

Research Article

Haplotypes of susceptibility to chronic periodontitis do not influence MMP-8 levels or the outcome of non-surgical periodontal therapy

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Introduction: Chronic periodontitis (CP) is a multifactorial condition, presenting immunoinflammatory reaction, in which a myriad of molecules including cytokines and matrix metalloproteinases (MMPs) interplays, making the system extremely intricate. There is scarce information regarding interconnections of biological influence among IL-4, IL-8 and MMP-8, mainly considering genetic polymorphisms, and also, whether this can influence the outcome of periodontal therapy. Previously, we reported that variants in the interleukin 4 (IL4) and interleukin 8 (IL8) genes were associated with CP in Brazilians. The aim of this study was to investigate, in individuals with different genetic backgrounds with regard to the IL4 or IL8 haplotypes, differences in the immunological levels of MMP-8 in gingival crevicular fluid (GCF) before and after non-surgical periodontal treatment. A total of 141 patients participated of this study, classified as susceptible or not to CP, according to the presence of haplotypes formed by polymorphisms in the IL4 or IL8 genes. All individuals received non-surgical periodontal therapy and follow-up continued for 45 days. The GCF samples were collected at baseline and on the 45th day. The MMP-8 levels were determined by ELISA.

Results: No association was found between genetic backgrounds and MMP-8 levels in GCF or the outcome of non-surgical periodontal therapy.

Conclusions: In this longitudinal clinical study, the presence of IL4 or IL8 haplotypes previously associated with CP did not influence the outcome of non-surgical periodontal therapy and the MMP-8 levels in the GCF. Additional studies are necessary to determine the mechanisms by which the IL4 or IL8 haplotypes affect individual susceptibility to CP.

Keywords: MMP-8; Chronic periodontitis; Disease Susceptibility; Gingival Crevicular Fluid

Introduction

Chronic periodontitis (CP) is a chronic infection-induced inflammatory and multifactorial disease. This disease is initiated by bacterial infection and can progress to the damage and destruction of the supporting tissues of the teeth [1]. The host response is influenced by both environmental (e.g. smoking, oral hygiene) and genetic factors [2]. Several cytokines involved in innate immunity have also been associated with periodontitis, such as interleukin 4 (IL-4) and IL-8.

Interleukin 4 is an anti-inflammatory cytokine secreted by T helper 2 lymphocytes (Th2), with potent down-regulation of macrophage function. Moreover, after stimulation with lipopolysaccharides (LPS) of periodontopathogenic bacteria, IL-4 acts as a mitogen of B cells and enhances the secretion of immunoglobulin G (IgG) and immunoglobulin E (IgE) [3]. Localized absence of this cytokine in diseased periodontal tissues has been associated with periodontal disease activity and progression [4, 5].

IL-8 is a key chemokine in the initiation and amplification of acute inflammatory reactions and in chronic inflammatory processes because it attracts and activates neutrophils in inflammatory regions [6-8]. IL-8 plays an important role in the pathogenesis of PD, with elevated levels in the gingival crevicular fluid from inflamed periodontal sites [9]. Excessive IL-8 in those sites may contribute to local periodontal destruction [10-12].

The injury of the supporting structures of the teeth, such as connective tissue attachment and alveolar bone is mainly developed by matrix metalloproteinases (MMP), which are a family of proteolytic enzymes classically implicated in matrix remodeling. Host cells present in the inflammation area induce the MMPs release [13]. The more abundantly and with major destructive power MMP present in periodontal disease and gingival crevicular fluid (GCF) is matrix metalloproteinase-8 (MMP-8) [14]. The polymorphonuclear neutrophils (PMN) are the mayor cells producing MMP-8, which is stored in secondary granules within neutrophils and is activated by autolytic cleavage [15, 16].

In the pathogenesis of chronic inflammatory periodontal disease, neutrophils are recognized as a major cellular component from the histopathology of the periodontal lesion around teeth. Neutrophils are recruited in vast numbers into the gingival crevice during periodontal inflammation, attracted by microbial plaque chemoattractants and chemokines, such as IL-8, released following microbial

perturbation of gingival epithelial cells [1]. An early event occurring in neutrophil activation is exocytosis of cytoplasmic granules, mainly MMP-8 which accounts for 80% of the total

collagenase protein found in the GCF of patients with CP [2]. Therefore, MMP-8 has been suggested as a good marker for detection of periodontal sites with active disease [17]. The literature presents a variety of studies demonstrating the reduction of MMP-8 levels and activity after non-surgical periodontal treatment, while sites that do not respond to treatment have been associated with elevated MMP-8 concentrations [18, 19]. Van der Steen et al. [20] showed that IL-8 can induce an increase of MMP-8 from neutrophils.

In the immunoinflammatory reaction, a myriad of molecules including cytokines and MMPs interplays, making the system extremely intricate. However, scarce information has been found regarding interconnections of biological influence among IL-4, IL-8 and MMP-8. Studies have shown that IL-4 inhibits the production of the MMP-3 in human conjunctival fibroblasts [21-23]. MMP-3 (stromelysin-1) possesses broad substrate specificity, being capable of degrading proteoglycans, laminin, fibronectin, and collagens. It is also capable of activating the precursor forms of other MMPs, including MMP-8, as well as inactivating plasminogen activator inhibitor I [24]. These studies provide biological mechanisms to support that the inhibition of MMP-3 by IL-4 could indirectly inhibit MMP-8 levels. Moreover, the relationship between MMP-8 (neutrophil collagenase) and IL-8 (neutrophil chemokine main attractive) has been demonstrated in some studies. Levels of IL-1 β , IL-8 and MMP-8 were found higher in GCF of patients with periodontitis compared with healthy individuals [19, 25, 26].

As aforementioned, the interplay between cytokines and MMPs remains scarcely explored, mainly considering genetic polymorphisms. Differences in cytokine levels can be attributed to polymorphisms in their respective genes [27-32]. Our group previously verified that polymorphisms in the IL4 gene (-590[T/C; promoter region, rs2243250], +33[C/T; 5'UTR region, rs2070874] and VNTR [variable number of tandem repeats; insertion/deletion of 70 bp in intron 3]) composed haplotypes associated with CP. Individuals carrying the haplotype (TCI/CCI) were five times more susceptible to CP (ORadjusted = 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 (ORadjusted = 0.29, 95% CI = 0.08-0.88) [33]. Alternatively, this means that individuals who carry the TTD/CTI haplotype were 71% genetically less susceptible to the development of periodontitis. In other study we investigated polymorphisms in the IL8 gene in five hundred individuals with or without CP, and demonstrated that the haplotype ATC/TTC, formed by the -251(T/A, rs4073), +396(T/G, rs2227307) and +781(C/T; rs2227306) polymorphisms in the IL8 gene, conferred two times more susceptibility to CP (ORadjusted=2.24, 95% CI=1.10-4.55) than the other haplotypes which were considered non-susceptible [34].

Recently, we investigated whether those haplotypes influence the IL-4 and IL-8 protein levels in GCF of patients with CP [35-37]. However, considering the potential biological relationship among IL-4, IL-8 and MMP-8, and their importance in the pathology of CP, we hypothesize that the mentioned haplotypes could influence the MMP-8 levels and the outcome to the non-surgical periodontal therapy.

Therefore, the aim of this study was to investigate differences in the MMP-8 levels in the GCF, before and after non-surgical periodontal treatment, in individuals with CP and different genetic backgrounds with regard to the IL4 or IL8 haplotypes.

Materials and Methods

Selection of participants

One hundred and forty one patients were enrolled in this study, divided into susceptible (S) or protected against (P) the development of CP. This classification was based on the genetic background of the patients, regarding to haplotypes formed by polymorphisms in the IL4 (n=62 individuals) or the IL8 gene (n=79 individuals) [33, 34].

All of the volunteers were informed about the aims and methods of the current study, and they provided their written consent to participate. This study was approved by the Committee for Ethical Affairs of the School of Dentistry at Araraquara, São Paulo State University (Protocol number 52/08).

The inclusion criteria were good general health, age between 30 and 60 years old, the 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 smokers with abstinence of less than 5 years), need for antibiotic prophylaxis, history of systemic or local disease with 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

All the clinical measurements, collection of GCF and non-surgical periodontal therapy were made according described in Anovazzi et al. [37], (for IL4 haplotypes) and in Corbi et al. [35], (for IL8 haplotypes). Patients carrying haplotypes in the IL4 gene were examined by a single previously calibrated periodontist (G.A./weighted Kappa=0.82, considering probing pocket depth as a clinical outcome), and those carrying

haplotypes in the IL8 gene were examined by another single periodontist (S.C.T.C), which was also previously calibrated (weighted Kappa=0.80, for probing pocket depth). Periodontists were blind about what haplotype the patient specifically carry, regarding gene and susceptibility to CP. Periodontal clinical examination recorded the following clinical parameters: probing pocket depth (PPD), percentage of the periodontal sites with PPD \geq 4 mm, clinical attachment loss (CAL), bleeding on probing (BOP), visible plaque index (VPI) [38] and gingival bleeding index (GBI) [38]. 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) in the whole mouth excluding the third molars. The cemento-enamel junction was accepted as a reference point in the measurement of CAL. The PPD and the CAL measurements were performed using a conventional Williams manual probe (Trinity - Campo Mourão, Brazil). The BOP (deemed positive if occurring within 15 seconds after the periodontal probing), the visible plaque index (VPI) and the gingival bleeding index (GBI) were recorded dichotomously. Patients were considered to have CP when two or more sites in non-adjacent teeth exhibited probing \geq 5 mm, \geq clinical 3 depths mm (moderate attachment chronic loss

periodontitis) [39] and bleeding on probing. Subjects without CP exhibited sites with probing \leq 3 mm pocket and depths \geq absence 3 mm. of clinical attachment loss

After the periodontal clinical evaluation of the genetically pre-screened subjects, they were distributed into the following four groups for each gene:

For the IL4 gene:

- Group IL4-SH: susceptible to CP conferred by IL4 haplotype, without CP (healthy);
- Group IL4-SCP: susceptible to CP conferred by IL4 haplotype, with CP;
- Group IL4-PH: protected against CP conferred by IL4 haplotype, without CP (healthy);
- Group IL4-PCP: protected against CP conferred by IL4 haplotype, with CP.

For the IL8 gene:

- Group IL8-SCP: susceptible to CP conferred by IL8 haplotype, with chronic periodontitis;
- Group IL8-NSCP: non-susceptible to CP conferred by IL8 haplotype, with chronic periodontitis;

- Group IL8-SH: susceptible to CP conferred by IL8 haplotype, without chronic periodontitis (healthy);

- Group IL8-NSH: non-susceptible to CP conferred by IL8 haplotype, without chronic periodontitis (healthy).

Collection of gingival crevicular fluid (GCF)

For the chronic periodontitis groups, GCF samples were collected from two proximal sites of non-adjacent ≥ 5 teethmm, with PPD CAL ≥ 3 mm and BOP. For the periodontally healthy groups, GCF samples were collected from two proximal sites of non-adjacent teeth with ≤ 2 mmPPD. Before GCF sampling, supragingival plaque was removed from the inter-proximal 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 30 seconds [40]. 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 (μ l) by reference to a standard curve. All strips with GCF were immediately and individually placed into sterile polypropylene tubes [41,42] and kept at -80°C until further analysis.

Non-surgical treatment

After collection of clinical parameters and GCF samples, all of the patients enrolled in the study received oral hygiene instructions and supragingival prophylaxis. Patients affected by CP received non-surgical periodontal therapy performed by calibrated periodontists (weighted Kappa=0.81, considering probing pocket depth as the clinical outcome), and blind to the genetic status of each patient (G.A for IL4 haplotype carriers and M.H.T for IL8 haplotype carriers). The therapy included motivation and instructions on oral hygiene and scaling and root planning over a 3- to 4-week period, using manual cures and ultrasonic instrumentation (Cavitron® Ultrasonics Inc., Long Island City, NY, USA). After non-surgical therapy, follow-up appointments were scheduled at 45 days for supportive periodontal therapy. Clinical periodontal measurements and GCF sampling were repeated 45 days after the completion of therapy.

GCF enzyme-linked immunosorbent assay (ELISA) analysis of MMP-8

The GCF samples were taken from -80°C storage and were thawed on ice. Subsequently, a solution of 50 μ l of phosphate buffered saline with 1% Tween 20 (PBS-T) [43] was added to each sample. GCF was extracted by centrifugation at 10.000 g

for 15 minutes at 4°C [44].

The concentration of MMP-8 in the GCF samples was assayed by a sandwich ELISA kit (R&D Systems Inc., Minneapolis, MN, USA). All assays were carried out according to the manufacturer's instructions. The ELISA plates were assessed spectrophotometrically at 450 nm (uQuant Bio-Tek Instruments, Winooski, VT/USA). All assays were conducted in duplicate. The levels of GCF MMP-8 in each sample were determined using the concentration values of standards included in the kit. GCF samples were assayed at a 1:4 dilution for MMP-8 according to previous standardization, and 100- μ l quantities of the dilutions were used in each well of the ELISA plates, as recommended by the manufacturer. The results for MMP-8 were obtained by multiplying the ELISA results by the dilution. These results were expressed in ng/ml and were multiplied by the initial sample volume (0.1 ml buffer + GCF volume) μ L. to obtain results in ng/L.

Statistical analysis

In order to assess clinical differences between individuals with haplotype of susceptibility to or protection against CP, for each gene analyzed (IL4 or IL8), a sample size calculation was performed utilizing a two-sided t test considering a 5% alpha error, with 1-mm clinical significant difference of the probing pocket depth between the groups, and a standard deviation of 0.5 mm [45, 46]. Then, with a total sample size of 24 subjects (6 subjects per group), the power of the study was calculated to be 95% [47]. Therefore, the number of subjects enrolled in the present study was large enough to detect clinical differences between the genetically pre-screened groups with an acceptable level of confidence for both haplotypes.

For the immunological evaluation, we utilized the MMP-8 (ng/L) protein values obtained from the collected sites (n=124) in a power calculation analysis (a two-sided t test considering a 5% alpha error). Then, with a total sample size of 10 subjects per group, the power of the study was calculated to be 90%.

The Shapiro-Wilk test was utilized to assess the normality of all of the data. The chi-squared test was used to determine whether the proportions of males and females were equal in the groups, and the Kruskal-Wallis test was used to determine whether patient age and total number of teeth were similar between the groups.

Comparisons between the groups formed by individuals who were genetically susceptible to or protected against CP with the same clinical conditions (IL4-SCPvsIL4-PCP; IL4-SHvsIL4-PH; IL8-SCPvsIL8-NSCP; and IL8-SHvsIL8-NSH) were accomplished using the Mann-Whitney test. Additionally, differences between the groups with and without CP with the same genetic conditions (IL4-SCPvsIL4-SH; IL4-PCPvsIL4-PH; IL8-SCPvsIL8-

SH; IL8-NSCPvsIL8-NSH) were also assessed. To determine the differences between the study periods (intra-group comparisons), the unpaired Wilcoxon test was utilized.

Spearman's correlations were used to investigate the associations among the clinical parameters and MMP-8 levels or GCF volume of each group at each time point. All of the data analyses were performed using statistical software package

Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA). Differences were considered significant when $p < 0.05$.

Results and Discussion

Clinical findings

No significant differences were found among subgroups of both IL4 and IL8 genetic background for gender (chi-square test; $p = 0.66$ for IL4 and $p = 0.38$ for IL8), age (Kruskal-Wallis test; $p = 0.57$ for IL4 and $p = 0.12$ for IL8), and number of teeth (Kruskal-Wallis test; $p = 0.064$ for both cytokines) (Table 1).

Table 1. Characteristics of Study Population.

Groups	n	Mean Age (years) (\pm Standard Deviation)	Gender		N° of teeth (\pm Standard Deviation)
			Female(%)	Male(%)	
IL4-SCP	16	47.94(\pm 10.46)	14(32.6)	2(11.1)	23.38(\pm 3.54)
IL4-SH	16	35.75(\pm 15.56)	9(20.9)	7(36.8)	26.63(\pm 1.26)
IL4-PCP	10	48.30(\pm 13.43)	8(18.6)	2(11.1)	22.80(\pm 4.21)
IL4-PH	20	42.75(\pm 9.57)	12(27.9)	8(44.4)	25.80(\pm 24.65)
Total	62	43.68(\pm 12.90)	43(70.5)	19(29.5)	24.65(\pm 3.33)
P value		0.57		0.66	0.064
IL8-SCP	21	46.57(10.24)	11(53.4)	10(47.6)	24.09(\pm 2.42)
IL8-SH	14	41.93(9.08)	7(50)	7(50.0)	24.85(\pm 4.20)
IL8-NSCP	20	46.55(8.26)	15(75)	5(25)	22.40(\pm 3.74)
IL8-NSH	24	41.63(8.80)	15(62.5)	9(37.5)	25.62(\pm 3.29)
Total	79	44.24(9.27)	48(60.7)	31(39.2)	24.26(\pm 3.55)
P value		0.12		0.38	0.064

n = individuals number; % = percentage of the number of individuals.

To investigate whether genetic susceptibility to CP was associated with clinical conditions, differences in clinical parameters among the groups were compared. Data from all patients carrying IL4 haplotype were previously published in Anovazzi et al. [37].

In summary, at baseline, we found significant differences between patients with CP and healthy patients, both carrying the IL4 haplotype of susceptibility to CP (SCP vs. SH), considering plaque index (PI) ($p = 0.00011$), gingival index

(GI) ($p = 0.0004$), bleeding of probing (BOP) ($p = 0.00003$), and periodontal probing depth (PPD) ($p = 0.00005$). Similarly, significant differences were found between patients with disease and healthy patients who carried the IL4 haplotype of protection against CP (PCP vs. PH) for PI ($p = 0.00075$), GI ($p = 0.002$), BOP ($p = 0.00013$) and PPD ($p = 0.000001$).

Therefore, the IL4 genetic carriage does not influenced the clinical periodontal parameters, even though after 45 days of the non-surgical periodontal therapy (data showed in Anovazzi et al. [37]). Clinical findings of all patients carrying IL8 haplotype were previously published in Corbi et al. [35]. In summary, all clinical parameters (PI, GI, BOP, and PPD) of the SCP and NSCP groups were similar at baseline, and all decreased significantly after non-surgical periodontal therapy (at 45 days), similarly for both groups. Importantly, patients with CP had worse periodontal condition than healthy patients, independently of the presence of IL4 or IL8 haplotypes.

Immunological analysis: GCF volume, MMP-8 levels and Correlation Analysis

Individuals with IL4 haplotypes

The immunological results were in agreement with the inflammatory status of the periodontal collection sites. Unsurprisingly, the GCF volume values were higher in groups with CP. At baseline and after 45 days of treatment, significant differences were observed between IL4-SCP and IL4-SH and between IL4-PCP and IL4-PH groups. Considering all of the studied time points, significant decreases in GCF volume were observed in the CP groups (results presented in Anovazzi et al. [37]).

Regarding MMP-8 levels (ng/ μ L) in GCF, no significant difference was observed between IL4-SCP and IL4-PCP groups at baseline and after 45 days of periodontal therapy (Figure 1). Significant differences in MMP-8 levels were only found in relation to the inflammatory status of the collected site (IL4-SCP versus IL4-SH groups and IL4-PCP versus IL4-PH, $p < 0.0001$) in the baseline and 45 days after periodontal treatment. Considering all of the studied periods, a significant decrease in the MMP-8 levels in the GCF was found in all of the groups: IL4-PCP ($p = 0.0002$), IL4-SCP ($p < 0.0001$), IL4-PH ($p = 0.0017$) and IL4-SH ($p < 0.0001$) (Figure 1).

Correlations among clinical parameters [37] and MMP-8 production or GCF volume revealed significant differences in IL4-SCP group. At baseline, the IL4-SCP group showed correlation between the MMP-8 concentration and PPD (0.51) (Table 2). After 45 days of completed periodontal therapy, a significant correlation was observed between PPD and GCF volume (0.68), and between PPD and MMP-8 concentration (0.57) (Table 2).

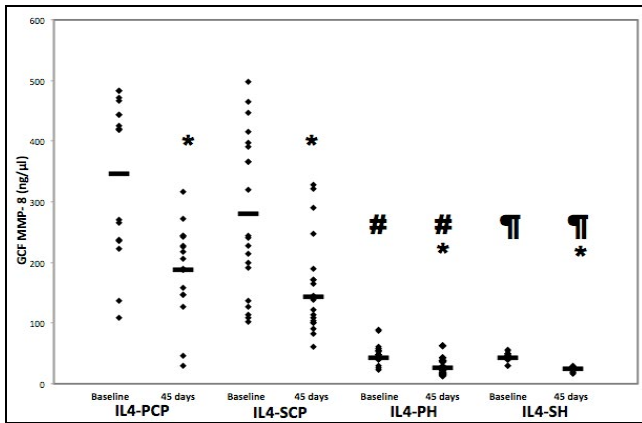


Figure 1- Comparison of MMP-8 concentration (pg/ μ L) in individuals with *IL4* haplotypes among the groups.
 (*) Significant differences when compared to baseline of the same group.
 (#) Significant differences when compared to group PCP in the same period.
 (¶) Significant differences when compared with the group SCP in the same period.
 Mann-Whitney test and Dunn test for evaluations between the groups in the same period.
 Wilcoxon test for evaluations between different periods in each group.

interfere with the MMP-8 levels, since no difference was found between the IL8-SCP and IL8-NSCP groups. Again, the difference in MMP-8 levels was significant when the presence or not of inflammation was considered (IL8-SCP [225 ng/ul] vs IL8-SH [50 ng/ul] groups, $p = 0.0001$)

(Figure 2). Considering all of the studied periods, a significant decrease in the MMP-8 levels in the GCF was found in all of the groups: IL8-NSCP ($p=0.000062$), IL8-SCP ($p=0.000301$), IL8-NSH ($p=0.00013$) and IL8-SH ($p=0.0014$).

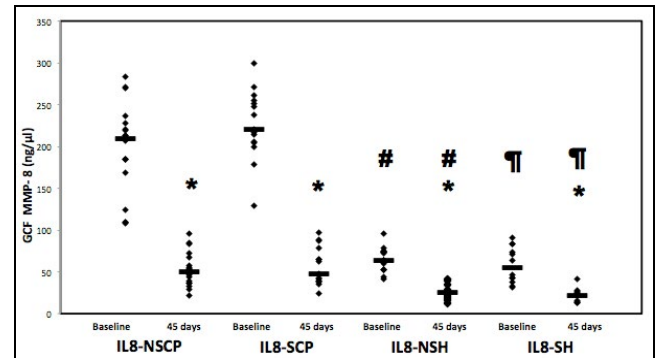


Figure 2- Comparison of MMP-8 concentration (pg/ μ L) in individuals with *IL8* haplotypes among the groups.
 (*) Significant differences when compared to baseline of the same group.
 (#) Significant differences when compared to group PCP in the same period.
 (¶) Significant differences when compared with the group SCP in the same period.
 Mann-Whitney test and Dunn test for evaluations between the groups in the same period.
 Wilcoxon test for evaluations between different periods in each group.

Table 2. Correlation coefficients (rho) of patients with *IL4* gene between clinical parameters and the immunological data or the GCF volume at baseline and at 45 days after periodontal treatment.

Period	Group/Parameter	MMP-8 Concentration (ng/ μ L)	GCF Volume	Period	Group/Parameter	MMP-8 Concentration (ng/ μ L)	GCF Volume	
Baseline	<i>IL4-PCP</i>		(rho)	45 days after periodontal therapy	<i>IL4-PCP</i>			
	PI	0.20	0.09		PI	0.06	0.36	
	GI	0.27	0.30		GI	0.32	0.26	
	BOP	0.06	0.35		BOP	0.15	0.06	
	PPD	0.24	0.42		PPD	0.48	0.27	
	<i>IL4-SCP</i>				<i>IL4-SCP</i>			
	PI	0.18	-0.49		PI	0.10	-0.09	
	GI	0.44	-0.21		GI	0.01	0.17	
	BOP	0.21	-0.41		BOP	0.43	0.10	
	PPD	0.51*	0.33		PPD	0.57*	0.68*	
	<i>IL4-PH</i>				<i>IL4-PH</i>			
	PI	0.04	0.20		PI	-0.29	-0.01	
GI	0.23	0.05	GI	-0.21	-0.19			
BOP	0.04	-0.20	BOP	0.01	0.24			
PPD	0.39	-0.23	PPD	0.11	-0.42			
<i>IL4-SH</i>			<i>IL4-SH</i>					
PI	0.11	-0.12	PI	0.10	0.48			
GI	0.18	-0.09	GI	0.07	0.31			
BOP	0.15	0.12	BOP	0.27	0.36			
PPD	0.07	-0.10	PPD	-0.26	0.09			

*Significant Spearman's correlation; PI: plaque index; GI: gingival index; BOP: bleeding on probing; PPD: periodontal probing depth.

Individuals with *IL8* haplotypes

Significant difference in GCF volume was found at baseline between IL8-SCP and IL8-SH groups, as well as between IL8-NSCP and IL8-NSH groups [36]. After 45 days of the non-surgical periodontal therapy, no difference was found between the IL8-SCP and IL8-NSCP [35].

Figure 2 shows the MMP-8 concentration determined by ELISA (ng/ul). The genetic background of patients did not

Correlations between clinical parameters [35] and MMP-8 production or GCF volume revealed at baseline that the IL8-SCP group showed a correlation between PPD and MMP-8 concentration (0.54). For the IL8-SH group a correlation was found between MMP-8 levels and BOP (0.51) (Table 3). After 45 days of completed periodontal therapy, in the IL8-SH group, a significant correlation was observed between plaque index (PI) and MMP-8 concentration (0.50) (Table 3).

We hypothesized that haplotypes in the IL4 or IL8 genes could influence MMP-8 levels, based on the fact that IL-8 and MMP-8 are related with the function of neutrophils and IL-4 could be part in the modulation of MMPs. However, no influence of the tested haplotypes on the MMP-8 levels was observed. This finding contributes to the knowledge about the potential biological interconnection among those molecules, since the literature is scarce of information about this. It is worth to mention that other genetic polymorphisms could influence MMP-8 levels, since there are about 14 millions of polymorphisms in the human genome [48].

The demographic data of the present study showed homogeneity in age, gender and number of teeth among subgroups of both IL4 and IL8 haplotype carriers (Table 1). Therefore, these

factors were not characterized as variables of influence in this study. Comparisons of clinical parameters that characterize the chronic periodontitis (PPD, CAL and BOP) showed no statistical significant differences between individuals with or without genetic susceptibility to CP for the IL8 haplotypes [35] neither between individuals susceptible or protect against to CP conferred by IL4 haplotypes [37].

Table 3. Correlation coefficients (rho) of patients with IL8 gene between clinical parameters and the immunological data or the GCF volume at baseline and at 45 days after periodontal treatment.

Period	Group/Parameter	MMP-8 Concentration (ng/ μ L)	GCF Volume	Period	Group/Parameter	MMP-8 Concentration (ng/ μ L)	GCF Volume
Baseline	IL8-NSCP	(rho)	(rho)	45 days after periodontal therapy	IL8-NSCP		
	PI	-0.30	0.26		PI	-0.41	0.30
	GI	-0.29	0.34		GI	-0.22	0.11
	BOP	-0.32	-0.12		BOP	0.04	0.16
	PPD	-0.32	0.40		PPD	-0.19	0.42
	IL8-SCP				IL8-SCP		
	PI	0.07	0.31		PI	0.01	-0.06
	GI	0.11	-0.27		GI	0.01	0.23
	BOP	0.10	-0.37		BOP	0.21	0.43
	PPD	0.54*	-0.01		PPD	0.31	0.11
	IL8-NSH				IL8-NSH		
	PI	-0.06	0.32		PI	0.07	-0.37
	GI	-0.006	0.23		GI	0.10	-0.38
	BOP	-0.07	-0.24		BOP	0.12	-0.21
	PPD	-0.03	-0.36		PPD	0.06	-0.44
IL8-SH			IL8-SH				
PI	0.47	-0.20	PI	0.50*	-0.23		
GI	0.37	0.17	GI	0.49	0.11		
BOP	0.51*	-0.11	BOP	-0.40	-0.33		
PPD	-0.45	0.16	PPD	-0.21	-0.12		

*Significant Spearman's correlation; PI: plaque index; GI: gingival index; BOP: bleeding on probing; PPD: periodontal probing depth.

Recently, we investigated whether those haplotypes influence the IL-4 and IL-8 protein levels in GCF of patients with CP [35-37]. The GCF volume at baseline showed that, independently of presence of IL4 or IL8 haplotypes, individuals affected by CP produced higher GCF volumes than individuals who were periodontally healthy. This finding is in accordance with previous studies, which showed higher GCF volumes in individuals with CP compared to controls [43, 49, 50].

MMP-8 levels were higher in individuals affected by CP than in patients periodontally healthy, independent of their haplotype carriage (IL4 or IL8 gene), for all of the studied time points (Figure 2, 4). These higher MMP-8 levels at diseased periodontal sites decreased significantly after non-surgical treatment which is in agreement with previous studies [18, 19]. The presence of MMP-8 in diseased periodontal tissues has been associated with periodontal disease activity and progression [18, 51]. Passoja et al. [52] has shown that MMP-8 levels in GCF of shallow crevices are associated with the extent of periodontal disease, and suggested that MMP-8 might be a valid prognostic marker for periodontal disease.

The correlation analysis in the present study revealed additional information that are also in agreement with the idea

that MMP-8 could be a valid prognostic marker for periodontal disease [52]. These results suggest that the periodontal parameter PPD increases as immunological MMP-8 concentration an increase, which agrees with the concept that MMP-8 is associated with the initiation and progression of periodontitis and reflects its severity [53-55].

Here we verified that patient's genetic background regarding IL4 and IL8 haplotypes do not influence MMP-8 levels or the outcome of non-surgical periodontal therapy. Similar results were found for IL-8 levels [35, 36], but not for IL-4. In a previous study, the PCP group demonstrated higher IL-4 levels than the SCP group after 45 and 90 days of the non-surgical therapy, in spite of similar clinical responses [37].

The present study contributes to the knowledge about the multifactorial character of the CP, since few studies have investigated different data such as clinical, immunological and genetic background simultaneously. Pirhan et al. [56], investigating the MMP13 gene and did not find a significant association between gene polymorphisms and MMP-13 protein levels in GCF or the outcome of periodontal therapy. Other studies have also not found an association between the outcome of periodontal therapy and genetic polymorphisms, such as polymorphisms in the mannose-binding lectin gene [57], the IL1A gene and the IL1B gene [58]. The IL-1 composite genotype is the most commonly investigated genotype in studies of periodontal disease, including the analysis of periodontal-treatment outcomes. Holla et al. [55] investigated the association of -799C/T and +17C/G polymorphisms in the MMP-8 gene and periodontitis, but not MMP-8 levels in GCF. Therefore more studies in this area are necessary.

Conclusions

Based on the results obtained in this study, we conclude that IL4 or IL8 haplotypes which were previously associated with susceptibility to CP did not influence MMP-8 levels or the outcome of non-surgical periodontal therapy.

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