RAQUEL MANTUANELI SCAREL CAMINAGA

Influência de Polimorfismos em genes de Citocinas na morbidade da Doença Periodontal Inflamatória Crônica

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para a obtenção do título de Doutor em Biologia e Patologia Buco-Dental.

Piracicaba 2002

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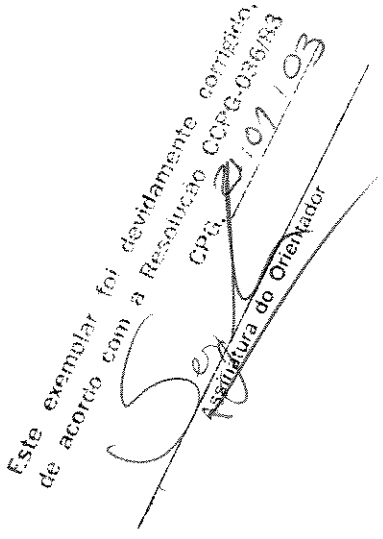

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Orientador: Prof. Dr. Sergio Roberto Peres Line

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Prof. Dr. Sergio Roberto Peres Line
Prof. Dr. Milton Ozório Moraes
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Prof. Dr. Luis Eduardo Aranha Camargo
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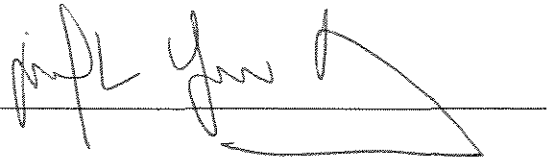
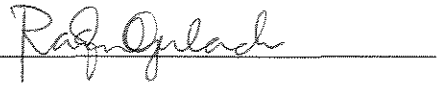
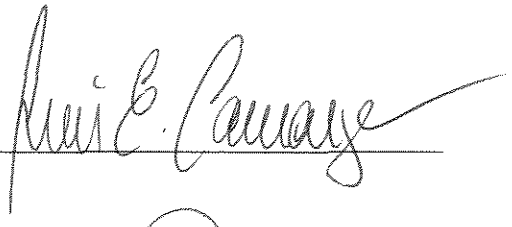
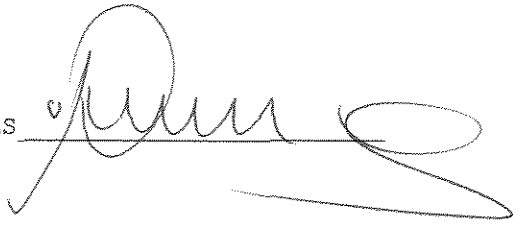
2. Prof. Dr. MILTON OZORIO MORAES 

3. Profa. Dra. RAQUEL FERNANDA GERLACH 

4. Prof. Dr. LUIS EDUARDO ARANHA CAMARGO 

5. Prof. Dr. SERGIO AUGUSTO CATANZARO GUIMARÃES 

DEDICATÓRIA

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SUMÁRIO

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RESUMO

A Doença Periodontal Crônica (DPC) caracteriza-se por um processo inflamatório nos tecidos de suporte do dente. É causada inicialmente por bactérias presentes na superfície dental (biofilme) e que adjacentes à gengiva, estimulam células do hospedeiro a gerarem uma resposta imunoinflamatória. Citocinas constituem importantes mediadores que influenciam o início e a progressão de diversas doenças inflamatórias crônicas, incluindo a doença periodontal. Polimorfismos gênicos têm sido relacionados ao aumento da expressão das citocinas correspondentes. O objetivo do presente estudo foi investigar polimorfismos nas regiões promotoras dos genes da *IL2*, *IL4* e *IL10* e relacioná-los com a doença periodontal crônica. Fizeram parte do estudo 113 indivíduos não-aparentados, não-fumantes, acima de 25 anos, subdivididos de acordo com o seguinte critério: 44 indivíduos saudáveis, 31 com periodontite moderada e 38 com periodontite severa. O DNA genômico foi obtido de células epiteliais da mucosa bucal através de um bochecho com 5 mL de solução de glicose a 3% e leve raspagem da mucosa jugal. Os polimorfismos *IL2* (-330), *IL4* (-590) e *IL10* (-819 e -592) foram investigados pela técnica PCR-RFLP e o polimorfismo *IL10* (-1082) foi investigado por Seqüenciamento Automático. Os polimorfismos no gene da *IL10* foram analisados também como haplótipos. Métodos estatísticos apropriados foram empregados para acessar diferenças entre os grupos estudados e as freqüências alélicas e genotípicas de cada polimorfismo, sendo que valores de $p<0,05$ foram considerados significativos. Diferenças significativas nas freqüências

alélicas e genotípicas foram encontradas nos polimorfismos *IL2* (-330) e *IL10* (-819 e -592), assim como na análise dos três polimorfismos no promotor do gene *IL10* arranjados como haplótipos. O estudo obteve as seguintes conclusões: (a) o polimorfismo *IL2* (-330) está associado à severidade da DPC na população estudada; (b) os polimorfismos *IL4* (-590) e *IL10* (-1082) não estão relacionados com a DPC; (c) os polimorfismos *IL10* (-819 e -592) estão associados à suscetibilidade da DPC; (d) determinados haplótipos no promotor do gene *IL10* estão associados à susceptibilidade da DPC principalmente no subgrupo formado por mulheres.

A análise dos polimorfismos *IL2* (-330) e *IL4* (-590) possibilitou um estudo genético adicional. Tais polimorfismos mostraram-se úteis em estudos de genética de populações, podendo complementar dados obtidos através de análise de DNA mitocondrial e regiões do cromossomo Y.

ABSTRACT

Chronic Periodontal Disease (CPD) is characterized by an inflammation in the supporting tissues of the teeth. It is primarily caused by bacteria present on tooth surfaces (biofilm), which adjacent to the gingiva, stimulate host cells to produce immunoinflammatory response. Cytokines represent important pathological mediators, which influence the onset and progression of several chronic inflammatory diseases, including CPD. Polymorphisms in cytokine genes have been associated with enhanced expression of the correspondent cytokines. The aim of this study was to investigate the relationship between polymorphisms in the promoter region of the genes *IL2*, *IL4* and *IL10* and CPD. One hundred and thirteen non related subjects, over 25 years, non smoking individuals, were divided according to the severity level of periodontal disease: 44 healthy individuals, 31 with moderate and 38 with severe periodontitis. DNA was obtained from epithelial cells through a mouthwash with 5 mL 3% glucose and scraping of oral mucosa. The polymorphisms *IL2* (-330), *IL4* (-590) and *IL10* (-819 e -592) were investigated using PCR-RFLP, and the polymorphism *IL10* (-1082) was investigated using DNA sequencing. Polymorphisms in the *IL10* gene were also analysed as haplotypes. Properly statistical methods were employed to access differences between the studied groups and allelic and genotypic frequencies of each polymorphism. *P* values <0.05 were considered significant. Significant differences were found in the *IL2* (-330) and *IL10* (-819 and -592) polymorphisms. The analysis of the three polymorphisms in the *IL10* gene arranged as haplotypes revealed significant

differences regarding CP. The conclusions of this study were: (a) the polymorphism *IL2* (-330) was associated with the severity of CP in the studied population; (b) polymorphisms *IL4* (-590) and *IL10* (-1082) were not related to CP; (c) polymorphisms *IL10* (-819 and -592) were associated with susceptibility to CP; (d) specific haplotypes in the promoter region of *IL10* gene are associated with susceptibility to CP, especially in female subjects.

The analysis of the polymorphisms *IL2* (-330) and *IL4* (-590) permitted an additional genetic study. These polymorphisms were useful as markers in population genetic studies, and could complement data obtained by mitochondrial DNA analysis and analysis of the non-recombining portion of the Y chromosome.

1. INTRODUÇÃO

A Doença Periodontal Crônica (DPC) é uma doença infecciosa que resulta em inflamação dos tecidos de suporte do dente (LINDHE *et al.*, 1999). A DPC é causada inicialmente por bactérias periodontopatogênicas como *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* e *Bacteroides forsythus* presentes na superfície dental (biofilme) (FLEMMIG, 1999). A presença dessas bactérias em adjacência à gengiva, estimulam as células (fibroblastos, macrófagos, leucócitos) do hospedeiro a secretarem citocinas pró-inflamatórias (KORNMAN & NEWMAN, 2000). A continuação do processo patológico pode resultar na formação de bolsas periodontais, contendo bactérias periodontopatogênicas, as quais irão manter o sistema imune do hospedeiro ativado. Isso resultará em destruição tecidual, como fibras de colágeno subjacentes à bolsa periodontal e perda de osso alveolar (OKADA & MURAKAMI, 1998). As características clínicas da DPC incluem perda de inserção clínica periodontal (Clinical Attachment Loss - CAL), inflamação e sangramento gengival, e em grau mais severo, aumento da mobilidade e exfoliação do dente (FLEMMIG, 1999).

Estudos epidemiológicos demonstram a ocorrência mundial da DPC, sendo amplamente difundida inclusive no Brasil (GJERMO *et al.*, 1984; DINI *et al.*, 1994). Embora existam dificuldades metodológicas e variabilidade nos valores de incidência da DPC, um estudo de KORNMAN *et al.* (1997) refere que a DPC afeta mais de 30% da população, sendo que em grau severo atinge 7-13% da

população. Dados recentes têm mostrado uma tendência maior de perda dental devido a razões periodontais do que devido à cárie (ONG, 1998). É válido acrescentar que a severidade da DPC e a perda dental são influenciadas pelo fumo (ONG, 1998; JOHNSON & SLACH, 2001). Assim, a DPC tem caráter multifatorial, pois não somente a presença de bactérias periodontopatogênicas é suficiente para levar à doença periodontal. Um estudo envolvendo 117 gêmeos adultos estimou que cerca de metade da variação encontrada na expressão da DPC deve-se a fatores genéticos (MICHALOWICZ *et al.*, 2000). O estresse psicossocial (LINDEN *et al.*, 1996), e doenças sistêmicas como diabetes (MEALEY, 2000) também podem influenciar a progressão da DPC. Principalmente durante a última década, um corpo crescente de evidências científicas sugere associação entre infecção oral e doenças sistêmicas (aterosclerose, doença cardiovascular, artrite, osteoporose, doenças pulmonares) (COHEN & SLAVKIN, 2000). Conseqüentemente, uma nova área, a Medicina Periodontal, tem surgido na Odontologia.

Nas doenças inflamatórias crônicas há fatores que não causam a doença mas amplificam alguns mecanismos que a tornam mais severa. Quando um determinado tecido apresenta uma resposta inflamatória a expressão de várias citocinas torna-se aumentada; posteriormente, na tentativa de controlar essa mesma resposta inflamatória local, a expressão de citocinas tende a diminuir. Desta maneira, há uma complexa interação entre citocinas pró e anti-inflamatórias atuando nos tecidos periodontais inflamados (OKADA & MURAKAMI, 1998). Nos últimos anos vários estudos têm procurado esclarecer como as citocinas

interferem na progressão da doença periodontal (WILSON *et al.*, 1996; YAMAMOTO *et al.*, 1997; OKADA & MURAKAMI, 1998).

Como na DPC ocorre uma resposta imunoinflamatória frente a microrganismos agressores, diferenças interindividuais na resposta imune do hospedeiro devem ser consideradas. Há uma tendência em explicar tais diferenças investigando a carga genética do indivíduo. Polimorfismos são variações genéticas encontradas na população, nas quais a freqüência do alelo mais raro de um determinado loco é maior que 1% (THOMPSON *et al.*, 1991). Um polimorfismo no gene da interleucina 1 (*IL1B*) mostrou que um dos genótipos promove a produção quadruplicada da proteína correspondente (POCIOT *et al.*, 1992). Estudos recentes têm demonstrado associação entre polimorfismos em genes de citocinas e suscetibilidade à doenças inflamatórias; por exemplo, polimorfismos nos genes da *IL1* foram associados à artrite reumatóide (BUCHS *et al.*, 2001) e à asma (KARJALAINEN *et al.*, 2002). O primeiro estudo investigando polimorfismos genéticos em relação a doença periodontal enfocou justamente o gene da *IL1*, onde foi observada associação à severidade da doença periodontal (KORNMAN *et al.*, 1997). A partir desse relato, muitos estudos têm sido realizados considerando não só a quantificação de citocinas nos sítios afetados pela doença periodontal mas também polimorfismos genéticos (SHIRODARIA *et al.*, 2000; YAMAZAKI *et al.*, 2001).

Interleucinas como a *IL2*, a *IL4* e a *IL10* têm sido investigadas com relação a doença periodontal, buscando-se entender papel que desempenham. Alguns dados importantes sobre essas interleucinas são referidos a seguir:

1.1 Interleucina 2

É uma citocina pró-inflamatória produzida por linfócitos T *helper* (Th1) e estimula macrófagos, células *natural killer* e células T citotóxicas que mediam a resposta imune celular (PARKES *et al.*, 1998). A *IL2* promove a ativação, proliferação e desenvolvimento dos linfócitos B (WILSON *et al.*, 1996). Assim como a *IL1* e *IL6* (GEMMELL & SEYMOUR, 1994), a *IL2* está envolvida em mecanismos de regulação óssea, pois estimula a atividade de osteoclastos na reabsorção óssea (RIES *et al.*, 1989). Apesar do papel pró-inflamatório da *IL2*, seu envolvimento na influência e progressão da DPC é pobremente entendido. Existem estudos conflitantes sobre os níveis da *IL2* no soro de indivíduos com doença periodontal (MCFARLANE & MEIKLE, 1991) e níveis da *IL2* presentes em cultura de células mononucleares de gengiva e de sangue periférico (FUJIHASHI *et al.*, 1993; TAKAHASHI *et al.*, 1997).

1.2 Interleucina 4

Produzida por linfócitos T *helper* (Th2), a *IL4* apresenta propriedades tanto pró-inflamatórias quanto anti-inflamatórias. Como característica pró-inflamatória, a *IL4* induz a proliferação de linfócitos B e a liberação de imunoglobulinas (Ig) IgG e IgE após estimulação por lipopolissacarídeos (LPS) de bactérias periodontopatogênicas (SNAPPER & PAUL, 1987; TEW *et al.*, 1989). Em sítios com periodonto inflamado foram encontrados níveis elevados de IgG e IgE (REINHARDT *et al.*, 1989; HYYPPÄ, 1984, respectivamente). Um dos papéis biológicos da *IL4* é promover supressão de citocinas pró-inflamatórias como a *IL1*

e fator de necrose tumoral α (*TNFα*) (ESSNER *et al.*, 1989; HART *et al.*, 1989). Foi sugerido que a ausência de *IL4* nos tecidos gengivais com periodontite poderia ser responsável pelo acúmulo de macrófagos na região, pois a *IL4* induz apoptose em macrófagos, inclusive isolados de tecido gengival inflamado (YAMAMOTO *et al.*, 1997).

1.3 Interleucina 10

É uma citocina relacionada a vários distúrbios, desde doenças auto-imunes como a artrite reumatóide (PEREZ *et al.*, 1995) e lúpus eritematoso sistêmico (LAZARUS *et al.*, 1997) a desenvolvimento de câncer (ESKDALE *et al.*, 1997). A *IL10* é produzida por linfócitos T *helper* (Th2) e assim como a *IL4* apresenta propriedades pró e anti-inflamatórias. Como característica pró-inflamatória, a *IL10* estimula a proliferação e diferenciação de linfócitos B (DE WAAL MALEFYT *et al.*, 1991*a*). A *IL10* inibe citocinas pró-inflamatórias como a *IL1* *TNFα*, *IL6* e *IL8* (DE WAAL MALEFYT *et al.*, 1991*b*). Um recente estudo demonstrou que a *IL10* inibiu a produção de *IL6* por fibroblastos gengivais humanos após estimulação por LPS de *Porphyromonas gingivalis* (WANG *et al.*, 1999).

1.4 Polimorfismos em genes de citocinas e a Genética de Populações

Polimorfismos em regiões variáveis do genoma humano como o HLA, DNA mitocondrial e regiões do cromossomo Y são comumente analisados na

tentativa de entender origem ou parentescos genéticos entre diferentes etnias ou populações (PROBST *et al.*, 2000; ALVES-SILVA *et al.*, 2000; CARVALHO-SILVA *et al.*, 2001). Há uma incidência crescente de relatos científicos sobre genes de citocinas que apresentam diferentes freqüências alélicas em populações de etnias diferentes e geograficamente distantes (REYNARD *et al.*, 2000; HIJAZI & HAIDER, 2000; YAMAZAKI *et al.*, 2001). Uma vez que determinados alelos em genes de citocinas podem influenciar certas doenças, conhecer as freqüências desses alelos em uma população pode ser de relevância clínica, pois pode informar sobre a suscetibilidade ou a severidade daquela doença na população (REYNARD *et al.*, 2000).

2. PROPOSIÇÃO

Este trabalho se propôs a investigar polimorfismos nas regiões promotoras dos genes *IL2*, *IL4* e *IL10* procurando relacioná-los com a doença periodontal crônica.

3. ARTIGOS

ARTIGO 1

Investigation of an IL-2 polymorphism in patients with different levels of chronic periodontitis

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Investigation of an IL-2 polymorphism in patients with different levels of chronic periodontitis

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Short title: *IL-2 polymorphism, chronic periodontitis*

Abstract.

Background: Interleukin-2 (IL-2) is a pro-inflammatory cytokine derived from Th1 cells. This cytokine is involved in the B-cell activation and stimulates macrophages, natural killer cells, T-cell proliferation and osteoclasts activity. Interleukin-2 has been also implicated in the stimulation of osteoclasts activity in bone resorption.

Objective: In this study the relationship between the polymorphism –330 (T→G) in the IL-2 gene and different levels of chronic periodontal disease was investigated.

Material & Methods: DNA was extracted from buccal epithelial cells of 113 unrelated adult individuals control and with different levels of periodontitis. The PCR-RFLP technique was used to investigate the polymorphism in the promoter of IL-2 gene.

Results: When comparing the data of three groups of patients (control, moderate and severe) we did not find significant differences between the studied IL-2 polymorphism and severity levels of periodontal disease. However, when the control and moderate phenotypes were grouped together and compared with genotypes TT versus TG/GG a significant difference was observed.

Conclusion: We conclude that the –330 (T→G) polymorphism in the IL-2 gene is associated with the severity of periodontal disease. The results presented in this study suggest an active role of IL-2 in the pathogenesis of periodontal disease.

Key words: Interleukin-2; polymorphism; chronic periodontitis; risk factors.

Periodontal disease (PD) is characterized by inflammatory cell accumulation in the periodontal tissues. Since most pathogenic bacteria reside in periodontal pockets and do not invade the periodontal tissues, the immune system can never efficiently eliminate the microorganisms (Okada & Murakami 1998). This unique situation leads to a chronic inflammation and continuous host response, resulting in tissue destruction (Page 1991). The local host response to bacteria includes the recruitment of leukocytes and the subsequent release of inflammatory mediators (Okada & Murakami 1998). The involvement of cytokines in PD has been extensively studied in the past few years, but the mechanisms by which they interfere in the disease progression are not completely understood.

Gemmell & Seymour (1994) have proposed that the stable periodontitis lesion is mediated principally by cells with the T-helper 1 (Th1) cytokine profile, while the progressive periodontitis lesion involves Th2 cells. Interleukin-2 (IL-2) is a cytokine produced by Th1 cells. This cytokine is involved in the B-cell activation and stimulates macrophages, natural killer cells and T-cell proliferation, which mediate the cellular immune response, being regarded as a pro-inflammatory cytokine (Tew et al. 1989, Wilson et al. 1996, Parkes et al. 1998). Interleukin-2 has been also implicated in the stimulation of osteoclasts activity in bone resorption (Ries et al. 1989). The levels of immunoreactive IL-2 in dental pulp have been shown to be a relevant parameter to determine the extend of pulpal inflammation (Rauschenberger et al. 1997). There is evidence indicating that IL-2 may also be a relevant factor in the pathogenesis of periodontal disease. Lymphocytes cultured from the chronically-inflamed periodontal tissues of patients with alveolar bone loss produced IL-2 (Seymour et al. 1985). The levels of IL-2 in the sera of periodontitis patients are elevated when compared to those of normal subjects (McFarlane & Meikle 1991). Due to its biological properties, IL-2 has been suggested to be a useful marker of pathologic inflammatory activity in systemic diseases

(John et al. 1998) and periodontal conditions (McFarlane & Meikle 1991, Rauschenberger et al. 1997, Takahashi et al. 1997).

Polymorphisms in cytokine genes are associated with chronic inflammatory diseases, such as interleukin-10 (IL-10), found in high levels in systemic lupus erythematosus (Lazarus et al. 1997) and in rheumatoid arthritis patients (Perez et al. 1995). Individual differences in levels of interleukin-1 (IL-1) related to susceptibility to PD are attributed to alleles of polymorphic genes. The allele 2 of the IL-1β (+3953) gene is more prevalent in patients with chronic periodontitis, which indicates a connection between this polymorphism and periodontal severity in adults (Kornman et al. 1997, Gore et al. 1998). In this paper we use the term *chronic* in the place of *adult* periodontitis, according to the criteria of the *1999 World Workshop for Classification of Periodontal Diseases and Conditions* by Armitage (1999).

The investigation of polymorphisms in the IL-2 gene concerning periodontal diseases is important because of the roles IL-2 plays in the inflammatory process. It is assumed that polymorphisms in the promoter region of genes can modify the level of expression of proteins (McGuire et al. 1994, John et al. 1998, Lazarus et al. 1997). A polymorphism in the position –330 (T→G) of IL-2 gene promoter was identified by John et al. (1998). The authors claim that this polymorphism could be useful as a marker to diagnose susceptibility to inflammatory diseases.

The purpose of this study was to investigate the relationship between the –330 (T→G) polymorphism in the IL-2 gene and severity of chronic periodontal disease, to verify if such polymorphism could serve as a marker of susceptibility to this disease.

Materials and Methods

Selection of subjects

A convenience sample of 113 unrelated, non-smoking subjects >25 years of age, were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba – UNICAMP. The patients are from the Southeastern region of Brazil. The baseline clinical parameters for the subject population are presented in Table 1. All subjects were in good general health and had at least 20 teeth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis; current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study. Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth, assessment of clinical attachment loss (CAL), tooth mobility and observation of bleeding on probing. Measurements of probing depth and attachment level were recorded at 6 points around each tooth. Subjects were included in clinical categories according to PD generalized severity:

1) *Control group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth >3 mm (n = 44)

2) *Moderate Periodontitis*: Patients with teeth exhibiting ≥ 3 mm and ≤ 6 mm CAL (n = 31)

3) *Severe Periodontitis*: Patients with teeth exhibiting ≥ 7 mm CAL (n = 38)

Analysis of genetic polymorphisms

Cells were obtained through a mouthwash with 3% glucose solution and light scrapings of the buccal mucosa with a sterile wood spatula. DNA was extracted from epithelial buccal cells with sequential phenol/chlorophorm solution and precipitated with salt/ethanol solution (Trevilatto & Line 2000, Scarel et al. 2000). The IL-2 gene promoter region (accession number AJ006884) was amplified by PCR utilizing the primers: Reverse - 5' CAT TGT GGC AGG AGT TGA GGT 3' and Forward - 5' TAT TCA CAT GTT CAG TGT AGT TCT 3'. The forward primer used has been described previously by John et al. (1998), with a T base in the –333 position altered to C, which creates a restriction site for the enzyme *Mae*I (C ^ T A G). This establishes an efficient and simple RFLP method to detect the –330 (T→G) polymorphism in the IL-2 gene.

The PCR temperature profile included an initial denaturation step at 95°C for 2 min, followed by 35 cycles each of 95°C (1 min), 59°C (1 min) and 72°C (1 min), with a final extension step of 72°C (5 min). RFLP was performed in a final reaction volume of 20 μL using 1.5 U *Mae*I (Boehringer-Mannheim) and 7 μL of PCR product (410 pb). The reactions took place overnight (ON) at 45°C. The products were analyzed in 5 % polyacrylamide gel electrophoresis. The gel was stained by rapid silver staining method (Sanguinetti et al. 1994)

The genotype and allele frequencies were calculated by direct counting and then dividing by the number of subjects to produce genotype frequency, or by number of chromosomes to produce allele frequency (Reynard et al. 2000). The significance of the differences in observed frequencies of polymorphisms in control and diseased groups was assessed by Chi-square (χ^2) test. Differences were considered significant when $p < 0.05$. In a separated analysis we compared groups control/moderate versus severe and calculated the risk associated with

individual alleles. It was also used the odds ratio (OR) with 95 % confidence intervals to obtain homozygous TT versus TG/GG genotypes (Table 3). The analysis was performed with the SAS statistical package.

Results

*Mae*I enzyme digestion cleaves the PCR products in two fragments: 387 pb + 23 pb (allele 2). The results were statistically analyzed with absolute (n) and percentage per column (%) of the genotypes and alleles. No significant differences among the three groups analyzed related to allelic (χ^2 = 5.385, p = 0.068) and genotypic (χ^2 = 6.105, p = 0.191) frequencies were found. The average frequency of allele T was homogeneous among the patients with different levels of PD (Table 2). In a separated analysis of the Caucasian subjects, no significant differences of genotypic (χ^2 = 3.295, p = 0.51) and allelic (χ^2 =2.733, p = 0.25) frequencies were found among groups control, moderate and severe.

However, when we compared groups control/moderate versus severe the allele distribution indicated an association of allele T with group control/moderate (χ^2 = 4.906, p = 0.027) as shown in Table 3. Individuals with the T allele seem to be approximately twice less likely to develop the severe PD (OR = 1.99; 95 % CI = 1.07-3.7). The frequency of genotype TT in the group control/moderate was also significantly different compared to the group formed by patients with severe PD (χ^2 = 5.479, p = 0.019). Individuals with the TT genotype seem to be 2.5 times less likely to develop the severe PD than individuals who are heterozygous or GG homozygous (OR = 2.57; 95 % CI = 1.15-5.73) (Table 3).

Discussion

In spite of the pro-inflammatory roles attributed to IL-2, its involvement and influence on the progression of periodontal disease are poorly understood. High levels of IL-2 were observed in the serum of patients with periodontal pocket depths greater than 5 mm (McFarlane & Meikle 1991). However, it was found that gingival mononuclear cells (GMC) culture supernatants from inflamed tissues of chronic periodontitis patients contained no detectable IL-2 at both mRNA and protein levels (Fujihashi et al. 1993). The assessment of *in vitro* IL-2 producing capacity of peripheral blood mononuclear cells (PBMC) and lymphocytes from patients with different forms of periodontitis showed no correlation between IL-2 production and disease types (Takahashi et al. 1997). Both the healthy and groups with periodontitis had subjects with PBMC exhibiting elevated and depressed IL-2 production. This finding may support the concept of "high or low IL-2 producers" in response to particular stimuli and genetic factors controlling IL-2 synthesis (Molvig et al. 1988, Takahashi et al. 1997). Perhaps this idea may explain the results of McFarlane & Meikle (1991), in which the several subjects might have been "IL-2 high producers" (Takahashi et al. 1997). The expression levels of IL-2 may be modulated by genetic polymorphisms in regulatory regions of the gene. These regulatory regions exist upstream the protein–coding region of genes and encode instructions deciphered by transcription factors, which recognize specific DNA motifs and enhance or repress the transcription (Stern 2000).

We have not found other reports in which a polymorphic marker in the promoter of the IL-2 gene was used with regard to severity of periodontal disease in adults. When comparing the data of three groups of patients (control, moderate and severe) we did not find significant differences between the studied IL-2 polymorphism and severity levels of PD. However, when the control and moderate phenotypes were grouped together and compared to severe group

regarding genotypes TT versus TG/GG, a significant difference was observed. This data suggest an association between the –330 (T→G) polymorphism in the IL-2 gene and the severity of periodontal disease.

It is interesting to note that TT is the most frequent genotype of IL-2 in the population presently studied, as well as in Caucasoid individuals from the United Kingdom (John et al. 1998). It is worth mentioning that the Brazilian population is highly heterogeneous, with Native American, African and European ancestry (Alves-Silva et al. 2000). As racial differences are common in polymorphic systems (Mourant et al. 1976), it is possible that the marker used in this study may present a different correlation regarding the progression of periodontal disease in other populations or racial groups. In the Brazilian southern region, the European population is predominant (IBGE, 1995 – accession http://www.ibge.gov.br). This is consistent with the predominance of Caucasoid individuals observed in our sample. The results presented in this study suggest an active participation of IL-2 in the pathogenesis of periodontal disease polymorphism was not suitable as a useful marker for chronic periodontal disease.

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Table 1. .Baseline clinical parameters of the subject population (n=113)

	control (n=44)	**moderate** (n=31)	**severe** (n=38)
AGE (years)			
mean (± SD)	43.2 (± 14.0)	36.9 (± 11.2)	43.6 (± 14.4)
GENDER %			
Female	68.2	80.6	84.2
Male	31.8	19.4	15.8
ETHNIC GROUP %			
Caucasoid	84.1	77.4	68.4
Afro-American	06.8	16.1	13.2
Mulatto	06.8	06.5	18.4
Japanese	02.3	0.0	0.0

Table 2. Allele and genotype frequencies of the –330 (T→G) polymorphism in the IL-2 gene promoter in healthy patients and patients with different levels of periodontitis (n=113).

	control	**moderate**	**severe**	**p***
ALLELE	n (%)	n (%)	n (%)	
T	68 (77.27)	51 (82.25)	50 (65.78)	0.068
G	20 (22.72)	11 (17.74)	26 (34.21)	
GENOTYPE				
TT	26 (59.09)	21 (67.74)	15 (39.47)	0.191
TG	16 (36.36)	09 (29.03)	20 (52.63)	
GG	02 (04.54)	01 (03.20)	03 (07.89)	

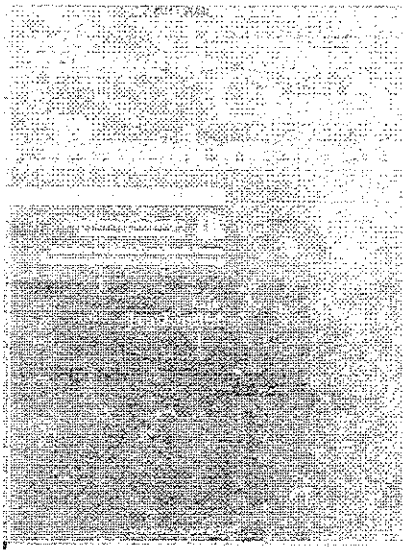
$p^* < 0.05$

Table 3. Allele and genotype frequencies of the –330 (T→G) polymorphism in the IL-2 gene promoter in control/moderate groups versus severe group (n=113).

	control/moderate	**severe**	**p***	**OR**
ALLELE	n (%)	n (%)		
T	119 (77.27)	50 (65.78)	0.027	1.99
G	31 (22.72)	26 (34.21)		(95% CI = 1.07-3.70)
GENOTYPE				
TT	47 (62.66)	15 (39.47)		
TG	25 (33.33)	20 (52.63)	0.062	---
GG	03 (04.00)	03 (07.89)		
GENOTYPE				
TT	47 (62.66)	15 (39.47)	0.019	2.57
TG/GG	28 (37.33)	23 (60.52)		(95% CI = 1.15-5.73)

p* < 0.05

ARTIGO 2



Investigation of *IL4* gene polymorphism in individuals with different levels of chronic periodontitis in a Brazilian population

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Investigation of *IL4* gene polymorphism in individuals with different levels of chronic periodontitis in a Brazilian population

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Short title: *IL4 gene polymorphism, chronic periodontitis*

Abstract

Background: Cytokines are key factors that mediate the inflammatory process during periodontal disease. Recent works have shown that the levels of cytokine expression are regulated by genetic polymorphisms, and that these variations can interfere with the progression of disease. The –590 (C→T) polymorphism of the *IL4* gene is associated with high levels of IgE in asthmatic families, and the frequency of the T allele was increased in asthmatic children. The concentration of IgE in gingival tissue was found to be elevated in patients with periodontitis.

Objective: In this study the relationship between the –590 (C→T) polymorphism in the *IL4* gene and different levels of chronic periodontal disease was investigated.

Material and Methods: DNA was extracted from buccal epithelial cells of 113 unrelated adult individuals with different levels of periodontitis. The PCR-RFLP technique was used to investigate the polymorphism in the promoter of *IL4* gene.

Results: No significant differences in the allele and genotype frequencies of the polymorphism were found between control and groups with periodontal disease.

Conclusion: We conclude that the –590 (C→T) polymorphism in the *IL4* gene is not associated with the susceptibility to chronic periodontal disease.

Key words: *IL4*; polymorphism; chronic periodontitis; risk factors.

Chronic periodontitis, the most frequent occurring form of periodontitis, is an infectious disease resulting in inflammation in the supporting tissues of the teeth, progressive attachment and bone loss. It is initiated and sustained by bacterial plaque, and is characterized by pocket formation and/or gingival recession (Lindhe et al. 1999). This form of periodontal disease (PD) can be considered as an immunological disease since localized chronic inflammation associated with gingiva exhibits several unique immunological features, which include elevated cellular and humoral immune responses (Fujihashi et al. 1993a). The local host response to bacteria includes the recruitment of leukocytes and the subsequent release of inflammatory mediators, like cytokines (Okada & Murakami 1998).

Periodontal lesions contain both T cells and macrophages, which can produce cytokines like interleukin-4 (*IL4*), interleukin-1 (*IL1*) and interleukin-6 (*IL6*). All of these cytokines have been shown to play a role in B cell activation, proliferation and differentiation (Tew et al. 1989). In established and advanced periodontitis lesions, the majority of infiltrating cells are B cells and plasma cells (Okada et al. 1983, Passo et al. 1988, Yamazaki et al. 1994). The proliferation of B cells is induced by mitogens, like *IL4* and lipopolyssaccharides (LPS) of periodontopathogenic bacteria especially *Actinobacillus actinomycetemcomitans* (*A.a.*). Interleukin-4 can induce resting B cells to enter S phase more promptly upon subsequent stimulation with LPS (Rabin et al. 1985, Tew et al. 1989). Interleukin-4 enhances immunoglobulin G (IgG) and immunoglobulin E (IgE) secretion by B cells stimulated with LPS (Snapper & Paul, 1987, Tew et al. 1989). IgG1 was shown to be the major IgG subclass secreted by isolated gingival mononuclear cells followed by IgG2 (Ogawa et al. 1989). Reinhardt et al. (1989) have shown that both IgG1 and IgG4 levels in gingival crevicular fluid (GCF) from periodontally active sites were significantly elevated

over stable areas. The concentration of IgE in gingival tissue was found to be elevated in patients with periodontitis (Hyyppä 1984). Another important role of the *IL4* in the immune response is the suppression of macrophage function (Essner et al. 1989, Hart et al. 1989). Yamamoto et al. (1997) showed that the addition of exogenous *IL4* induced apoptosis in macrophages isolated from inflamed gingival tissues. Several studies have investigated *IL4* at both mRNA and protein levels in periodontal lesions (Fujihashi et al. 1993a, Fujihashi et al. 1993b, Yamazaki et al. 1994) and serum of patients with periodontal disease (MacFarlane & Meickle 1991).

Polymorphisms in the promoter region of genes can modify the level of expression of proteins (McGuire et al. 1994, Lazarus et al. 1997, John et al. 1998). Individual differences in levels of interleukin-1 (*IL1*) related to susceptibility to PD are attributed to alleles of polymorphic genes. The allele 2 of the *IL1β* (+3953) gene was more prevalent in patients with chronic periodontitis, which indicates a connection between this polymorphism and periodontal severity in adults (Kornman et al. 1997, Gore et al. 1998). A polymorphism in the promoter of *IL2* gene was also associated with the severity of periodontal disease (Scarel-Caminaga et al. 2002). A polymorphism at position –590 (C→T) of the *IL4* gene promoter has been identified (Rosenwasser *et al.* 1995). This polymorphism was shown to increase luciferase activities and is associated with elevated levels of IgE phenotype in asthmatic families. Noguchi *et al.* (1998) showed that the –590 (C→T) polymorphism in *IL4* gene promoter is associated with the development of asthma in Japanese children. Since *IL4* seems to play a role in periodontal diseases, the investigation of genetic polymorphisms that affect its transcriptional activity may provide important information on

its function in periodontal diseases. Therefore, the purpose of this study was to investigate the –590 (C→T) polymorphism of *IL4* gene in individuals with different levels of chronic periodontitis.

Materials and Methods

Selection of subjects

Chronic periodontitis.

A convenience sample of 113 unrelated, non-smoking subjects >25 years of age, were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba – UNICAMP. The patients were from the Southeast region of Brazil. The baseline clinical parameters for the subject population are presented in Table 1. All subjects were in good general health and had at least 20 teeth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis; current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study. Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth, assessment of clinical attachment loss (CAL), tooth mobility and observation of bleeding on probing. Measurements of

probing depth and attachment level were recorded at 6 points around each tooth. Subjects were included in clinical categories according to PD generalized severity:

1. *Control group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth >3 mm (n = 44)
2. *Moderate Periodontitis*: Patients with teeth exhibiting ≥ 3 mm and ≤ 6 mm CAL (n = 31)
3. *Severe Periodontitis*: Patients with teeth exhibiting ≥ 7 mm CAL (n = 38)

Analysis of genetic polymorphisms

Cells were obtained through a mouthwash with 3% glucose solution and light scrapings of the buccal mucosa with a sterile wood spatula. DNA was extracted from epithelial buccal cells with sequential phenol/chlorophorm solution and precipitated with salt/ethanol solution (Trevilatto & Line 2000, Scarel et al. 2000). The *IL4* gene promoter region (accession number M23442) was amplified by PCR as described previously by Noguchi *et al.* (1998). The primer pair used was: Forward - 5' TAA ACT TGG GAG AAC ATG GT 3' and Reverse - 5' TGG GGA AAG ATA GAG TAA TA 3'. The original base in the position –594 of the *IL4* promoter gene is "T" which was altered to "G", creating a restriction site for the enzyme *Ava* II, which can cleave the PCR products in two fragments: 177 pb + 18 pb (allele 2, -590C). This establishes an efficient and simple RFLP method to detect the –590 (C→T) polymorphism in the *IL4* gene.

The PCR temperature profile included an initial denaturation step at 95°C for 5 min, followed by 35 cycles each of 95°C (1 min), 51°C (1 min) and 72°C (1 min), with a final extension step of 72°C (5 min). RFLP was performed in a final reaction volume of 20 μL using 1.5 U *Ava* II (Amersham Pharmacia Biotech) and 10 μL of PCR product (195 pb). The reactions took place overnight (ON) at 37°C. The products were analyzed in 10 % polyacrylamide gel electrophoresis. Gels were stained by rapid silver staining method (Sanguinetti et al. 1994).

The genotype and allele frequencies were calculated by direct counting and then dividing by number of chromosomes to produce allele frequency, or by the number of subjects to produce genotype frequency (Reynard et al. 2000). The allele and genotype distributions in the different groups were assessed by Chi-square (χ^2) test. Differences were considered significant when $p < 0.05$. In a separate analysis we compared groups control/moderate versus severe. In order to verify whether in different ethnic groups any allele or genotype could be associated with different levels of periodontitis, we also investigated the allele and genotype frequencies in Caucasian subjects separate from African-American, Mulatto and Asian subjects.

Results

Controls and groups with periodontal disease were genotyped according to the cleavage pattern of the *Ava* II enzyme restriction. The statistical analysis revealed no significant differences among the groups related to genotypic ($\chi^2 = 5.797$, $p = 0.215$) and allelic frequencies ($\chi^2 = 0.0231$, $p = 0.989$) (Table 2). When grouped together the control and

moderate groups to compare with the severe group, the genotypic ($\chi^2 = 0.401$, $p = 0.818$) and allelic frequencies ($\chi^2 = 0.001$, $p = 0.982$) showed no association between the *IL4* polymorphism and the severity of the disease. The analysis of the Caucasian subjects ($n = 87$) revealed the genotypic ($\chi^2 = 2.806$, $p = 0.591$) and allelic ($\chi^2 = 0.678$, $p = 0.713$) frequencies. Despite the fewer number of individuals in the subgroup formed by Afro-American, Mulatto and Asian subjects ($n = 26$), a separated analysis was made, and the results of the genotypic ($\chi^2 = 6.282$, $p = 0.179$) and allelic ($\chi^2 = 2.363$, $p = 0.307$) frequencies showed similar results when compared to the whole sample. This fact indicates that regardless of the ethnic group there were no associations between the –590 (C→T) *IL4* gene polymorphism and chronic periodontitis.

The Hardy-Weinberg analysis of both Causasian subjects ($p > 0.05$; $\chi^2 = 0.1067$) and the whole group (including Afro-American, Mulatto and Asian, $p > 0.05$; $\chi^2 = 1.3591$) showed that the frequencies fell into the expected equilibrium and were randomly distributed.

Discussion

Gene polymorphisms may cause significant changes in function by altering the levels or activity of specific proteins. Therefore, disease-associated genetic polymorphisms may reveal which elements within a complex network of proteins are critical in determining the risk and the severity of disease (Shirodaria et al. 2000). Recent studies have demonstrated an association of the allele T at position –590 from the open reading frame of the *IL4* gene with asthma/atopy in USA and Japanese populations (Rosenwasser et al. 1995, Noguchi et al. 1998). Atopic allergy and asthma might be comparable to periodontal disease since they are multifactorial diseases that elicit the immune response and are determined by

interactions between several genes and important non-genetic factors, like the environment. In atopy and asthma, the level and the frequency of exposure to numerous environmental allergens, such as mold, are key components of the complex mosaic that determines the expression of these allergic diseases (Barnes & Mash 1998). In the case of periodontal disease, the environmental factors that influence the inflammatory response in periodontal tissues are periodontopathogenic bacteria accumulated in the subgingival region (Wilson et al. 1996). Asthma is an immune disorder characterized by an enhanced IgE production. Asthmatic patients have elevated values of IgE in their whole saliva and in gingival tissue (Hyyppä 1984). Children with allergic asthma showed salivary high IgE concentrations and signs of more severe gingival inflammation than their healthy controls (Hyyppä 1980, Hyyppä et al. 1979). Interleukin-4 plays a crucial role in asthma, due to its action on B cells to switch the Ig isotype from IgM to IgE and to the maturation of T-helper cells to the Th2 phenotypes (Noguchi et al. 1998, Hijazi & Haider 2000). An increase of IgE has been observed in gingival tissue of individuals with chronic periodontitis (Hyyppä 1984). In periodontal lesions *IL4* can activate B cells to produce IgE and IgG against LPS of periodontal bacteria (Tew et al. 1989). Due to these aspects, seemingly the immune mechanisms of allergic disorders may be similar to periodontal disease. Therefore the investigation of –590 (C→T) polymorphism in the *IL4* gene, was directed with the aim to verify if such a polymorphism could serve as a marker of susceptibility to periodontal diseases.

This is the first study in which a polymorphic marker in the promoter of the *IL4* gene was used with regard to severity of periodontal disease. We did not find an association

between the investigated polymorphism and severity levels of chronic periodontitis in Brazilian patients. It is possible that different results might be encountered in other populations or racial groups. As racial differences are common in polymorphic systems (Mourant et al. 1976), variant alleles or groups of interacting genes lead to the expression of the clinical phenotype (Barnes & Marsh 1998) in different populations. Perhaps this concept could explain the different results regarding *IL4* promoter polymorphism and susceptibility to asthma. Despite the reports of the association of the –590T polymorphism in the *IL4* gene in US and Japanese asthmatic subjects (Rosenwasser et al. 1995, Noguchi et al. 1998), different results are presented by Walley & Cookson (1996) and Hijazi & Haider (2000) in studies with UK/Australian families and Kuwaiti Arab population, respectively. The separated analysis of Caucasians subjects and the subgroup formed by Afro-American, Mulatto and Asian subjects, indicate that the racial background does not influence the negative association between periodontal disease and the –590 polymorphism of *IL4* gene. It is worth mentioning that the Brazilian population is highly heterogeneous, with Native American, African and European ancestry (Alves-Silva et al. 2000). Europeans are the predominant ancestry in the Brazilian Southern region (IBGE, 1995 – accession http://www.ibge.gov.br) and it is consistent with the predominance of Caucasoid individuals in our sample. This ethnic heterogeneity may contribute through several events of recombination, to the complex genetic network involved in immune diseases.

Although the polymorphism studied in this work does not appear to be associated with periodontal diseases, there are other polymorphisms in the *IL4* gene that could be investigated, like a variation in the intron 2 which is associated with severe asthma (Chouchane et at. 1999). We conclude that the restriction fragment length polymorphism

(RFLP) of the *IL4* gene at the position –590 (C →T) is not associated with the susceptibility to chronic periodontal disease in the Brazilian population.

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Table 1. Baseline clinical parameters of the subject population (n=113)

	control (n=44)	**moderate** (n=31)	**severe** (n=38)
AGE (years)			
mean (± SD)	43.2 (± 14.0)	36.9 (± 11.2)	43.6 (± 14.4)
GENDER %			
Female	68.2	80.6	84.2
Male	31.8	19.4	15.8
ETHNIC GROUP %			
Caucasoid	84.1	77.4	68.4
Afro-American	06.8	16.1	13.2
Mulatto	06.8	06.5	18.4
Asian	02.3	0.0	0.0

Table 2. Allele and genotype frequencies of the –590 (C →T) polymorphism in the *IL4* gene promoter in healthy patients and patients with different levels of periodontitis (n=113 – whole sample).

	control	**moderate**	**severe**	**p**
ALLELE	n (%)	n (%)	n (%)	
T	33 (37.5)	24 (38.71)	29 (38.16)	0.989
C	55 (62.5)	38 (61.29)	47 (61.84)	
GENOTYPE				
TT	08 (18.18)	02 (06.45)	04 (10.53)	
TC	17 (38.64)	20 (64.52)	21 (55.26)	0.215
CC	19 (43.18)	09 (29.03)	13 (34.21)	

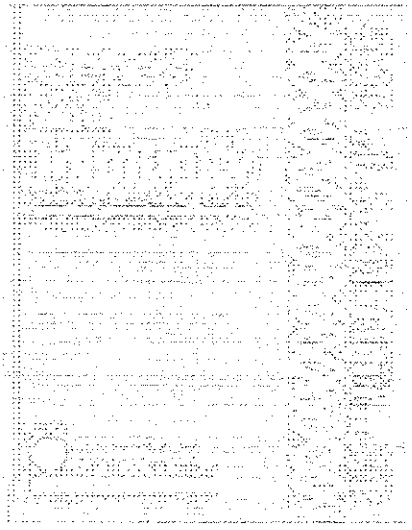
Table 3. Allele and genotype frequencies of the –590 (C →T) polymorphism in the *IL4* gene promoter in the Caucasoid subjects (n=87).

	control	**moderate**	**severe**	**p**
ALLELE	n (%)	n (%)	n (%)	
T	23 (31.08)	18 (37.5)	16 (30.76)	0.713
C	51 (68.91)	30 (62.5)	36 (69.23)	
GENOTYPE				
TT	04 (10.81)	02 (08.33)	01 (03.84)	
TC	15 (40.54)	14 (58.33)	14 (53.84)	0.591
CC	18 (48.64)	08 (33.33)	11 (42.30)	

Table 4. Allele and genotype frequencies of the –590 (C →T) polymorphism in the *IL4* gene promoter in the group formed by Afro-American, Mulatto and Asian subjects (n=26).

	control	**moderate**	**severe**	**p**
ALLELE	n (%)	n (%)	n (%)	
T	10 (71.42)	06 (42.85)	13 (54.16)	0.307
C	04 (28.57)	08 (57.14)	11 (45.83)	
GENOTYPE				
TT	04 (57.14)	00 (00)	03 (25.0)	
TC	02 (28.57)	06 (85.71)	07 (58.33)	0.179
CC	01 (14.28)	01 (14.28)	02 (16.66)	

ARTIGO 3



Interleukin10 gene Promoter Polymorphisms are associated with Chronic Periodontitis

Genes and Immunity, submitted

Interleukin10 gene Promoter Polymorphisms are Associated with Chronic Periodontitis

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Summary

Chronic periodontitis (CP) is characterized by an inflammation in the supporting tissues of the teeth caused primarily by bacterial infection. Interleukin 10 (IL10) is an anti-inflammatory cytokine that can stimulate the proliferation and differentiation of B cells. It has been demonstrated that polymorphisms in the promoter region of the *IL10* gene may influence the expression of this gene and can be transmitted in a haplotype fashion. The aim of the present study was to investigate the relationship between three single-nucleotide polymorphisms (SNPs), located at –1082, –819 and –592 base pair from the transcriptional start site of the *IL10* gene, and Chronic Periodontitis. DNA was extracted from buccal epithelial cells of 110 unrelated adult individuals control and with different levels of periodontitis. DNA sequencing was employed in the investigation of –1082 polymorphism, and PCR-RFLP in the –819 and –592 polymorphisms. Significant differences in the –819 and –592 SNPs were obtained comparing Control and CP groups in the total sample. When the same analysis was made only with female subjects the association between the those SNPs and CP was stronger. The genotype frequency of haplotypes showed that the GCC/ACC was predominant in the Control group for the total sample and especially for women (OR = 8.83; 95% CI=1.69-46.03, p = 0.009). The ACC haplotype was prevalent in the Control and the ATA haplotype in the CP group. In female subjects the ATA haplotype seemed to increase susceptibility to CP (OR = 2.57; 95% CI=1.10-5.95, p = 0.043).We conclude that specific haplotypes and the SNPs –819 and –592 in the promoter of *IL10* gene could serve as useful markers of susceptibility to Chronic Periodontitis in Brazilian patients.

Introduction

Chronic periodontitis (CP) is an infectious disease resulting in inflammation in the supporting tissues of the teeth (Lindhe et al. 1999). CP has been reported to affect more than 30% of the population, with severe disease reported in 7-13% (Kornman et al. 1997). The primary cause of the CP is a bacterial infection since several bacterial species reside in a biofilm on tooth surfaces, referred to as dental plaque (Flemmig 1999). Over past decade, a growing body of scientific evidence suggests an association between oral infection and systemic diseases (ie, atherosclerosis, cardiovascular disease, arthritis, diabetes, pulmonary diseases) (Cohen and Slavkin 2000). Consequently, a new discipline, periodontal medicine has emerged in dentistry.

Bacteria present in a biofilm adjacent to the gingiva stimulate host cells (ie, fibroblasts, macrophages and polymorphonuclear leukocytes) to secrete pro-inflammatory cytokines (Kornman and Newman 2000). The process of disease can continue by the formation of periodontal pocket which contains pathogenic bacteria. The bacteria maintain the immune system activated resulting in tissue destruction, such as loss of collagen fibers subjacent to the pocket epithelium and alveolar bone loss (Page 1991; Okada and Murakami 1998). The clinical features of CP include clinical attachment loss of gingival epithelium (CAL), gingival inflammation, bleeding of the gingiva following application of pressure, and in a more severe level, increased mobility and tooth exfoliation (Flemmig 1999).

When inflammatory responses are generated in any given tissue, the expression of a variety of cytokines is generally increased at the site and then down-regulated to control the local inflammatory response. There is a complex network of pro and anti-inflammatory

cytokines acting in the inflamed periodontal tissues (Okada and Murakami 1998). Many efforts have been done to elucidate the cytokine mechanisms which interfere in the periodontal disease progression. One important cytokine which has been investigated in periodontal disease and in other inflammatory diseases is interleukin10 (IL10) (Perez et al. 1995; Nozaki et al. 1997).

IL10 is an anti-inflammatory cytokine produced by T-helper 2 cells (Th2), macrophages and B cells and may play a modulatory role because it inhibits cytokine synthesis by Th1 cells (IL2 and interferon-γ) (Fiorentino et al. 1989). IL10 also inhibits inflammatory cytokines such as IL1, tumor necrosis factor (TNF-α), IL6 and IL8 (de Waal Malefyt et al 1991*a*). However, IL10 is a B cell stimulator, enhancing B cell proliferation and differentiation (de Waal Malefyt et al 1991*b*). Therefore, IL10 can play important roles in the regulation of cellular and humoral immune responses, representing a good candidate gene in autoimmunity (Hajeer et al. 1998).

The gene encoding IL10 has been mapped to chromosome 1 (1q31-q32 [MIM 124092], Kim et al. 1992) and several polymorphisms in the 5' –promoter region of the *IL10* gene have been identified. These promoter polymorphisms are associated with altered synthesis of IL10 in response to inflammatory stimuli (Turner et al. 1997; Barnes 2001). The single-nucleotide polymorphisms in the promoter region of the *IL10* gene which have been more studied are located at positions –1082, –819 and –592 from the transcriptional start site. The polymorphism at position –1082 is a G to A substitution and lies within a putative Ets transcription factor binding site (Kube et al. 1995). The –1082G allele is known to be associated with high *in vitro* IL10 production (Turner et al. 1997). The position –819 of the

IL10 gene presents a dimorphic polymorphism, a C to T substitution, and may affect an estrogen responsive element (unpublished data in Lazarus et al. 1997). The –592 polymorphism is a C to A substitution and lies within a region with a negative regulatory function (Kube et al. 1995). The three dimorphisms exhibit strong linkage disequilibrium and appear in three preference potential haplotypes: GCC (G at position –1082, C at position –819 and C at position –592), ACC and ATA (Turner et al. 1997). The GCC haplotype has been more frequent in individuals with systemic lupus erithematosus (SLE) (Lazarus et al. 1997). The ACC haplotype has significantly been increased in patients with rheumatoid arthritis (RA), who are positive for IgA rheumatoid factor (Hajeer et al. 1998). These facts suggest that IL10 may play a key role in promoting disease severity and this may be genetically determined (Barnes 2001).

The inflammatory process in SLE and RA has a multifactorial character, which is similar to CP, and IL10 has been thought to play a role in all of them. It is worth mentioning that the epidemiological studies indicate that periodontitis is widespread among the Brazilian population (Gjermo et al. 1984; Dini et al. 1994). The purpose of this study was to investigate the cited polymorphisms in the *IL10* gene and the haplotypes in individuals with different levels of chronic periodontitis, to verify if such polymorphisms could serve as markers of susceptibility to or severity of Chronic Periodontitis.

Subjects and Methods

Subjects

A convenience sample of 110 unrelated, non-smoking subjects >25 years of age, were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of

Piracicaba – UNICAMP. The patients are from the Southeastern region of Brazil. The baseline clinical parameters for the subject population are presented in table 1. All subjects were in good general health and had at least 20 teeth in the mouth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis; current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board (85/2000), signed a consent form after being advised of the nature of the study. Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth, assessment of clinical attachment loss (CAL), tooth mobility and observation of bleeding on probing. Measurements of probing depth and attachment level were recorded at 6 points around each tooth. Subjects were included in clinical categories according to PD generalized severity:

1. *Control group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth >3 mm (n = 43)
2. *Chronic Periodontitis group*: formed by two subgroups with different levels of the disease. The subgroups are: *Moderate Periodontitis* - Patients with teeth exhibiting

≥ 3 mm and ≤ 6 mm CAL (n = 31), and *Severe Periodontitis* - Patients with teeth exhibiting ≥ 7 mm CAL (n = 36)

Analysis of genetic polymorphisms

Cells from subjects were obtained through a mouthwash with 3% glucose solution and light scrapings of the buccal mucosa with a sterile wood spatula. DNA was extracted from epithelial buccal cells with sequential phenol/chlorophorm solution and precipitated with salt/ethanol solution (Trevilatto and Line 2000, Scarel et al. 2000). The *IL10* gene promoter region (Genbank accession number X78437) was amplified by PCR (total volume of 50 μL) in two fragments:

Promoter region of *IL10* (Fragment 1): utilizing the Primers Reverse - 5' TTC TGT GGC TGG AGT CTA AAG TT 3' and Forward - 5' TTC CTC CCA GTT ACA GTC TA 3' (1 μM of each primer), amplification reactions were performed with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM of each dNTP, 500 ng genomic DNA and 2.5 units *Taq* DNA Polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). The PCR temperature profile included an initial denaturation step at 95°C for 5 min, followed by 35 cycles each of 95°C (1 min), 59°C (1 min) and 72°C (1 min), with a final extension step of 72°C (5 min).

Promoter region of *IL10* (Fragment 2): amplification reactions were performed utilizing the Primers Reverse - 5' GGT CTC TGG GCC TTA GTT TCC 3' and Forward - 5' AAC TTT AGA CTC CAG CCA CAG AA 3' in the same conditions described above. After an

initial denaturation at 95°C for 5 min, the 35 cycle profile was as follows: 95°C (1 min), 62°C (1 min) and 72°C (1 min), with a final extension step of 72°C (5 min).

The amplification of 395 bp (fragment 1) of *IL10* promoter region (containing the –1082 SNP) was submitted to purification by eletrophoresis on 2% agarose gel followed by the use of a DNA purification Kit (GFX PCR DNA and Gel Band Purification Kit - Amersham Pharmacia Biotech). Polymorphisms were investigated by sequencing in a total reaction of 10 μL containing 400 ng purified PCR-products, 4 μL DYEnamic ET Terminator (Amersham Pharmacia Biotech) and 3.2 pmol reverse primer, using the ABI 377 DNA Sequencer (Perkin-Elmer Corporation, Foster City, CA).

In order to verify the –819 and –592 SNPs, the fragment 2 of *IL10* promoter region (492 bp) was amplified and the RFLP (Restriction Fragment Length Polymorphism) method was performed similarly as described by Eskdale et al. (1997), but with different band lengths due to the different primers synthesized. Briefly, with respect to the –819 SNP, it was used 1 U *Mae*III (Boehringer-Mannheim) and 6 μL of PCR product in a final volume of 20 μL, which was digested overnight (ON) at 55°C. The products were analyzed in 10 % polyacrylamide gel electrophoresis and stained by rapid silver staining method (Sanguinetti et al. 1994). The digestion with *Mae*III yielded 2 bands (368 and 124 bp) where –819 base was T, and where base –819 was C, 3 bands were generated (217, 151 and 124 bp). Similarly, to analyze the –592 SNP, a reaction volume of 15 μL, containing 2 U of the enzyme restriction *Rsa* I, was used to digest 10 μL of PCR product (492 bp) ON at 37°C. It was generated 3 bands (350, 92 and 50 bp) when the base –592 was C and 4 bands (240, 110, 92 and 50 bp) when the base –592 was A.

Statistical Analysis

The genotype and allele frequencies of the SNPs were calculated by direct counting and then dividing by the number of subjects to produce genotype frequency, or by number of chromosomes to produce allele frequency (Reynard et al. 2000). The frequencies of haplotypes formed by the SNPs were calculated by direct counting, considering the three putative haplotypes described in Turner et al. (1997). In order to calculate gene heterozygosity, to verify the fit to Hardy-Weinberg expectations and linkage disequilibrium, it was used the computer program package ARLEQUIN (v. 2.0 - Schneider et al. 2000). This computer program was also employed to calculate haplotype frequencies because we noticed different putative haplotypes. The computer program package ARLEQUIN calculated the maximum-likelihood haplotype frequencies using an Expectation-Maximization algorithm (Dempster et al. 1977; Excoffier and Slatkin 1995), and it was able to estimate the haplotype frequencies from multi-locus genotype data when the gametic phase was unknown (missing parental genotypes).

The significance of the differences in observed frequencies of each polymorphism in Control and subgroups with different levels of CP (Moderate and Severe) was assessed by standard Chi-squared (χ^2) or Fisher's exact tests using the computer program BioEstat (v 1.0 – Ayres 1998) and SAS statistical package (v.6.11; SAS Institute, 1996). Differences were considered significant when $p < 0.05$, and the risk associated with individual alleles or genotypes was calculated as the odds ratio (OR) with 95% confidence intervals (CI). Considering the data between groups Control and CP (Moderate + Severe) it was used the CLUMP program (Sham and Curtis 1995) to analyze the genotype frequencies. This

program is designed for use in genetic case-control studies where multiple alleles are being considered and the observed frequencies of some alleles are rare. The significance of differences in allele frequencies is assessed in a 2xN table using Monte Carlo simulations. This program is able to "clump" columns together when they are greater than 2 columns table and produces maximal chi-squared values. The statistic produced is the T4 value, which is equivalent to the chi-squared value of the difference in frequencies of all the alleles clumped together. The use of Monte Carlo method avoids the need for a Bonferroni correction and the difficulty of assessing the significance of rarer alleles. One thousand simulations were performed for each SNP and haplotypes data. A p-value of <0.05 was taken to be statistically significant. It is worth mentioning that the CLUMP program was used in a study focusing individuals with periodontitis and polymorphisms in the *IL1* gene (Hodge et al. 2001).

Results

Frequency Analysis in the Control and subgroups with different levels of CP

Table 2 shows the allele and genotype frequencies of the three *IL10* gene promoter polymorphisms in the Control and subgroups with CP (moderate and severe levels). No significant differences were found in the –1082 position of promoter of *IL10* gene. The allelic frequency of the –819 polymorphism suggests an association with CP ($p = 0.064$) which is clearly seen in the genotypic frequency ($p = 0.028$). The –592 polymorphism was statistically different in the genotype frequency ($p = 0.000$) using the Fisher's exact test.

Frequency Analysis in the Control and Chronic Periodontitis group

The data of total sample (n = 110) and only female subjects (n = 83) were analyzed in table 3. The Chronic Periodontitis (CP) group was formed by moderate and severe subgroups together. No significant results were found in the total and female samples regarding –1082 *IL10* polymorphism.

Table 3 showed that the –819 SNP of the *IL10* gene, in the total sample, had a significant difference in the genotype frequency (p = 0.017) which was confirmed when the CC genotype was confronted with CT/TT and the p value changed to 0.013. Individuals with CC genotype seemed to be three times less susceptible to Chronic Periodontitis (OR = 3.04; 95% CI = 1.34-6.91). Since Lazarus et al. (1997) commented that the –819 polymorphism may affect an estrogen receptor element, the same analysis was made only in female subjects (table 3). Interestingly, the –819C allele was more prevalent in the Control group (75%; p = 0.017) with an OR = 2.5 (95% CI = 1.23-5.1). Therefore women with the C allele at –819 SNP seemed to be 2.5 times less susceptible to develop CP, and when the homozygous CC genotype was confronted with CT/TT, the OR increased to 4.13 (95% CI = 1.55-11.02).

The dimorphic locus –592 of *IL10* gene revealed the same tendency observed in the analysis for the –819 locus. The genotype frequencies of –592 locus were significantly different for the total sample (p = 0.007) and for the female sample (p = 0.012) (table 3), where it was possible to note the predominance of the CC genotype in the Control group. In the total sample, individuals with CC genotype seemed to be over than twice less likely to develop Chronic Periodontitis than individuals with CA/AA genotypes (OR = 2.41; 95%

CI = 1.08-5.36). The odds ratio increased to 3.38 when only female subjects were analysed (OR = 3.38; 95% CI = 1.29-8.82).

Haplotype Frequencies

The distribution of the haplotypes arranged as alleles is shown in table 4. For the total sample (n=110) there was no difference statistically significant (p = 0.179) between Control and CP groups. In the same table 4, the distribution of the haplotypes in female subjects (n=83) suggested a significant difference (p = 0.061). Figure 1 shows, in female subjects, the predominance of haplotype ACC in the Control group and the predominance of haplotype ATA in the Chronic Periodontitis group. In female subjects the haplotype ATA seemed to increase susceptibility to Chronic Periodontitis in 2.5 times (OR = 2.57; 95% CI = 1.10-5.95, p = 0.043). In table 4, it was possible to note the presence of rare haplotypes: GTA, ATC, GTC and ACA. The GTA haplotype was found in a Southern Chinese population in a frequency of 4% (Mok et al. 1998). In this study, if the total sample (n=110) were taken as a whole group (due to the absence of statistical difference between Control and CP groups), the frequency of GTA haplotype was 1.8%. Table 4 also showed a new haplotype, since its presence could not be seen in the literature. The ATC haplotype was present in the Southeast Brazilian population in a frequency of 4%.

Considering the three SNPs independently investigated, the genotype distributions were consistent with the assumption of Hardy-Weinberg equilibrium in the Control group of the total sample (n=110). Regarding the CP group, the –1082 locus was in the expected Hardy-Weinberg equilibrium; however, the loci –819 and –592 failed to fit to Hardy-Weinberg equilibrium, due to an excess of heterozygotes (locus –819, observed frequency 62.6% and

expected frequency 55%, p = 0.04; locus –592 observed frequency 67% and expected frequency 52.8%, p = 0.0004). The computer program ARLEQUIN assesses the percentage of gene heterozygosity by the formula of Nei (1987). In our population, considering the total sample, there was high percentage of gene heterozygosity (Control = 0.7012 +/- 0.0163; Chronic Periodontitis = 0.7330 +/- 0.0152). These results reveal the importance of the organization of the data as genotypes.

Table 5 shows haplotypes arranged as homozygous or heterozygous fashion in the Control and CP groups. There was significant difference in the frequency of the haplotypes in the total sample (p = 0.011) and in female sample (p = 0.051). The graphical distribution of haplotypes arranged as genotypes for female subjects can be observed in figure 2. The heterozygous haplotype GCC/ACC was increased in Control groups when compared to CP groups. In order to assess the biological importance of this fact, the haplotype GCC/ACC was compared to other haplotypes taken together, to obtain the "p" and OR values. Considering the total sample, there was a significant difference of haplotype GCC/ACC between Control and CP groups (OR = 8.26; 95% CI = 2.17-31.4, p = 0.001). When only female subjects were analyzed it was obtained p = 0.009 and the OR increased to 8.83 (95% CI = 1.69-46.03).

Discussion

Allelic variations in cytokine genes and factors regulating their expression result in phenotypic differences in cytokine responses between individuals, which can be important in disease susceptibility and progression (Bidwell et al. 1999; Yamazaki et al. 2001). The

first report showing association between severity of periodontitis and cytokine genes was published by Kornman et al. (1997), who investigated polymorphisms in the *IL1* gene cluster. After that, other cytokine genes were investigated, as previously demonstrated by our group, which revealed positive associations between variations in cytokine genes and periodontal disease (Scarel-Caminaga et al. 2002*a*; Souza et al. 2003; Trevilatto et al. 2003). In the present study, it was demonstrated that polymorphisms in the promoter region of the *IL10* at positions –819 and –592 are associated with susceptibility to Chronic Periodontitis, especially in women (table 3). The –819 polymorphism lies within a DNA motif forming a putative estrogen responsive element (unpublished data in Lazarus et al. 1997). A previous study about Systemic Lupus Erythematosus (SLE) showed a strong association between the production of anti-Ro autoantibodies and a Cytosine at positions –819 and –592 of *IL10* gene. For each position, the frequency of C was 93% and the OR was 6.2 (95% CI = 1.7-27.1; $p = 0.003$) in Ro+ patients compared with the Ro- group (Lazarus et al. 1997). These results were obtained in a group with SLE showing male: female ratio of 1:12. The authors commented that it may not be coincidental that SLE is almost 10 times more prevalent in women than in men, and that women taking oral contraceptives are more prone to SLE. However, the real role of the estrogen in the regulation of the IL10 production is not clear.

It is interesting to observe that in both studies (SLE and this study with CP), the patients were predominantly women, and that the genetic status can influence the predisposition of women to disease. However, while the C alleles in the –819 and –592 positions of *IL10* gene promoter seem to be associated with SLE disease, the same C alleles in homozygous fashion seem to "protect" individuals against CP. This apparent discrepancy may be

explained by the fact that the –1082, –819 and –592 polymorphisms are frequently inherited together because their high proximity in the chromosome, so that it is referred to as a haplotype (Lewin 1997). Also, the dual role of IL10 must be remembered: it can act as an anti-inflammatory cytokine but it stimulates B cell proliferation and immunoglobulin secretion. The haplotype GCC has been associated with high production of IL10 (Turner et al. 1997) and individuals with SLE had the production of autoantibodies only in the presence of GCC haplotype. As the SLE is an autoimmune disease whose pathogenesis is attributed to the production of autoantibodies, individuals with the high producer haplotype of IL10 are more prone to develop SLE. Regarding Chronic Periodontitis, although there was no statistical association between the –1082 SNP and CP, the predominance of CC genotype both in –819 and –592 positions in the Control group may indicate a genetic combination that could collaborate to high production of IL10. Individuals who are high producers of IL10 might be more protected against CP due to the anti-inflammatory role of IL10. A periodontal lesion begins with typical acute inflammation, dominated by polymorphonuclear leukocytes, and continues with the presence of T-helper lymphocytes, which secrete inflammatory cytokines such as IL1, IL2 and IL6 (Tew et al. 1989). Therefore, a genetically determined increase of anti-inflammatory IL10 cytokine would down regulate the immune response against periodontopathogenic bacteria which are involved in periodontitis.

Previous studies about the expression of human IL10 defined the location of transcription factor binding motifs in the immediate promoter of this gene (Kube et al. 1995; Eskdale et al. 1997). Polymorphisms that influence transcription factor interaction with gene promoters modify promoter "strength", thereby the gene expression can be

modulated. The –592 *IL10* polymorphism is located between consensus sequences for DNA binding by two types of transcription factors: members of the Ets family and Sp1 (Hobbs et al. 1998). Individuals with Adenine at –592 locus showed increased production of IgE in a study of families with asthmatic probands (Hobbs et al. 1998). Accordingly, patients who had inherited the C allele at –592 seemed to present a better prognosis than others, similarly to what happened in Chronic Periodontitis. However, the cited polymorphism and total IgE cannot predict whether this polymorphism acts to promote or inhibit IL10 production (Hobbs et al., 1998). The –592 polymorphism was located within a region identified as negative regulatory sequence (Kube et al. 1995).

There is a tendency to clarify the involvement of IL10 in periodontal disease. It was demonstrated that mRNA expression for *IL10* in periodontitis lesions was significantly higher compared to autologous peripheral blood mononuclear cells (PBMC) (Yamazaki et al. 1997). Recently, an analysis of microsatellite polymorphisms failed to find a relationship between *IL10* and early-onset periodontitis (EOP) (Kinane et al. 1999). Japanese patients with chronic and generalized early-onset periodontitis were genotyped to the –1082, –819 and –592 SNPs in *IL10* gene, and no significant differences were found in allele or haplotype frequencies between patients and controls (Yamazaki et al. 2001). In our study, it was observed a significant relationship between the CC genotype both at –819 and –592 loci in *IL10* gene in the Control group compared to the Chronic Periodontitis group, with a relevant OR values (table 3). Moreover the haplotype analysis also showed interesting results. Due to the high percentage of heterozygosity in both Control and CP groups, we investigated the frequency of haplotypes arranged as genotypes. We found a predominance of the heterozygous haplotype GCC/ACC in the Control group compared to

other genotypes with significant statistical differences and OR values in the total sample and especially in female subjects (Table 5). Again, our results revealed genetic variations in Chronic Periodontitis with regard to the gender, and do not seem to be a coincidence. Actually, the GCC/ACC haplotype configures a heterozygosity with respect to the –1082 locus (G/A). It is worth mentioning that PBMC of individuals with –1082G are higher producers of IL10 than those with –1082A (Turner et al.1997). In order to explain the majority of GCC/ACC haplotype in control individuals compared to other genotypes, we speculate that the GCC/ACC haplotype would secrete intermediate levels of IL10 than homozygous GCC, what was suggested by Lazarus et al (1997). Very high levels of IL10 instead of producing positive behavior of immune response might have inhibited this response (Romagnani 1991).

Considering haplotypes arranged as alleles (Table 4), we observed especially in women, that the ACC haplotype was predominant in the Control group (39.3%) and the ATA haplotype was predominant in the Chronic Periodontitis group (37.3%). Among women, the ATA haplotype seemed to be 2.5 times more likely to develop CP than ACC haplotype. It is known that ATA/ATA genotype is associated with lower IL10 production following microbial lipopolysaccharide (LPS) stimulation than other genotypes (Crawley et al. 1999; Yamazaki et al. 2001). Therefore, it is biologically understandable that in the Control group there is a predominance of ACC haplotype, which secretes higher levels of anti-inflammatory IL10 compared to the ATA haplotype. Individuals in the diseased group with ATA haplotype would have lower levels of IL10 which could explain the excess of inflammatory cytokines present in periodontal lesions of the chronic form of the disease.

Other studies (Kinane et al. 1999;Yamazaki et al. 2001) have failed to show an association between polymorphisms in *IL10* gene and periodontal diseases probably due to two reasons: (i) genotypic differences in cytokine genes are differently inherited to ethnically different populations (Scarel-Caminaga et al. 2002*b*); (ii) the presence of high number of transcription factor binding motifs in the promoter of *IL10* gene, many of them are potential cytokine response elements (Eskdale et al. 1997). It is suggested that the expression of *IL10* gene may be influenced by other inflammatory cytokines. In this way, the IL10 does not act in isolation but its important roles in inflammatory diseases function within a cytokine network.

The haplotype analysis revealed rare haplotypes as GTA, only described in Southeastern Chinese populations (Mok et al. 1998), and a high percentage of gene heterozygosity for our population. These facts could indicate the genetic heterogeneity of the Southeastern Brazilian population, probably due to the high miscegenation of the races (Alves-Silva et al. 2000; Carvalho-Silva et al. 2001). It is worth mentioning the finding of the ATC haplotype in our population since it had never been reported in the literature.

We conclude that the –819 and –592 SNPs in the promoter of *IL10* gene serve as markers of susceptibility to Chronic Periodontitis in the studied population. Regarding to haplotypes in the promoter of *IL10* gene, there was a predominance of GCC/ACC in the Control group, and the ATA haplotype was associated to the susceptibility of the Chronic Periodontitis in Brazilian patients.

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Eletronic-Database Information

Accession numbers and URLs for data in this article are as follows:

David Curtis, http://www.mds.qmw.ac.uk/statgen/dcurtis/software.html (for CLUMP computer program)

GenBank, http://www.ncbi.nlm.nih.gov/GenBank/ (for accession number X78437)

Genetics and Biometry Laboratory, http://anthro.unige.ch/arlequin (for ARLEQUIN computer program)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for IL10 [MIM 124092])

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Table 1

Baseline clinical parameters of the subject population (n=110)

	control (n=43)	**moderate** (n=31)	**severe** (n=36)	**total** (n=110)
AGE (years)				
mean (± SD)	43 (± 14.0)	37 (± 11)	44 (± 10.5)	41.2 (12.5)
GENDER	n (%)	n (%)	n (%)	n (%)
Female	28 (65.1)	25 (80.6)	30 (83.4)	83 (75.5)
Male	15 (34.9)	6 (19.4)	6 (16.6)	27 (24.5)
ETHNIC GROUP	n (%)	n (%)	n (%)	n (%)
Caucasoid	36 (83.7)	24 (77.4)	24 (66.7)	84 (76.3)
Afro-American	3 (7.0)	5 (16.1)	5 (13.9)	13 (11.8)
Mulatto	3 (7.0)	2 (6.5)	7 (19.4)	12 (10.9)
Japanese	1 (2.3)	0.0	0.0	1 (0.9)

Table 2

Allele and genotype frequencies of the polymorphisms in the *IL10* gene promoter in Control and patients with different levels of periodontitis (n=110).

SNP[a]	Control n[b] (%)[c]	Moderate n[b] (%)[c]	Severe n[b] (%)[c]	p[d]
-1082				
Allele	**n = 86**	**n = 62**	**n = 72**	0.385
A	55 (64.0)	46 (74.2)	47 (65.3)	
G	31 (36.0)	16 (25.8)	25 (34.7)	
Genotype	**n = 43**	**n = 31**	**n = 36**	
AA	17 (39.5)	17 (54.8)	17 (47.2)	0.546[#]
AG	21 (49.0)	12 (38.7)	13 (36.1)	
GG	05 (11.6)	02 (06.5)	06 (16.7)	
-819				
Allele	**n = 86**	**n = 62**	**n = 72**	0.064
C	58 (67.4)	30 (48.4)	44 (61.1)	
T	28 (32.6)	32 (51.6)	28 (38.9)	
Genotype	**n = 43**	**n = 31**	**n = 36**	
CC	21 (48.8)	05 (16.1)	11 (30.6)	0.028[#]
CT	16 (37.2)	20 (64.5)	22 (61.1)	
TT	06 (14.0)	06 (19.4)	03 (08.3)	
-592				
Allele	**n = 86**	**n = 62**	**n = 72**	0.249
C	59 (68.6)	35 (56.6)	49 (68.0)	
A	27 (31.4)	27 (43.5)	23 (32.0)	
Genotype	**n = 43**	**n = 31**	**n = 36**	
CC	21 (48.8)	06 (19.4)	13 (36.1)	0.000[#]
CA	17 (39.5)	23 (74.2)	23 (63.9)	
AA	05 (11.6)	02 (06.4)	00 (00.00)	

[a] Single-Nucleotide Polymorphism.

[b] n = absolute number.

[c] (%) = relative number.

[d] p values marked by ([#]) were calculated by Fisher's exact test and p values not marked were calculated by Chi-squared test.

Table 3

Frequencies of the *IL10* gene polymorphisms in Control and Chronic Periodontitis individuals.

SNP[a]	Total (n = 110) Control n[d] (%)[e]	Total (n = 110) CP[b] n[d] (%)[e]	p^c	Female Subjects (n = 83) Control n[d] (%)[c]	Female Subjects (n = 83) CP[b] n[d] (%)[e]	p^c
-1082						
Allele	**n = 86**	**n = 134**	0.488	**n=56**	**n=110**	0.748
A	55 (64.0)	93 (69.4)		35 (62.5)	73 (66.4)	
G	31 (36.0)	41 (30.6)		21 (37.5)	37 (33.6)	
Genotype	**n = 43**	**n = 67**		**n = 28**	**n = 55**	
AA	17 (39.5)	34 (50.7)	0.462*	11 (39.3)	25 (45.5)	0.844*
AG	21 (49.0)	25 (37.3)		13 (46.4)	23 (41.8)	
GG	05 (11.6)	08 (12.0)		04 (14.3)	07 (12.7)	
-819						
Allele	**n = 86**	**n = 134**	0.096	**n = 56**	**n = 110**	0.017
C	58 (67.4)	74 (55.2)		42 (75.0)	60 (54.5)	
T	28 (32.6)	60 (44.8)		14 (25.0)	50 (45.5)	
OR[f]		----		OR=2.5 (95% CI = 1.55-11.02)		
Genotype	**n = 43**	**n = 67**		**n = 28**	**n = 55**	
CC	21 (48.8)	16 (23.9)	0.017	15 (53.6)	12 (21.8)	0.011*
CT	16 (37.2)	42 (62.7)		12 (42.8)	36 (65.5)	
TT	06 (14.0)	09 (13.4)		01 (03.6)	07 (12.7)	
Genotype						
CC	21 (48.8)	16 (23.9)	0.013	15 (48.8)	12 (23.9)	0.007
CT/ TT	22 (51.2)	51 (76.1)		13 (51.2)	43 (76.1)	
OR[f]	OR =3.04 (95% CI = 1.34-6.91)			OR=4.13 (95% CI = 1.55-11.02)		
-592						
Allele	**n = 86**	**n = 134**	0.451	**n = 56**	**n = 110**	0.127
C	59 (68.6)	84 (62.7)		42 (75.0)	68 (61.8)	
A	27 (31.4)	50 (37.3)		14 (25.0)	42 (38.2)	
Genotype	**n = 43**	**n = 67**		**n = 28**	**n = 55**	
CC	21 (48.8)	19 (28.4)	0.007*	15 (53.6)	14 (25.5)	0.012*
CA	17 (39.5)	46 (68.6)		12 (42.8)	40 (72.7)	
AA	05 (11.6)	02 (03.0)		01 (03.6)	01 (01.8)	
Genotype						
CC	21 (48.8)	19 (28.4)	0.048	15 (48.8)	14 (25.5)	0.022
CA/ AA	22 (51.2)	48 (71.6)		13 (51.2)	41 (74.5)	
OR[f]	OR = 2.41 (95% CI = 1.08-5.36)			OR=3.38 (95% CI = 1.29-8.82)		

[a] Single-Nucleotide Polymorphism.

[b] Chronic Periodontitis.

[c] *P* values marked by (*) were obtained by the T4 values of computer program CLUMP and *p* values not marked were calculated by Chi-squared test.

[d] Absolute number.

[e] Relative number.

[f] Odds Ratio.

Table 4

Frequency of the haplotypes of the *IL10* gene promoter in Control and with Chronic Periodontitis.

Haplotypes	Total (n = 110)		Female Subjects (n = 83)	
	Control	CP[a]	Control	CP[a]
	n[b] (%)[c]	n[b] (%)[c]	n[b] (%)[c]	n[b] (%)[c]
-1082 –819 – 592	**n = 86**	**n = 134**	**n = 56**	**n = 110**
G C C	28 (32.5)	37 (27.6)	20 (35.7)	33 (30.0)
A C C	30 (34.9)	36 (27.0)	22 (39.3)	27 (24.5)
A T A	24 (27.9)	48 (35.8)	13 (23.2)	41 (37.3)
G T A	03 (03.5)	01 (0.7)	01 (01.8)	01 (01.0)
A T C	01 (01.2)	08 (06.0)	00	05 (04.5)
G T C	00	03 (02.2)	00	03 (02.7)
A C A	00	01 (0.7)	00	00
p[d]	0.179*		0.061*	
Haplotype				
A C C	30 (34.9)	36 (27.0)	22 (39.3)	27 (24.5)
A T A	24 (27.9)	48 (35.8)	13 (23.2)	41 (37.3)
p[d]	0.199		0.043	
OR[e]	---		2.57 (95% CI = 1.10-5.95)	

[a] Chronic Periodontitis.

[b] Absolute number.

[c] Relative number.

[d] *P* values marked by an asterisc (*) were obtained by the T4 values of computer program CLUMP and *p* values not marked were calculated by Chi-squared test.

[e] Odds Ratio.

Table 5

Genotype frequencies of putative *IL10* gene promoter haplotypes in Control and with Chronic Periodontitis.

Genotypes	Total (n = 110)		Female Subjects (n = 83)	
	Control	CP[a]	Control	CP[a]
	n[b] (%)[c]	n[b] (%)[c]	n[b] (%)[c]	n[b] (%)[c]
-1082 –819 – 592 / -1082 –819 – 592	**n = 43**	**n = 67**	**n = 28**	**n = 55**
G C C / G C C	03 (06.9)	07 (10.4)	03 (10.6)	06 (11.0)
A C C / A C C	06 (14.0)	05 (07.5)	05 (17.9)	04 (07.3)
A T A / A T A	04 (09.3)	02 (03.0)	01 (03.6)	01 (01.8)
G C C / A C C	12 (28.0)	03 (04.5)	07 (25.0)	02 (03.6)
G C C / A T A	09 (20.9)	19 (28.3)	06 (21.4)	18 (32.7)
A C C / A T A	06 (14.0)	19 (28.3)	05 (17.9)	16 (29.1)
G C C / G T A	01 (02.3)	01 (01.5)	01 (03.6)	01 (01.8)
A T C / A T A	01 (02.3)	04 (06.0)	00	03 (05.5)
G T A / G T A	01 (02.3)	00	00	00
A C C / A C A	00	01 (01.5)	00	00
G T C / A T C	00	01 (01.5)	00	01 (01.8)
G T C / A T A	00	02 (03.0)	00	02 (03.6)
A C C / A T C	00	03 (04.5)	00	01 (01.8)
p[d]	0.011*		0.051*	
Genotype				
G C C / A C C	12 (28.0)	03 (04.5)	07 (25.0)	02 (03.6)
Others	31 (72.0)	64 (95.5)	21 (75.0)	53 (96.4)
p[d]	0.001		0.009	
OR[e]	8.26 (95% CI = 2.17-31.4)		8.83 (95% CI = 1.69-46.03)	

[a] Chronic Periodontitis.

[b] Absolute number.

[c] Relative number.

[d] *P* values marked by an asterisc (*) were obtained by the T4 values of computer program CLUMP and *p* values not marked were calculated by Chi-squared test.

[e] Odds Ratio.

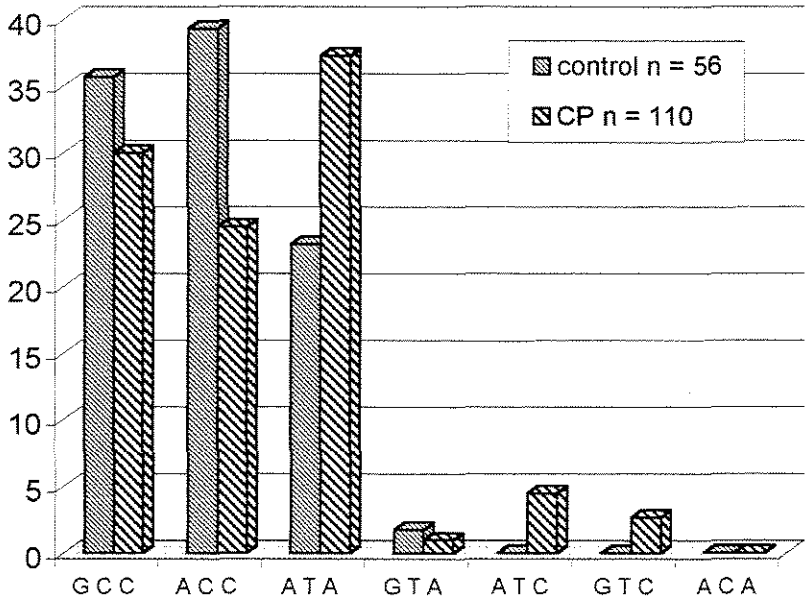

Figure 1 Comparison of *IL10* haplotypes arranged as alleles for female subjects divided in Control and CP (Chronic Periodontitis). *P* value = 0.061 obtained by CLUMP. For details see table 4.

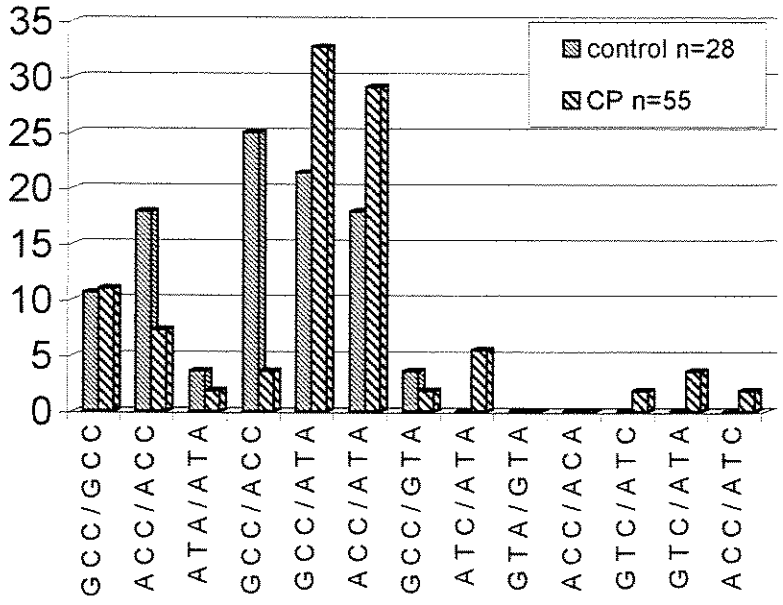

Figure 2 Comparison of *IL10* haplotypes arranged as genotypes for female subjects divided in Control and CP (Chronic Periodontitis). *P* value = 0.051 obtained by CLUMP. For details see table 5.

ARTIGO 4

Frequencies of the –330 (T→G) *IL2* and –590 (T→C) *IL4* gene polymorphisms in a South-eastern Brazilian population.

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Frequencies of the –330 (T→G) *IL2* and –590 (T→C) *IL4* gene polymorphisms in a South-eastern Brazilian population.

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Summary

Polymorphisms in promoter regions of cytokine genes, may affect their transcription. A T/G substitution at position -330 of interleukin-2 (*IL2*) gene and a T/C substitution at position -590 in the interleukin-4 (*IL4*) gene have been described previously. The –590 (T→C) *IL4* gene polymorphism was associated with asthma and atopy in US and Japanese populations. Population genetics is a useful tool for determination of the biological significance of genetic polymorphisms. The aim of this study was to investigate the frequencies of polymorphisms in the promoter regions of *IL2* and *IL4* genes in a South-eastern Brazilian population and compare them to those published for other populations. Allele frequencies were estimated in 114 unrelated individuals from São Paulo State. These subjects had an average age of 41.2 years (± 12.4) and their ethnic composition comprised: white (78.07 %), black (11.4 %), mulatto (10.53 %). DNA from subjects was extracted from epithelial buccal cells, and PCR-RFLP technique was employed to investigate the –330 (T→G) *IL2* and –590 (T→C) *IL4* gene polymorphisms. The allele frequency of *IL2* gene polymorphism obtained in our study was similar to UK Caucasoid groups. The T allele frequency of *IL4* gene polymorphism observed in the Brazilian White group was similar to the UK and Australian populations, while the frequency observed for the Brazilian Black group was similar to Japanese and Kuwaiti Arab populations. The results of –330 (T→G) *IL2* and –590 (T→C) *IL4* polymorphisms demonstrate the high contribution of European lineages to the South-eastern Brazilian population.

Introduction

A single base change variation at position –330 (T→G) has been characterized in the 5' region of the human interleukin-2 gene (John *et al.*, 1998). That report evaluated the frequencies for the T and G alleles in 79 unrelated healthy Caucasians from Manchester, UK. The authors claimed that this polymorphism could be useful as a marker to diagnose susceptibility to inflammatory diseases. The same polymorphism was investigated in 76 unrelated healthy blood donors from South-east of England (Reynard *et al.*, 2000).

Rosenwasser *et al.* (1995) described a T to C single base exchange at position –590 bp from the open reading frame in the interleukin-4 gene. This polymorphism was associated with asthma and atopy in US (Rosenwasser *et al.*, 1995) and in the Japanese population (Noguchi *et al.*, 1998). Studies in UK, Australian families (Walley & Cookson, 1996) and Kuwaiti Arab populations (Hijazi & Haider, 2000) have failed to find this association.

The knowledge of the frequencies of the interleukin polymorphisms in a particular population may also contribute to understand the evolution of inflammatory diseases and indicate the susceptibility to a kind of disease (Rosenwasser *et al.*, 1995; Lazarus *et al.*, 1997; Kornman *et al.*, 1997). To determine the biological significance of genetic polymorphisms, a useful tool is population genetic studies (Faucz *et al.*, 2000). Besides, genetic polymorphisms can be used as genetic markers in anthropological analysis (Constans, 1988).

Brazilians form one of the most heterogeneous populations in the world with Native American, African and European ancestry (IBGE, 2000). According to the official Brazilian Census (IBGE), between 1992 and 1999, the racial distribution of the São Paulo

population is respectively: white, 71.8 %; black, 4.2 %; mulatto, 22.6 %; yellow and Amerindians, 1.5 %. The high number of white individuals can be explained by the predominance of European ancestry in the South and South-east of Brazil (IBGE, 2000). According to Callegari-Jacques & Salzano (1999), 58 % of the immigrants who arrived in Brazil between 1500 and 1972 were Europeans. The state of São Paulo is located in the South-eastern region of Brazil. Its economy is based mainly on industrial and commercial sectors, but agriculture is also considerably developed, with the population living predominantly in urban centers (IBGE).

The aim of this study was to investigate the frequencies of polymorphisms in the promoter regions of –330 (T→G) *IL2* and –590 (T→C) *IL4* genes in a South-eastern Brazilian population, and compare the results to data published for other populations.

Material and Methods

A convenience sample of 114 individuals living in the State of São Paulo, were recruited for study from the patient pool at the Dental Clinic of the Faculty of Dentistry of Piracicaba – UNICAMP. The unrelated subjects had a median age of 41.2 years (± 12.4). The major part of the population studied belongs to the middle class, and their ethnic composition comprises: white (78.07 %), black (11.4 %), mulatto (10.53 %).

In concomitant studies, we investigated the relationship of the cited polymorphisms and chronic periodontal disease (PD). In those studies the individuals were divided into three groups (control, moderate and severe PD). Statistical analysis showed no association between the *IL2* and *IL4* polymorphisms and severity of PD when the three groups were

analysed separately. Regarding *IL-2* investigation, when individuals of the control and moderate groups were pooled and compared to the severe group of PD, we could observe that patients with the T allele seem to be 1.99 times less likely to develop the severe PD (Scarel-Caminaga *et al.*, 2002). Therefore, in this study, individuals with severe PD were excluded from the *IL2* polymorphism investigation and we have performed the analysis in a group with 76 individuals.

DNA was extracted from epithelial buccal cells as previously described (Scarel *et al.*, 2000; Trevilatto & Line, 2000). Polymerase chain reaction (PCR) amplifications were carried out in a total volume of 50 μL containing 300 ng genomic DNA, 2 U *Taq* polymerase, 200 μM of each dNTP, 1 pmol of each PCR primer, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3) and 50 mM KCl. To amplify the region of interest in the promoter of *IL2* gene (Genbank accession number AJ006884) the following primers were used: forward - 5' TAT TCA CAT GTT CAG TGT AGT TCT 3' and reverse - 5' CAT TGT GGC AGG AGT TGA GGT 3'. The forward primer used has been described previously by John *et al.* (1998), with T base at –333 position altered to C, which creates a restriction site for the enzyme M*ae*I (C ^ T A G). The conditions to amplify the *IL2* gene were 1 cycle at 95°C for 2 min followed by 35 cycles of 95°C (1 min), 59°C (1 min) and 72°C (1 min). The restriction fragment length polymorphism (RFLP) for *IL2* gene was carried out in a 20 μL final volume reaction using 1.5 U M*ae*I (Boehringer-Mannheim) and 7 μL PCR product. The reactions were incubated at 45°C overnight (O.N.).

The primers used to amplify the *IL4* gene (Genbank accession number M23442), were previously described by Noguchi *et al.* (1998). A mismatch was introduced in the forward

primer with a T base at –594 position altered to G, creating a restriction site for the enzyme *AvaII*. The following primers were used: forward - 5' TAA ACT TGG GAG AAC ATG GT 3' and reverse - 5' TGG GGA AAG ATA GAG TAA TA 3'. After an initial incubation at 95°C for 5 min, 35 cycles of 95°C (1 min), 51°C (1 min) and 72°C (1 min) were performed. The reactions were completed by a final extension cycle of 72°C for 5 min. The digestion of *IL4*-PCR products was performed using 1.5 U *AvaII* and 10 μL PCR product in a 20 μL final volume reaction, incubated at 37°C O.N.

Statistics

The allele and genotype frequencies were calculated for the –330 (T→G) *IL2* and –590 (T→C) *IL4* polymorphisms, by direct counting and then dividing by the number of chromosomes to produce allele frequency, or by the number of subjects to produce genotype. The goodness of fit to the Hardy-Weinberg equilibrium calculating the expected frequencies of each genotype and comparing them to the observed values was performed using a Chi-square (χ^2) test (Soares & Siqueira, 1999). Frequency data for alleles in each ethnic group and total Brazilian sample were compared to those of other populations by the use of a Chi-square test for equal proportions. Differences were considered significant when $p < 0.05$. The analysis was performed with the SAS statistical package and the software R.

Results and Discussion

Genotype distribution of *IL2* polymorphism in the 76 individuals was consistent with the assumption of Hardy-Weinberg equilibrium ($p > 0.05$; $\chi^2 = 0.0008$). The Chi-square test applied to the *IL2* gene polymorphism showed that the allele frequency in the Brazilian population (all ethnic groups included) was similar to that for Manchester, UK (John *et al.*, 1998) and South-east of England (Reynard *et al.*, 2000) populations ($p > 0.05$; $\chi^2 = 2.424$) (Table 1). When the Brazilian White group was compared to Manchester and South-east of England, the association was stronger than the previous analysis ($p > 0.05$; $\chi^2 = 0.961$). This result indicates that the allele frequency of the *IL2* gene polymorphism in the White population of South-east of Brazil does not differ from Caucasian populations of UK. The Black and Mulatto groups were also compared to the White Brazilians (Black: $p > 0.05$; $\chi^2 = 2.229$), (Mulatto: $p > 0.05$; $\chi^2 = 0.825$) and UK populations (Black: $p > 0.05$; $\chi^2 = 3.459$), (Mulatto: $p > 0.05$; $\chi^2 = 1.619$) where no statistical differences were observed. This result might have been different if we had more individuals in those groups, but the proportion of Black and Mulatto individuals included in our analysis is similar to their distribution within the population of South-east of Brazil.

With respect to the *IL4* polymorphism ($n = 114$), the Hardy-Weinberg analysis of the genotype data indicated that the frequencies fell into the expected equilibrium and were thus randomly distributed ($p > 0.05$; $\chi^2 = 0.5459$). The frequencies of the *IL4* polymorphism in Brazilian and other populations can be observed in the Table 2. The Chi-square test for equal proportions showed no statistical differences in the T allele frequency between Brazilian (all ethnic groups together – 37.3 %) and UK (31 %) populations ($p >$

0.05; χ^2 = 2.595). When the frequency of the T allele in Brazilian White group (32.1 %) was compared to the UK population (31 %) ($p > 0.05$; χ^2 = 0.073), we could observe an even closer association than when the analysis was made with the different Brazilian ethnic groups together. The strong similarity between the *IL4* T allele frequency in Brazilian White group and UK populations is explained by the predominance of individuals of European ancestry in that population (IBGE, 2000). Our results are in agreement with HLA polymorphism studies in a White population from Paraná State (South of Brazil, with a similar ethnical composition), whose alleles are predominantly of European origin (80.6 %) (Probst *et al.*, 2000). The Brazilian White group showed no statistical difference with the Australian population ($p > 0.05$; χ^2 = 2.08) that was also formed mainly by individuals of European ancestry.

The frequency of the T allele in Brazilian White group (32.1 %) was different from Japanese (70 % - $p < 0.05$; χ^2 = 74.99) and Kuwaiti Arab (75.5 % - $p < 0.05$; χ^2 = 46.74) populations. We think that the low frequency of the T allele in the Brazilian White group in comparison to Asian's refers to: (i) Asian immigration, mainly from Japan, Lebanon and Syria was more recent than European immigration (Alves-Silva *et al.*, 2000); (ii) the proportion of immigrants from Asia (2 %) is the lowest in comparison to others (Callegari-Jacques & Salzano, 1999). Probably, due to these reasons, the rate of admixture between Asian and other groups, which form the Brazilian population, is not highly expressive, in comparison to Europeans.

Although the sample size of the Brazilian Black group is small, there was a significant increase of the T allele frequency in this group (69.2 %) in comparison to the Brazilian

White group (32.1 %). Interestingly, the allele frequency matched well those of Asian groups. The reported T allele frequency of *IL4* was 70 % for Japanese ($p > 0.05$; $\chi^2 = 0.007$) and 75.5 % for Kuwaiti Arab ($p > 0.05$; $\chi^2 = 0.422$) populations (Noguchi *et al.*, 1998; Hijazi & Haider, 2000). It would be interesting to obtain data about allele frequency in African populations, since all Brazilian ethnical groups may exhibit some degree of racial admixture (Arpini-Sampaio *et al.*, 1999; Callegari-Jacques & Salzano, 1999; Alves-Silva *et al.*, 2000). Regarding the Brazilian Mulatto group, the comparison of the *IL4* allele frequency to other populations revealed results not statistically different from UK ($p > 0.05$; $\chi^2 = 1.215$) and Australian ($p > 0.05$; $\chi^2 = 2.581$) populations. The *IL4* allele frequency in the Brazilian Mulatto group was more similar to the Brazilian White group ($p > 0.05$; $\chi^2 = 0.887$) than to the Brazilian Black group ($p < 0.05$; $\chi^2 = 3.848$). However, it is important to mention that the Mulatto group was composed of only 12 individuals. The analysis of larger sample of this sub-population might give a more precise indication of the alleles distribution. In our sample there are no Amerindians and Japanese individuals. It would be interesting to investigate the frequencies of *IL2* and *IL4* polymorphisms in these populations, since studies with other genes were already performed (Faucz *et al.*, 2000; Probst *et al.*, 2000).

PCR-RFLP studies of interleukin polymorphisms can be used as a rapid screening of population, providing data on allele and genotype frequencies. Our data showed that the interleukin polymorphisms might be used as markers in population genetic studies, and can complement data obtained by mitochondrial DNA analysis (Chen *et al.*, 1995; Alves-Silva *et al.*, 2000) and of nonrecombining portion of the Y-chromossome (Carvalho-Silva *et al.*,

2001). These findings indicate that the allelic frequencies of –330 (T→G) *IL2* and –590 (T→C) *IL4* gene polymorphisms in the South-eastern population of Brazil approach those of the European population.

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Table 1. Allele and genotype frequencies of –330 (T→G) *IL2* gene in 76 unrelated South-eastern Brazilian individuals.

IL-2						
	Brazilian n (%)				Manchester, UK * n (%)	South-east England # (%)
	Total	White	Black	Mulatto		
ALLELE	n = 152	n = 126	n = 16	n = 10	n = 158	n = 152
T	122 (80.3)	98 (77.8)	15 (93.7)	09 (90)	115 (72.8)	(75.6)
G	30 (19.7)	28 (22.2)	1 (6.3)	01 (10)	43 (27.2)	(24.3)
GENOTYPE	n = 76	n = 63	n = 8	n = 5	n = 79	n = 76
TT	49 (64.5)	38 (60.3)	07 (87.5)	04 (80)	42 (53.2)	(55.3)
TG	24 (31.6)	22 (34.9)	01 (12.5)	01 (20)	31 (39.2)	(40.8)
GG	03 (03.9)	03 (04.8)	--	--	06 (07.6)	(03.9)

* Data from John *et al.* (1998); # Reynard *et al.* (2000).

Table 2 Allele and genotype frequencies of –590 (T→C) *IL4* polymorphism in 114 unrelated South-eastern Brazilian individuals compared to other populations.

	IL-4							
	Brazilian n(%)				Japanese[&]	Kuwaiti Arab[#]	Australian [%]	UK [%]
	Total	White	Black	Mulatto	n (%)	n (%)	(%)	(%)
Allele	n = 228	n = 178	n = 26	n = 24	n = 430	n = 94	n = 2008	n = 366
T	85 (37.3)	57 (32.1)	18 (69.2)	10 (41.7)	301 (70.0)	71 (75.5)	(27)	(31)
C	143 (62.7)	121 (67.9)	08 (30.8)	14 (58.3)	129 (30.0)	23 (24.5)	(73)	(69)
Genotype	n = 114	n = 89	n = 13	n = 12	n = 215	n = 47	n = 1004	n = 183
TT	14 (12.3)	07 (07.9)	05 (38.5)	02 (16.7)	101 (46.9)	27 (57.4)	ND	ND
TC	57 (50.0)	43 (48.3)	08 (61.5)	06 (50.0)	97 (45.1)	17 (36.2)	ND	ND
CC	43 (37.7)	39 (43.8)	--	04 (33.3)	17 (07.9)	03 (06.4)	ND	ND

[&] Data from Noguchi *et al.*, 1998; [#] Hijazi & Haider, 2000; [%] Walley & Cookson, 1996; ND = not determined

4. CONCLUSÕES

ARTIGO 1. O polimorfismo –330 (T→G) no gene *IL2* está associado com a severidade da DPC. Os resultados apresentados sugerem um papel ativo da interleucina 2 na patogênese da doença periodontal.

ARTIGO 2 O polimorfismo –590 (T→C) no gene *IL4* não está associado com a suscetibilidade à DPC.

ARTIGO 3 Haplótipos específicos e os polimorfismos –819 e –592 na região promotora do gene *IL10* podem ser marcadores genéticos úteis da suscetibilidade à DPC em indivíduos brasileiros.

ARTIGO 4 A técnica da PCR-RFLP é um método rápido para investigar os polimorfismos –330 (T→G) *IL2* e –590 (T→C) *IL4* que se mostraram úteis como marcadores em estudos de Genética de Populações, podendo complementar resultados a partir de outras regiões polimórficas do genoma humano. A análise dos polimorfismos –330 (T→G) *IL2* e –590 (T→C) *IL4* demonstraram a alta contribuição de linhagens Européias na população do Sudeste do Brasil.

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ANEXOS

I - Outros Trabalhos Aceitos para Publicação.

Trevilatto, P.C.; **Scarel-Caminaga, R.M.**; Brito Jr, R.B., De Souza, A.P.; Line, S.R.P. Polymorphism at position –174 of IL-6 gene is associated with susceptibility to chronic periodontitis in a Caucasian Brazilian population. **J Clin Periodontol,** v.30, n.5, 2003, *in press*.

De Souza, A.P.; Trevilatto, P.C.; **Scarel-Caminaga, R.M.**; Brito Jr, R.B.; Line, S.R.P. MMP1 promoter polymorphism: association with chronic periodontitis severity in Brazilian population. **J Clin Periodontol,** v.30, n.2, 2003, *in press*.

De Souza, A.P.; Trevilatto, P.C.; **Scarel-Caminaga, R.M.**; Brito Jr, R.B.; Line, S.R.P. Analysis of TGF-β1 promoter polymorphism (C-509T) in patients with chronic periodontitis. **J Clin Periodontol,** v.30, n.6, 2003, *in press*.