concentration of *A. actinomycetemcomitans* in the analyzed sites between individuals carrying the different haplotypes (ATC/TTC) *IL8*+ and (AGT/TTC) *IL8*–. Table 1 shows comparisons of only CP patients carrying the opposite haplotypes of each gene, considering the periods before and after periodontal treatment. A decrease in the *A. actinomycetemcomitans* levels after the periodontal treatment regardless of *IL8* haplotypes ($P \leq 0.0001$) was observed, demonstrating the efficiency of the non-surgical periodontal therapy independently of the genetic carriage. The levels of *A. actinomycetemcomitans* at baseline were significantly lower in the control sites in patients carrying the *IL8*– haplotype ($P = 0.03$), when compared with the diseased and healthy sites.

Referring to the haplotypes in the *IL4* gene, considering the healthy and control sites at baseline, there was a significantly higher concentration of *A. actinomycetemcomitans* in patients

carrying the haplotype of susceptibility to CP (TCI/CCI) *IL4*+ than in those carrying the protected (TTD/CTI) *IL4*– haplotype (healthy sites, $P = 0.01$; control sites, $P = 0.02$). The levels of *A. actinomycetemcomitans* at baseline were significantly lower in the healthy and control sites in patients carrying the *IL4*– haplotype ($P = 0.04$), when compared with the diseased sites (Table 1). Indeed, considering the diseased sites of patients carrying the *IL4* haplotype, comparing the *A. actinomycetemcomitans* levels of diseased sites before and after the periodontal treatment, it was observed that for both haplotypes TCI/CCI (*IL4*+, susceptible) and TTD/CTI (*IL4*–, protected) there was a decrease in the levels of this periodontopathogen after periodontal treatment, but with a significant difference only associated to the TTD/CTI *IL4*– (protected). In other words, after the non-surgical periodontal treatment a significantly higher amount of *A. actinomycetemcomitans* was found in *IL4*– (protected) patients in comparison with the *IL4*+ (susceptible) individuals with CP ($P = 0.0002$; Mann-Whitney test, Table 1).

Table 1. Demographic characteristics of patients, clinical parameters of studied groups and 16S rRNA gene copies of *A. actinomycetemcomitans* [median (min—max)] in samples obtained from diseased, healthy and control sites.

Demographic characteristics and clinical parameters				
	Baseline groups (*IL8* haplotype)			
Characteristics (mean values ± SD)	*IL8*+_CP $n = 14$	*IL8*+_H $n = 14$	*IL8*–_CP $n = 16$	*IL8*–_H $n = 21$
Age (years) (±)	43.78 (±10.39)[a]	41.93 (±9.08)[a]	47.94 (±7.78)[a]	43.01 (±8.41)[a]
Gender (M/F)	6/8[a]	7/7[a]	3/13[a]	6/15[a]
Probing depth (mm) (±)	5.04 (±0.19)[a]	1.72 (±0.16)[b]	5.34 (±0.60)[a]	1.80 (±0.26)[b]
Attachment level (mm) (±)	5.29 (±0.76)[a]	1.33 (±0.61)[b]	5.75 (±1.11)[a]	1.83 (±0.54)[b]
% of sites with plaque	67.15 (±16.56)[a]	33.72 (±21.40)[b]	59.93 (±24.71)[a]	43.67 (±27.39)[b]
Gingival bleeding	27.02 (±15.42)[a]	14.36 (±10.71)[b]	25.58 (±13.48)[a]	19.49 (±13.22)[b]
Bleeding on probing (Y/N)	28/0[a]	4/24[b]	32/0[a]	6/36[b]
	Baseline groups (*IL4* haplotype)			
Characteristics (mean values ± SD)	*IL4*+_CP $n = 6$	*IL4*+_H, $n = 12$	*IL4*–_CP $n = 9$	*IL4*–_H $n = 12$
Age (years) (±)	53.33 (±7.37)[a]	38.91 (±5.79)[b]	48.33 (±14.24)[a]	40.75 (±10.96)[a]
Gender (M/F)	2/4[a]	6/6[a]	2/7[a]	5/7[a]
Probing depth (mm) (±)	4.92 (±0.90)[a]	1.52 (±0.23)[b]	4.78 (±0.73)[a]	1.57 (±0.31)[b]
Attachment level (mm) (±)	5.75 (±0.91)[a]	1.55 (±0.20)[b]	4.72 (±0.57)[a]	1.77 (±0.32)[b]
% of sites with plaque	73.73 (±21.48)[a]	18.62 (±8.29)[b]	60.78 (±25.28)[a]	16.65 (±10.00)[b]
Gingival bleeding	38.31 (±20.12)[a]	7.55 (±3.06)[b]	26.69 (±13.11)[a]	6.00 (±3.14)[b]
Bleeding on probing (N/Y)	3/9[a]	1/21[b]	4/14[a]	1/23[b]
	After treatment Groups (*IL8* haplotype)		After treatment groups (*IL4* haplotype)	
Characteristics (patients with CP) (diseased sites) (mean values ± SD)	*IL8*+_CP ($n = 14$) (28DS)	*IL8*–_CP ($n = 16$) (32DS)	*IL4*+_CP ($n = 6$) (12DS)	*IL4*–_CP ($n = 9$) (18DS)
Probing depth (mm) (±)	3.68 (±1.09)	3.84 (±1.14)	3.00 (±0.60)	2.78 (±0.81)
Attachment level (mm) (±)	3.96 (±1.04)	4.19 (±1.47)	3.89 (±1.59)	2.56 (±0.62)
% of sites with plaque	22.18 (±12.53)	16.02 (±10.04)	21.19 (±9.24)	20.00 (±9.49)
Gingival bleeding	13.23 (±12.15)	7.52 (±4.87)	12.29 (±6.23)	11.61 (±9.69)
Bleeding on probing (Y/N)	8/20	10/22	8/4	5/13
	Comparison baseline vs after treatment			
	Groups (*IL8* haplotype)		groups (*IL4* haplotype)	
Clinical parameters (patients with CP) (diseased sites) (mean values ± SD)	*IL8*+_CP ($n = 14$) (28DS)	*IL8*–_CP ($n = 16$) (32DS)	*IL4*+_CP ($n = 6$) (12DS)	*IL4*–_CP ($n = 9$) (18DS)
PPD (mm)				
Baseline	5.04 (±0.19)	5.34 (±0.60)	4.92 (±0.90)	4.78 (±0.73)
After treatment	3.68 (±1.09)*	3.84 (±1.14)*	3.00 (±0.60)*	2.78 (±0.81)*
CAL (mm)				
Baseline	5.29 (±0.76)	5.75 (±1.11)	5.75 (±1.91)	4.72 (±.0.57)
After treatment	3.96 (±1.04)*	4.19 (±1.47)*	3.89 (±1.59)*	2.56 (±0.62)*
BOP (Y/N)				
Baseline	28/0	32/0	12/0	18/0
After treatment	8/20*	10/22*	8/4*	5/13*

Table 1. (*Continued*.)

	16S rRNA gene copies of *A. actinomycetemcomitans*			
	Groups (*IL8* haplotype)		groups (*IL4* haplotype)	
Aggregatibacter actinomycetemcomitans [median (min—max)]	*IL8+_CP*	*IL8–_CP*	*IL4+_CP*	*IL4–_CP*
Baseline				
Diseased sites	367.2 (8.7–3221654.2)^{Aa}	102.2 (17.1 -887626,9)^{Aa}	15,442.68 (16.3–667948.4)^{Aa}	208.7 (25.0–373.673,8)^{Aa}
Healthy sites	609.0 (10.8 -158251.8)^{Aa}	59.3 (17.1–14771.2)^{Ab}	57.9 (15.3–70478.2)^{Aa}	17.1 (3.9–1425.1)^{Bb}
Control sites	52541.3 (1.8–167272.4)^{Aa}	10.9 (4.8–381201.5)^{Ab}	125.6 (7.8–184967.5)^{Aa}	42.1 (19.7–42420.3)^{Bb}
After treatment				
Diseased sites	11.2 (3.6–7707.9)$^{A\#}$	0.5 (0.0–227.6)$^{A\#}$	25.3 (0.0–179.6)$^{A\#}$	170.1 (93.7–430.9)^{B}

Patients genetically susceptible (+) or non-susceptible/protected (–) regarding each *IL8* or *IL4* gene;
IL8+_CP = ATC/TTC; *IL8-_CP* = AGT/TTC; *IL4+_CP* = TCI/CCI; *IL4-_CP* = TTD/CTI
CP = patients with chronic periodontitis; H = patients without chronic periodontitis (healthy).
HS = healthy sites; DS = diseased sites
Age evaluated by analysis of variance (statistical difference between groups; Tukey's test, $p < 0.05$).
Gender evaluated by the χ2test (no statistical difference among groups).
Different letters denote significant differences ($P < 0.05$) when comparing the diseased groups with the healthy groups (CP vs H) for each genetically susceptible (+) or non-susceptible/protected (–) groups, *P*-value < 0.0001(Mann–Whitney U-test).
PPD = probing pocket depth; CAL clinical attachment loss; BOP bleeding on probing (Y = yes/N = no)
Comparison baseline versus after treatment: Mann-Whitney test for evaluations of CP patients between the *IL8+_*vs *IL8–_*groups, or *IL4+_*vs *IL4–* groups. *significant difference compared to the baseline period by the Wilcoxon test to determine difference between data of the baseline and after treatment (of each haplotype group alone, intragroup analysis).
For 16S rRNA gene copies of *A. actinomycetemcomitans* [median (min—max)] in samples obtained from diseased, healthy and control sites. Capital letters = Mann–Whitney U-test for evaluation between susceptible and non-susceptible (or protected) groups for each type of site (e.g. *IL8+_*CP vs *IL8–_*CP; *IL4+_*CP vs *IL4–_*CP). Small letters = Kruskal–Wallis test (followed by Dunn's post test) for the evaluation of different sites within the same haplotype group (e.g. diseased sites vs healthy sites vs control sites). # = significant difference ($p < 0.05$) of diseased sites between baseline and after periodontal treatment within the same haplotype group (e.g. *IL8+_*CP, each haplotype group alone, intragroup analysis) by the Mann–Whitney U-test.

Multiple logistic regressions and correlation analyses between clinical, immunological and microbiological data

Multifaceted evaluations made by multiple logistic regressions and correlation analyses produced massive results that are presented in the supplementary material. Multiple logistic regressions considering demographic, clinical, immunological and microbiological data of CP patients carrying each *IL8+* or *IL4+* haplotypes before or after the periodontal treatment demonstrated that there were no association of genetic carriage of *IL8* nor *IL4* haplotypes with any of potential CP covariant investigated here (Tables 2 and 3).

Results of correlation analyses are presented in Tables S1–S8, and here we highlighted some of the most relevant findings. In general, for both *IL4* haplotype groups there were significant correlations among *A. actinomycetemcomitans*, other periodontopathogens levels, clinical and immunological data (before and after the periodontal treatment) (Tables S1–S4). Correlation analyses of the *IL4* haplotypes showed that for CP groups, considering the *IL4-* haplotype (protected), as the PPD before and after periodontal treatment increases, the anti-inflammatory IL-4 level also increases (Table S3, $\rho = 0.9$ and 0.8). In contrast, for the *IL4+* patients, as the PPD before periodontal treatment increases, the IL-4 level decreases (Table S4, $\rho = -1.0$). Considering control groups, we observed that in individuals carrying the *IL4–* haplotype (protected), after the prophylactic treatment, as the levels of bacteria from red complex increases, the IL-4 level increases (Table S1; $\rho = 1.0$), demonstrating an active immune response. This was not observed for subgingival sites of individuals with the *IL4+* haplotype (susceptible). This indicates that patients considered 'genetic protected' against development of CP (*IL4–* haplotype) seems to be able to produce the necessary immune response according to the microbiological challenge.

Table 2. Multiple logistic regression considering demographic, clinical, immunological and microbiological data of patients with chronic periodontitis carrying the haplotypes of *IL8* gene before and after the periodontal treatment.

Variables	OR	95%IC	*p*-value
IL8 gene before treatment			
Gender	10.42	0.11–964.23	0.19
Age	0.99	0.8–1.23	
Bleeding on probing	1.09	0.85–1.40	0.91
Probing pocket depth	0.53	0–435.07	0.4
Clinical attachment loss	1.15	0–306.53	0.97
Volume CGF (μl)	0.1	0–2.75	0.08
Concentration IL -8 (ng/μl)	0.99	0.88–1.12	0.83
Aggregatibacter actinomycetemcomitans	1	1–1	0.85
Porphyromonas gingivalis	1	1–1	0.78
Treponema denticola	1	1–1	0.56
IL8 gene after treatment			
Tannerella forsythia	1	1–1	0.73
Gender	2.46	0.17–35.9	0.42
Age	1.03	0.84–1.27	0.69
Bleeding on probing	1.12	0.87–1.45	0.26
Probing pocket depth	20.06	0–632.32	0.56
Clinical attachment loss	0.02	0–642.83	0.36
Volume CGF (μl)	1.53	0.07–35.55	0.74
Concentration IL -8 (ng/μl)	1.01	0.92–1.1	0.79
Aggregatibacter actinomycetemcomitans	0.99	0.97–1.01	0.32
Porphyromonas gingivalis	1	1–1	0.90
Treponema denticola	1	1–1	0.36
Tannerella forsythia	1	1–1	0.44

CGF volume = volume of gingival fluid (μl). *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* = quantification of the levels of each periodontopathogen

Table 3. Multiple logistic regression considering demographic, clinical, immunological and microbiological data of patients with chronic periodontitis carrying the haplotypes of *IL4* gene before and after the periodontal treatment.

Variables	OR	95%IC	*p*-value
IL4 gene before treatment			
Gender	0.12	0–246.8	0.62
Age	1.04	0.87–1.25	0.49
Bleeding on probing	2.28	0–3.37	0.17
Probing pocket depth	3.74	0–64.6	0.75
Clinical attachment loss	1.49	0–24.78	0.88
Volume CGF (μl)	5.14	0.02–130.30	0.40
Concentration IL -4 (ng/μl)	0	0–52.42	0.37
Aggregatibacter actinomycetemcomitans	1	1–1	0.46
Porphyromonas gingivalis	1.03	0.93–1.2	0.81
Treponema denticola	1.01	0.81–1.2	0.72
Tannerella forsythia	1	1–1.1	0.81
IL4 gene after treatment			
Gender	0.04	0–261.83	0.74
Age	1.09	0.56–2.13	0.72
Bleeding on probing	0	0–10.61	0.53
Probing pocket depth	0	0–1.13	0.56
Clinical attachment loss	1.06	0–1.10	0.57
Volume CGF (μl)	0	0–1.11	0.59
Concentration IL -4 (ng/μl)	1.01	0.93–1.12	0.78
Aggregatibacter actinomycetemcomitans	0.98	0.87–1.1	0.56
Porphyromonas gingivalis	1.0	0.99–1.4	0.88
Treponema denticola	0.96	0.93–1.5	0.82
Tannerella forsythia	1.03	0.91–1.2	0.79

CGF volume = volume of gingival fluid (μl). *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* = quantification of the levels of each periodontopathogen.

In regard to the *IL8* haplotypes, in patients without CP a positive correlation before periodontal treatment between the IL-8 concentration and the GCF volume as well with the *A. actinomycetemcomitans* levels was observed for the *IL8*– patients (Table S5; $\rho = 0.6$ and $\rho = 0.5$). A positive correlation between the *A. actinomycetemcomitans* and *Treponema denticola* levels in control group carrying the *IL8*– haplotype ($\rho = 0.6$) was found, while for the *IL8*+ haplotype patients it was not found (Table S6). Among CP patients, those who carry the *IL8*– haplotype (Table S7) seemed to develop a clearly and better orchestrated immune response, denoted by a positive correlation between the amount of *T. denticola* and clinical periodontal parameters of BOP ($\rho = 0.8$), PPD ($\rho = 0.6$) and CAL ($\rho = 0.6$), while in the *IL8*+ haplotype patients, there was a negative correlation between *T. denticola* and BOP (Table S8; $\rho = -0.6$). Another antagonistic behavior between patients with different genetic background was observed regarding the immune response: in *IL8*– haplotype patients, there was a positive correlation between *A. actinomycetemcomitans* levels and IL-8 cytokine ($\rho = 0.8$) after periodontal treatment, while for the *IL8*+ haplotype patients no significant result was found (Tables S7 and S8).

DISCUSSION

Although there is no study in the literature with different populations that enables comparison of *IL4* and *IL8* haplotypes results with this study, it can be noted increasing research investigating the combined influence of different factors related to the development of the CP (Nibali *et al.* 2007, 2008, 2010; Holla *et al.* 2008; Repeke *et al.* 2009; Teles *et al.* 2010; Corbi *et al.* 2012). Some studies have evaluated the contribution of genetic, microbial and inflammatory factors on periodontitis and found no difference in the frequency or periodontal load of periodontopathogens in patients with different genotypes (Repeke *et al.* 2009; Trombone *et al.* 2009). In contrast, positive results were found in individuals carrying a haplotype called *IL1*-positive gene (Agerbaek, Lang and Persson 2006), or associated with an *IL4RA* (interleukin 4 receptor alpha) polymorphism (Reichert *et al.* 2011), or *TNFA* (–857) and *IL1A* (–889) SNPs with the presence of *Tannerella forsythia, Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans* (Laine *et al.* 2013). In regard to the influence of the host genetic carriage in the non-surgical periodontal treatment outcome, few studies had focused on this approach, in which some of them found positive association in *MMP1* (matrix metalloproteinase 1) (Pirhan *et al.* 2008) and *MMP13* (Pirhan *et al.* 2009), while negative association was found in others *IL1A* and *IL1B* (Huynh-Ba *et al.* 2007), binding lectin to mannose (Ozcaka *et al.* 2010), *IL8* (Finoti *et al.* 2013c; Corbi *et al.* 2014) and *IL4* (Anovazzi *et al.* 2013; Finoti *et al.* 2013a).

We observed in this study that patients who carried the *IL8*+ haplotype of CP susceptibility showed no difference in the *A. actinomycetemcomitans* levels compared to patients with the *IL8*– haplotype of non-susceptibility to CP (Table 1). Moreover, the non-surgical periodontal treatment similarly decreased the *A. actinomycetemcomitans* levels for both *IL8* haplotypes. Thus, the genetic load of the patient did not influence the effectiveness of the non-surgical periodontal treatment. Previous analysis of these *IL8* haplotypes showed no differences between haplotypes for periodontal clinical indices and concentration of IL-8 cytokine, both before (Corbi *et al.* 2012) and after the periodontal treatment (Corbi *et al.* 2014). In contrast, CP patients with *IL8*– haplotype showed significantly higher levels of gingival fluid than patients with *IL8*+ haplotype. By linear regression, it was found that the haplotype in *IL8* gene contributed with 6% of the gingival fluid volume (Corbi *et al.* 2012). Here, in this study, the positive correlation between the levels of GCF and IL-8 cytokine found in patients with *IL8*– haplotype reinforces the relationship between these data.

The multiple logistic regression analysis performed here showed no association of any of the studied demographic, microbiological, immunological and clinical variables with the *IL8* or the *IL4* haplotypes. This indicates that any of these characteristics are associated with the different haplotypes in the *IL8* and *IL4* genes, that there was no bias in the sample investigated here and that the haplotypes are occurring randomly, without any selective pressure. Considering the results of correlation analyses, in general, for both haplotypes in the *IL8* and *IL4* genes significant correlations among *A. actinomycetemcomitans*, other periodontopathogens levels, clinical and immunological data (before and after the periodontal treatment) were observed. Taking together the haplotypes not associated with susceptibility to CP, it can be noted that patients with the *IL4*– haplotype 'CP genetically protected' produced an equilibrated immune response according to the microbiological challenge, and patients with the *IL8*– haplotype (non-susceptible to CP) seemed to develop a clearly and better orchestrated immune response. Positive association between the presence of periodontopathogens and genetic load of the patients was also demonstrated in other studies using multilevel analysis focusing polymorphism in the *IL6* gene (Nibali *et al.* 2007, 2008, 2010).

Referring to the results obtained here for the haplotype in the *IL4* gene, it is worth to emphasize that before the

periodontal treatment there was a significantly higher amount of *A. actinomycetemcomitans* in healthy and control sites of *IL4+* patients (Table 1). In a previous study with the same patients, there was also a higher amount of *P. gingivalis, Ta. forsythia* and *Treponema denticola* in diseased and healthy sites of *IL4+* patients (Finoti *et al.* 2013a). That study followed up the patients after 45 and 90 days of finalized non-surgical periodontal treatment and demonstrated gradual reduction of the microorganisms levels in all sites of patients carrying both investigated haplotypes. This demonstrated that the *IL4* gene haplotypes did not influence the response to non-surgical periodontal treatment as well as observed for the haplotypes in the *IL8* gene. In this study, 45 days after periodontal treatment the levels of *A. actinomycetemcomitans* were significantly higher in patients with *IL4–* haplotype (protected against CP). In compliance with Anovazzi *et al.* (2013), patients with the *IL4–* haplotype were detected with higher levels of *P. gingivalis* in the same sites after 45 days of periodontal treatment, especially after 90 days (but without statistical difference comparing to the other haplotype). Moreover, higher levels of *T. denticola* in some samples of patients carrying this *IL4–* haplotype were detected, although there was no significant difference when compared to patients with *IL4+* haplotype. Interestingly, in GCF of patients carrying the *IL4–* haplotype, there was significant increased concentration of IL-4 cytokine, as shown by another study (Anovazzi *et al.* 2013). It is possible that those patients carrying the *IL4–* haplotype had higher levels of the anti-inflammatory IL-4, contributing to less periodontal destruction by presenting better control of the inflammatory response triggered by subgingival microorganisms, even their amount is significantly higher in comparison to patients with *IL4+* haplotype.

It can be noted that significantly higher levels of *A. actinomycetemcomitans* were found in the *IL4–* (protected) haplotype patients (Finoti *et al.* 2013a) as well *P. gingivalis* and *Ta. forsythia* in patients with *IL8–* haplotype (non-susceptible) (Finoti *et al.* 2013c) after periodontal treatment. Together, these data indicate that patients carrying the *IL4–* or *IL8–* haplotypes (not susceptible to CP) present higher levels of periodontopathogens. Those patients seem to need a higher microbial challenge to develop the CP (Finoti *et al.* 2013b). Interestingly, patients carrying the opposite haplotype *IL8+* or *IL4+* (genetically susceptible to CP) seem to develop CP face to a lower bacterial challenge when compared to patients without genetic susceptibility CP. Furthermore, considering the immunological factor, the smaller amount of bacteria in patients with *IL8+* haplotype is capable of eliciting the same IL-8 cytokine levels found in patients who carry the *IL8–* haplotype (Finoti *et al.* 2013a).

Patients carrying the *IL4–* haplotype, even with higher levels of *A. actinomycetemcomitans*, have increased production of the anti-inflammatory cytokine IL-4 (compared to *IL4+* haplotype patients) favors no development of periodontitis (Anovazzi *et al.* 2013). This agrees with the idea of Shapira, van Dyke and Hart (1992) that the absence of IL-4 might trigger PD. We were recently able to demonstrate *in vitro* that subjects with the *IL4–* haplotype showed higher IL-4 mRNA expression by live *A. actinomycetemcomitans*-stimulated monocytes and lymphocytes, indicating that this haplotype is functional and increases the transcriptional activity of the *IL4* gene in immune cells (Anovazzi *et al.* 2017). Moreover, after stimulation with *A. actinomycetemcomitans,* the cytokine levels of IL-4, IL-10 and IL1-RA in whole blood from *IL4–* haplotype individuals were also significantly higher. This immunomodulatory effect is supported by the increased proportion of Th2 cells in the *IL4–* haplotype after stimulation with *A. actinomycetemcomitans* (Anovazzi *et al.* 2017). This agrees with the idea that these patients are genetically 'protected' against the development of CP.

We concluded that the haplotypes in the *IL4* gene (but not the *IL8* haplotype) influenced the levels of *A. actinomycetemcomitans* in subgingival sites before and after the non-surgical periodontal treatment. Also by the correlation analyses, we concluded that there were significant correlations between genetic, microbiological and immunological factors, with *IL8* and *IL4* haplotypes. This study contributes with the better understanding of the interrelationship of genetic, immunological and microbiological factors of CP, reiterating the multifactorial nature of this disease.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSPD* online.

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