# Analysis of IL-1A(-889) and TNFA(-308) gene polymorphism in Brazilian patients with generalized aggressive periodontitis

Nívea Maria de Freitas[1], Ana Vitória Imbronito[1], Adriana Costa Neves[2], Fábio Daumas Nunes[3], Francisco Emilio Pustiglioni[1], Roberto Fraga Moreira Lotufo[1]

[1] Department of Periodontology, Faculty of Odontology, University of São Paulo, Brazil
[2] Butantan Institute, São Paulo, Brazil
[3] Department of Oral Pathology, Faculty of Odontology, University of São Paulo, Brazil

**Correspondence :** N.M. de Freitas, av. Prof Lineu Prestes 2227, Cidade Universitária, São Paulo, Brazil
<nmfreita@usp.br>
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**ABSTRACT. Generalized aggressive periodontitis (GAP) comprises a group of periodontal diseases characterized by the rapid destruction of periodontal tissues which affect young individuals who generally present no systemic disorders. Polymorphisms in the interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) genes have been associated with an increased severity of chronic periodontitis. The objective of the present study was to evaluate the association between IL-1A (-889) and TNFA (-308) gene polymorphisms and GAP. One hundred nonsmoking subjects were selected, including 30 with GAP and 70 without periodontal disease. Gene polymorphisms were analyzed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. For IL-1 (-889), the frequency of genotype 1/1 was 54.3% in the control group and 56.7% in the study group. The frequency of genotype 1/2 was 37.1% in the control group and 40% in the study group. Genotype 2/2 was detected at a frequency of 8.6% and 3.3% in the control and study groups, respectively. For TNFA, genotype 1/1 was present in 68.6% of control subjects and in 80.0% of patients with GAP, while the frequency of genotype 1/2 was 27.1% in the control group and 20% in the study group. Genotype 2/2 was present in 4.3% of control subjects and was not detected in the study group. The frequencies of allele 1 and allele 2 of the IL-1A (-889) gene were 72.9% and 27.1%, respectively, in the control group and 76.7% and 23.3% in the GAP group. For the TNFA (-308) gene, the frequency of allele 1 was 82.15% in the control group and 90% in the study group, whereas the frequency of allele 2 was 17.85% in the control group and 10% in the study group. Statistical analysis revealed no significant difference in allele distribution for either gene between the two groups. No association was observed between GAP and IL-1A (-889) and TNFA (-308) gene polymorphisms in Brazilian patients.**

**Keywords:** polymorphism, periodontitis, interleukin-1, tumor necrosis factor, periodontal disease

Aggressive periodontitis (AP) comprises a group of rare periodontal diseases characterized by frequently severe clinical manifestations that affect young individuals, which progress rapidly and can involve multiple family members. The diagnosis of AP requires the exclusion of the presence of systemic diseases that may severely compromise host defenses and cause premature tooth loss.

Although the presence of bacteria is essential for the onset of the disease, the number and type of these microorganisms are not sufficient to explain differences in the inflammatory and immune responses and, consequently, in the severity of the periodontal disease [1]. It has currently become evident that in the case of most chronic diseases other factors exist that do not cause disease but that modify the course of the disease, rendering it more severe. Among these factors are genetic alterations, called polymorphisms, which are commonly found in the population [2]. Gene polymorphisms are locations within the genome that vary in sequence between individuals and are very prevalent, affecting at least 1% of the population [3]. Many

genes responsible for cytokine production exhibit polymorphisms [4] that can modify the production of proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [5, 6]. In all cases, it has been suggested that the host response to bacterial etiological agents may be exaggerated or deficient, resulting in periodontal breakdown at an early age. Polymorphisms in various cytokine genes can influence the level of secretion of these substances and explain the variations in individual immune-inflammatory responses to a bacterial aggression [2]. Recent studies have indicated that the IL-1 gene polymorphism might be associated with a greater severity of the disease in patients with chronic periodontitis and AP [7-9].

IL-1 is the cytokine most frequently found in inflammatory processes and is involved in the onset and progression of connective tissue and bone destruction. Bacterial products such as lipopolysaccharide increase the synthesis of IL-1 [10]. IL-1 mediates the recruitment of inflammatory cells to the sites of infection, promotes bone resorption,

stimulates the production of fibroblasts, induces the synthesis of prostaglandin E2 by macrophages and fibroblasts, stimulates the production of metalloproteinases that degrade extracellular matrix proteins, and participates in the host immune response [11]. The role of the IL-1α and IL-1β gene polymorphisms was evaluated in patients with generalized or localized, early-onset periodontitis (EOP) by Diehl and coworkers [7]. In the case of generalized juvenile periodontitis (GJP), allele 1 at the IL-1 locus was transmitted more frequently than allele 2. The results of linkage disequilibrium analysis suggested that the IL-1β polymorphism may be more important for the association with localized juvenile periodontitis (LJP) [7]. On the other hand, Walker *et al*. (2000) [12] ruled out the importance of allele 1 of gene IL-1β in the African-American population and Hodge *et al*. 2001 [13] concluded that there was no association between the IL-1 polymorphism and GJP of European origin.

TNF-α is a proinflammatory cytokine produced mainly by macrophages, with a biological role similar to that of IL-1. It induces the secretion of collagenase, prostaglandin E2 and IL-6 by human fibroblasts and in human bone cell culture, and possesses important immunologic activities. TNF-α also stimulates bone resorption by osteoclasts, but is 500 times less potent than IL-1 [5]. TNF-α has also been detected in gingival fluid from patients with gingivitis and periodontitis [14]. Several studies have reported an association between the TNFA gene polymorphism (allele 2) and infectious and autoimmune diseases such as cerebral malaria, rheumatoid arthritis, lupus erythematosus and diabetes [15]. Few studies are available in the literature regarding the association between the TNFA gene polymorphism and periodontal disease. Kinane *et al*. [16], studying the association between EOP and TNFA (-308) and IL-10 polymorphisms, did not observe any association between these polymorphisms and GJP. In addition, no association was observed between the TNFA (-308) gene polymorphism and AP in patients with generalized or localized AP [17].

The association between the TNFA (-308) gene polymorphism and GJP was evaluated in a Japanese population by Endo *et al*. [18]. The results showed no difference in polymorphism frequency between patients with GJP and healthy controls, and indicated that its frequency is low in the Japanese population. It is possible that the prevalence of these polymorphisms varies among different races as observed in studies evaluating the IL-1A and IL-1B polymorphism in Chinese subjects [19] and in individuals of Hispanic origin [20] without periodontal disease.

The objective of the present study was to evaluate the association between the IL-1A (-889) and TNFA (-308) gene polymorphisms and generalized AP in Caucasian Brazilian patients.

## METHODS

### *Patients and controls*

The study was approved by the Research Ethics Committee of the Dental School, University of São Paulo. All patients received detailed information about the objective of the study and signed a free, informed consent form to participate.

This case-control study involved 100 individuals from the city of São Paulo in the southeastern region of Brazil. Patients with aggressive periodontitis and the control individuals were recruited from the Dental School at University of São Paulo. Patients and healthy controls came from the same geographical region and had a similar socio-economic status.

The AP group comprised 30 Caucasian patients (patients with parents and grandparents of different European origins) (six males and 24 females), with a mean age of $25.93 \pm 3.27$ (range 21 to 30 years). The patients presented attachment loss > 4 mm in at least three permanent teeth, in addition to molars and incisors. The control group consisted of 70 white, age-matched subjects (seven males and 63 females), mean age of $26.4 + 3.16$ years (range 21 to 30 years) without clinical evidence of attachment loss in any tooth. These individuals had at least 24 teeth.

A complete medical and dental history was obtained for the patients and control subjects, with all individuals showing good general health. Excluded from the study were patients with orthodontic devices, insufficient restorations margins, patients with a history of diabetes, pregnant or breast-feeding women, patients chronically using anti-inflammatory drugs, smokers, and subjects with a history of hepatitis and HIV infection.

The following clinical parameters were recorded: bleeding index upon probing, probing pocket depth at six sites/teeth, clinical attachment level at 6 sites/teeth, observation of clinical mobility, bleeding upon probing and the presence of plaque/calculus. All patients in the AP group had severe, generalized aggressive periodontitis, based on the International Workshop for a Classification of Periodontal Disease and Conditions in 1999 [21]. The clinical examination was performed by only one examiner (NMF) which was calibrated (kappa value = 0.82).

Fourteen periapical radiographs were taken from patients with AP. The patients were submitted to periodontal treatment and enrolled in a periodontal control and maintenance program.

### *Collection of saliva, enzymatic digestion and DNA extraction*

Non-stimulated saliva samples were collected into a sterile universal container, transferred to 2 mL tubes and stored in a freezer at -20°C until the time of DNA extraction. The patients were instructed not to eat or brush their teeth for 30 min before saliva collection.

A 500 μL aliquot of saliva was transferred to a 2 mL tube and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with 1 mL 1X PBS. The material was again centrifuged at 2000 rpm for 5 min and the supernatant was discarded. The pellet was washed with 1 mL 1X PBS, centrifuged and the supernatant was again discarded. The pellet was then submitted to enzymatic digestion with proteinase K.

For digestion, 200 to 400 μL of sterile lysis buffer containing 1 M NaCl, 1 M Tris [tris(hydroxymethyl)aminomethane], pH 8, 0.5 M EDTA (ethylenediaminetetraacetic acid), pH 8, 10% SDS (sodium dodecyl sulfate) (Sigma Chemical Co., St. Louis, MO, USA) and

proteinase K (Invitrogen, Carlsbad, CA, USA) at a final concentration of 500 µg/mL were added to the tube. The tubes were shaken in a water bath at 55°C for 3 to 7 days until complete dissolution of the pellet. Proteinase K solution (200-400 µg/mL) was added at 24h intervals and the tubes were inverted at least once a day. After complete dissolution of the material, the tubes were incubated at 95°C for 10 min for proteinase K inactivation.

DNA was extracted from the samples by the ammonium acetate and isopropanol method standardized at the Laboratory of Molecular Biology, Dental School, University of São Paulo [22]. Briefly, 200 µL 4 M ammonium acetate (Synth, BR) were added to the tube containing the lysate for protein precipitation. The material was homogenized for 20 s, incubated on ice for 5 min and centrifuged at 13,000 x *g* for 3 min. Precipitated protein was observed at the bottom of the tube and the supernatant containing DNA was transferred to another tube. For precipitation of DNA, 600 µL 100% isopropanol was added and the mixture was homogenized and centrifuged at 16,000 x *g* for 5 min. The supernatant was discarded and the DNA pellet was washed with 600 µL 70% ethanol and centrifuged at 16,000 x *g* for 2 min. The alcohol was removed and the sample was evaporated to dryness at room temperature. The DNA pellet was dissolved in 30 to 50 µL TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8) and stored at 4°C until quantification in a spectrophotometer.

### Genotyping of IL-1A (-889) and TNF-a (-308)

The extracted DNA was amplified by PCR using primers specific for each gene whose sequences were obtained from the literature and verified using the GenBank [accession number X03833.1 for IL-1A (-889) and AF247608.1 for TNFA (-308)]. IL-1A (-889): 5′-AAG CTT GTT CTA CCA CCT GAA CTA GGC- 3′ [23] and 5′-TTA CAT ATG AGC CTT CCA TG-3′. TNFA (-308) 5′- AAG CAA TAG GTT TTG AGG GCC AT-3′ and 5′- TCC TCC CTG CTC CGA TTC CG -3′ [24]. All reactions were carried out in 0.5 mL PCR tubes containing 1% formamide (Invitrogen), 1X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen), 0.3 mM dNTP mix (2'-deoxynucleotide 5'-triphosphates: dATP, dTTP, dCTP, dGTP) (Invitrogen), MgCl$_2$ (Invitrogen), sense and antisense primers (Invitrogen), 2 U Taq DNA polymerase (Invitrogen), 100 to 300 ng genomic DNA, and sterile water in final volume of 25 µL. The MgCl$_2$ concentration was 2 mM for the IL-1A (-889) gene and 2.5 mM for the TNFA (-308) gene. The primer concentration was 500 pM for the IL-1A (-889) gene and 250 pM for the TNFA (-308) gene.

The PCR conditions were optimized for the different primers using as a basis one initial denaturation cycle at 94°C for 3 min, followed by 38 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 50 s for the IL-1A (-889) gene and at 61°C for 50 s for the TNFA (-308) gene and extension at 72°C for 1 min, and a final extension step at 72°C for 7 min.

### Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis is based on the pattern of DNA fragments produced by digestion of the PCR product with an endo-nuclease specific for the determination of polymorphisms. The RFLP technique involves the cleavage of DNA molecules with restriction enzymes, separation of the generated fragments by gel electrophoresis and their visualization in the form of bands.

IL-1A polymorphism in the position -889 has a cytosine (allele 1) substituted by thymine (allele 2), and the cytosine allele completes a NcoI site. Allele 1 yielded products of 83bp and 16bp, and allele 2 resulted in a product of 99bp. The three bands were shown in heterozygous individuals. TNFA (-308) polymorphism in the position -308 had a guanine (allele 1) substituted by adenine (allele 2) and the guanine allele completes a NcoI site. Allele 1 yielded products of 87bp and 20bp, and allele 2 resulted in a product of 107bp. Heterozygous individuals had the three bands.

For RFLP analysis of the IL-1A (-889) and TNFA (-308) genes, 5 µL of the PCR product was digested with 2 U of the NcoI restriction enzyme in 1X specific buffer and sterile water to a final volume of 20 µL at 37°C for 5 h. The resulting fragments were separated by size on 15% poly-acrylamide gel containing 5% glycerol (2.6 mL 10X TAE, 5.2 mL 50% glycerol, 25 mL 30% acrylamide, 520 µL 10% APS, 52 µL TEMED, and Milli-Q water, q.s.p. 50 mL). The PCR product was mixed with running buffer (10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). Two molecular weight standards were used for the determination of fragment size: low DNA Mass Ladder (Invitrogen) and 10-bp DNA Ladder (Invitrogen). The gels were run in 1X TAE buffer for approximately 6 h at room temperature.

After the run, the gel was stained with silver and developed for visualization of the DNA bands. First, the gel was fixed in 10% ethanol and 0.75% acetic acid for 20 min, followed by staining with 0.2% silver for 30 min, washing in Milli-Q water for 2 min and development with a solution of 3% NaOH and 0.03% formaldehyde for 20 min. Finally, the gel was incubated with 10% acetic acid for 12 min and washed with Milli-Q water for 12 min. All steps were performed in the dark and under shaking [25]. The gels were then scanned for subsequent analysis.

### Statistical analysis

Statistical analysis was performed using the standard statistical software (Stat View J-4.5 application program, SAS Institute Inc., NC, USA). The chi-square test was used to compare the sex distribution between control and aggressive periodontitis group. Hardy-Weinberg equilibrium in both groups was tested for genotyping frequencies by a chi-square test with one degree of freedom (d.f.). The associations of allele and distributions in aggressive periodontitis patients and healthy controls were assessed in 2 X 2 contingency tables (d.f. = 1). The 2 X 2 tables were analyzed using chi-square test or Fisher's exact test. A p-value < 0.05 was considered significant.

## RESULTS

The GAP group and control group were similar regarding age and gender distribution (p > 0.05). GAP patients had a mean of 15.53 (± 4.42) teeth with a probing depth greater

**Table 1**
Frequency of IL-1A (-889) genotype and alleles in generalized aggressive
periodontitis patients and healthy controls

|  |  | Controls | | GAP patients | |
|---|---|---|---|---|---|
|  |  | N | (%) | N | (%) |
| Genotype | 1/1 | 38 | (54.3) | 17 | (56.7) |
|  | 2/1 | 26 | (37.1) | 12 | (40.0) |
|  | 2/2 | 6 | (8.6) | 1 | (3.3) |
|  | Total | 70 | (100) | 30 | (100) |
| Alleles | Allele 1 | 102 | (72.9) | 46 | (76.7) |
|  | Allele 2 | 38 | (27.1) | 14 | (23.3) |
|  | Total | 140 | (100) | 60 | (100) |

Genotype: p-value = 0.791 (Fisher's exact test). Allele: p-value = 0.574 (Qui-square test).

than 5 mm, a mean probing depth of $5.35 \pm 0.9$ and clinical attachment loss of $6.33 \pm 0.7$; control patients had a mean probing depth of $2.11 \pm 0.3$ and clinical attachment loss of $2.45 \pm 0.25$.

Examination of polyacrylamide gels showed fragments of 99 bp (allele 2) and 83 bp and 16 bp (allele 1) for patients and controls heterozygous for IL-1A (-889). For the TNFA (-308) polymorphism fragments of 87 bp and 20 bp (allele 1) and 107 bp (allele 2) were visible for heterozygotes.

The genotype and allele frequencies of the two genes are shown in *tables 1* and *2*. With respect to the IL-1A (-889) gene, genotype frequencies in the control group were 54.3% for genotype 1/1, 37.1% for genotype 2/1 (heterozygous) and 8.6% for genotype 2/2. Statistically similar genotype frequencies were obtained for the study groups: 56.7% for genotype 1/1, 40% for genotype 2/1, and 3.30% for genotype 2/2. The frequency of allele 1 and allele 2 was 72.9% and 27.1% in control group and in the AP group the frequency for allele was 76.7% 1 and for allele 2 was 23.3%. Both groups were in Hardy-Weinberg equilibrium (aggressive periodontitis group, $\chi^2 = 0.41$; healthy group, $\chi^2 = 0.26$, d.f. = 1, p > 0.05).

The homozygous TNFA (-308) 1 allele was present in 68.6% of the control group and in 80% of AP group, while the heterozygous TNFA (-308) 2 allele was present in 27.1% of the control group and 20% of AP patients. The homozygous TNFA (-308) 2 allele was present in 4.3% of the control group and it was not detected in the AP group. The frequency of allele 1 and allele 2 was 82.15% and 17.85% respectively in the control group, and in the AP group the frequency for allele 1 was 90% and for allele 2 was 10%. Both groups were in Hardy-Weinberg equilib-

rium (aggressive periodontitis group, $\chi^2 = 0.37$; healthy group, $\chi^2 = 0.39$, d.f. = 1, p > 0.05).

Comparison of the IL-1A (-889) and TNFA (-308) gene polymorphisms between the AP and control groups revealed no significant association with AP, *i.e.*, the two groups did not differ in terms of changes in the genes analyzed (p > 0.05). The association with any allele or combination of alleles of the polymorphisms and AP are presented in *table 3*.

## DISCUSSION

Our results demonstrated that there were no significant association between GAP and the IL-1A (-889) and TNFA (-308) gene polymorphisms in nonsmoking patients with GAP and nonsmoking subjects without periodontal disease. As shown in *table 1* and *table 2*, the distribution of the IL-1A (-889) and TNFA (-308) frequencies did not reveal any significant differences between the groups.

The two groups were age-matched, and no smokers or former smokers were included since smoking has been identified as the major environmental risk factor associated with increased incidence and severity of periodontitis [26-28]. In addition, patients with the localized form of AP were also excluded in order to obtain a homogenous study group.

The findings presented here disagree with the results provided by Kornman *et al*. [8] for nonsmoking European patients with chronic periodontitis, which pointed to strong evidence of association between IL-1A (-889) gene polymorphism and the severity of periodontal disease. In

**Table 2**
Frequency of TNFA (-308) genotype and alleles in generalized aggressive
periodontitis patients and healthy controls

|  |  | Controls | | GAP patients | |
|---|---|---|---|---|---|
|  |  | N | (%) | N | (%) |
| Genotype | 1/1 | 48 | (68.6) | 24 | (80.0) |
|  | 2/1 | 19 | (27.1) | 6 | (20.0) |
|  | 2/2 | 3 | (4.3) | 0 | (0) |
|  | Total | 70 | (100) | 30 | (100) |
| Alleles | Allele 1 | 115 | (82.15) | 54 | (90.0) |
|  | Allele 2 | 25 | (17.85) | 6 | (10.0) |
|  | Total | 140 | (100) | 60 | (100) |

Genotype: p-value = 0.511 (Fisher's exact test). Alleles: p-value = 0.159 (Qui-square test).

**Table 3**
Associations between IL-1A (-889) and TNFA (-308) genes

|      |     |     | IL-1A |     |     |
|------|-----|-----|-------|-----|-----|
|      |     |     | **1/1** | **2/1** | **2/2** |
| TNFA | 1/1 | N   | 40    | 26  | 6   |
|      |     | (%) | (72.7) | (68.4) | (85.7) |
|      | 2/1 | N   | 14    | 10  | 1   |
|      |     | (%) | (25.5) | (26.3) | (14.3) |
|      | 2/2 | N   | 1     | 2   | 0   |
|      |     | (%) | (1.8) | (5.3) | 0   |
| Total |    | N   | 55    | 138 | 7   |
|      |     | (%) | (100) | (100) | (100) |

Kappa = 0.014 (p-value = 0.868).

that study, patients carrying allele 2 presented a 19-fold higher risk of developing periodontal disease than patients carrying allele 1.

Few studies have evaluated this association for various forms of periodontitis in different countries. The present results agree with those reported by other investigators studying patients with AP. Diehl *et al*. [7] reported a more frequent transmission of allele 1 of the IL-1A (-889) gene in Afro-American and Caucasian patients with EOP compared to allele 2. Hodge *et al*. [13] and Gonzalez *et al*. [29] also did not find any association between the IL-1A (-889) gene polymorphism and juvenile periodontitis. Thus, the different forms of the disease, as well as possible population differences, should be taken into account.

With respect to the TNFA (-308) gene, the genotype frequencies obtained in the present study were 68.6% for genotype 1/1, 27.1% for genotype 2/1 and 4.3% for genotype 2/2 in the control group, and 80% for genotype 1/1 and 20% for genotype 2/1 in the study group. The frequency of allele 1 was 82.15% in the control group and 90% in the study group. Allele 2 was observed in 17.85% of control subjects and in 10% of patients with AP.

This result agrees with Kinane *et al*. [16] who also did not observe any association between TNFA gene polymorphism and generalized AP. Shapira *et al*. [17] observed a slight, but not significant, increase in the prevalence of genotype 1/1 in patients with AP, contrary to the results obtained in this study. Although the detection of genotype 2/2 in the control group was not statistically different from the absence of this genotype in the AP patients, maybe a larger group of patients would provide more convincing results. The frequencies of TNFA genotype 2/2 are relatively low both for control and diseased patients. The calculation of sample size to have 80% power to detect a difference between AP patients and healthy controls at the = 0.05 level of significance, shows that approximately 234 patients in each group would need to be evaluated.

AP is a multifactorial disease resulting from the complex interactions between the host, microbiota and environment. The difficulty in associating a gene polymorphism with AP might be explained by the lack of higher expression of a single gene in the disease. Probably, there are other genes altering the expression of genes and influencing the clinical expression of the disease. Furthermore, multiple polymorphisms might be necessary for an increase in the severity of the disease. In addition, specific genes may vary among different populations and/or ethnic groups, and true heterogeneity in the susceptibility to the disease might be present.

In conclusion, the present study revealed no association between the IL-1A (-889) and TNFA (-308) gene polymorphisms and generalized AP.

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