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CAMILA SCHMIDT STOLF

FUNCIONALIDADE DO *IL10* SNP rs6667202 EM FIBROBLASTOS GENGIVAIS DE PACIENTES COM PERIODONTITE GRAU C

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Mestra em Clínica Odontológica, na Área de Periodontia.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of requirements for the degree of Master in Clinical Dentistry, in Periodontology area.

Orientador: Prof. Dr. Renato Corrêa Viana Casarin

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Steve Jobs

RESUMO

A suscetibilidade de indivíduos à periodontite grau C, estágio 3 ou 4 em jovens (Perio4C) está associada principalmente a fatores genéticos, como a presença de polimorfismos de nucleotídeo único (SNPs). Estudos anteriores demonstraram uma associação entre o SNP rs6667202 (C>A), região promotora do *IL10* e a Perio4C na população brasileira, onde o alelo alterado A foi detectado em maior frequência nesses pacientes. Assim, o objetivo desse estudo foi avaliar a funcionalidade do SNP rs6667202 em fibroblastos gengivais (FGs) de indivíduos com Perio4C e saúde periodontal (SP) estimulados com extrato proteico de *Aggregatibacter actinomycetemcomitans (Aa*PE*).* Nove pacientes com SP e oito com Perio4C foram divididos de acordo com o seu genótipo (AA, AC ou CC) para esse SNP, e uma biópsia foi realizada para estabelecer a cultura primária de FGs. Após a exposição dos FGs a 5 µg/ml durante 1,5 horas, o RNA total das células foi extraído para análises de expressão gênica do *IL10* por qPCR e alíquotas do sobrenadante foram submetidas a análises imunoenzimáticas (Luminex/MAGpix) para detecção da citocina IL-10. Em SP, a presença do alelo alterado A (genótipos AA e AC) promoveu menor expressão de *IL10* ($p = 0,027$ e $p < 0,0001$) e, da mesma forma, uma menor produção de IL-10 ($p = 0.002$ e $p = 0.001$) após o estímulo com *Aa*PE, quando comparados ao genótipo CC. Entretanto, em Perio4C não houve diferença estatística entre os genótipos ($p > 0.05$), apesar de terem mostrado menor expressão e produção de IL-10 que SP ($p = 0.033$ e $p < 0.001$), indicando alterações na resposta ao estímulo ocorrendo por vias diferentes. Conclui-se que este SNP apresenta uma ação funcional, em SP, diminuindo a expressão e produção de IL-10, sendo um potencial indicador de risco biológico. Além disso, em Perio4C, uma produção aberrante de IL-10 foi observada, não diretamente associada a esse SNP.

Palavras-chave: Polimorfismo de nucleotídeo único. Citocinas. Interleucina-10. Periodontite Agressiva.

ABSTRACT

The susceptibility of individuals to grade C, stage 3 or 4 periodontitis in young people (Perio4C) is mainly associated with genetic factors, such as the presence of single nucleotide polymorphisms (SNPs). Previous studies have demonstrated an association between SNP rs6667202 (C > A), *IL10* promoter region, and Perio4C in the Brazilian population, where the altered A allele was detected more frequently in these patients. Thus, the objective of this study was to evaluate the functionality of SNP rs6667202 in gingival fibroblasts (GFs) of individuals with Perio4C and periodontal health (PH) stimulated with protein extract of *Aggregatibacter actinomycetemcomitans* (*Aa*PE). Nine patients with SP and eight with Perio4C were divided according to their genotype (AA, AC, or CC) for this SNP, and a biopsy was performed to establish the primary culture of GFs. After exposing the GFs to 5 µg/ml for 1.5 hours, the total RNA was extracted for analysis of *IL10* expression by qPCR and aliquots of the cell's supernatant were subjected to immunoenzymatic analyzes (Luminex/MAGpix) for IL-10 detection. In PH, the presence of the altered allele A (genotypes AA and AC) promoted less expression of *IL10* ($p = 0.027$ and $p < 0.0001$) and, likewise, a lower production of IL-10 ($p = 0.002$ and $p = 0.001$) after stimulation with *Aa*PE, when compared to the CC genotype. However, in Perio4C there was no statistical difference between the genotypes ($p > 0.05$), although they showed less expression and production of IL-10 than PH ($p = 0.033$ and $p < 0.001$), indicating changes in the response to the stimulus occurring by different pathways. It is concluded that this SNP has a functional action, in PH, by decreasing the expression and production of IL-10, being a potential indicator of biological risk. In addition, in Perio4C, aberrant IL-10 production was observed, not directly associated with this SNP.

Keywords: Single nucleotide polymorphism. Cytokines. Interleukin-10. Aggressive periodontitis.

SUMÁRIO

1. INTRODUÇÃO

A periodontite é uma doença inflamatória de etiologia multifatorial, desencadeada pela resposta imune-inflamatória do hospedeiro aos periodontopatógenos presentes no biofilme subgengival, caracterizada clinicamente pela destruição óssea e perda de inserção conjuntiva, que se não tratada pode levar a perda dental (Armitage, 1999). Desde o Workshop Mundial de 2017 sobre a Classificação de Doenças e Condições Periodontais e Peri - implantares, a periodontite passou a ser classificada em estágios e graus. Os estágios relacionam-se com a gravidade e complexidade do tratamento, ou seja, a severidade da doença, e são divididos em quatro. Já os graus, classificados em três, representam a evidência ou risco de progressão da doença e o risco potencial de impacto sistêmico da periodontite no paciente (Caton et al., 2018).

Considerando a atual classificação, a antiga Periodontite Agressiva, forma particularmente grave de doença periodontal, que acomete indivíduos jovens sistemicamente saudáveis, e é caracterizada por início precoce, rápida progressão, agregação familiar dos casos e pobre resposta as abordagens terapêuticas, (Albandar, 2014; Armitage, 1999; Deas and Mealey, 2010) pode ser classificada como Periodontite estágios 3 ou 4 Grau C (Perio4C) (Caton et al., 2018). Embora sua prevalência seja relativamente baixa na população brasileira, variando de 0,3% a 5,5% (Susin et al., 2014), a Perio4C possui grande impacto na qualidade de vida dos pacientes, pois frequentemente leva à múltiplas perdas dentais de maneira precoce (Albandar, 2014).

A susceptibilidade dos indivíduos à Perio4C é determinada por uma complexa interação entre microbiota, sistema imune e fatores comportamentais, e é também regulada por fatores genéticos (Loos et al., 2015). Além disso, outros mecanismos, como aqueles que atuam na regulação da expressão gênica, por exemplo, os microRNAs, também podem influenciar nessa susceptibilidade (Loos et al., 2015; De Souza et al., 2014; Stoecklin-Wasmer et al., 2012). Porém, ainda não é possível explicar profundamente o perfil genético associado a Perio4C, os marcadores inflamatórios que influenciam na resposta do hospedeiro ao desafio microbiano, e que, consequentemente, influenciam o resultado do tratamento periodontal (Duarte et al., 2015; Kebschull et al., 2013; Vieira and Albandar, 2014).

A evidência atual indica que a Perio4C é poligênica, a exemplo de outras doenças complexas, com o envolvimento de múltiplos genes de pequeno efeito (Vieira and Albandar, 2014; Laine et al., 2012). Recentemente, alguns estudos utilizaram uma abordagem de avaliação de larga escala, como os estudos de associação genômica ampla (GWAS – genome wide association study), reportando associação de polimorfismos em genes até então não associados

a Perio4C, mas que necessitam de validação em populações independentes em outros estudos (Loos et al., 2015).

Utilizando essa abordagem, um estudo recente do nosso grupo de pesquisa validou alterações genéticas previamente descritas em outras populações (majoritariamente europeias) com a Perio4C. Taiete et al., 2018, avaliando 200 indivíduos com Perio4C, 200 indivíduos com Periodontite crônica (PC) e 200 indivíduos periodontalmente saudáveis (PH) refutou a ligação dos "single nucleotide polymorphisms" (SNPs) rs1537415 (*GLT6D1*) e rs1333048 (*ANRIL*) com a população de Perio4C analisada. Entretanto, em relação ao SNP rs6667202 (*IL10*), os resultados mostraram-se confirmatórios. O alelo alterado A de rs6667202 (C>A) foi mais frequentemente detectado em pacientes com Perio4C (76,5%) quando comparado aos demais grupos (PH = 65,8% e PC = 69,7%; $p<0,01$), associando esse SNP à ocorrência da Perio4C (Taiete et al., 2019).

Ao analisar a região promotora do *IL10* e a posição gênica do SNP rs6667202, pode-se supor um impacto nos níveis de expressão (mRNA) de *IL10*. Este SNP está localizado em uma região gênica *upstream* (região de 1,3 kb), em uma sequência *enhancer* distal de DNA, que atua regulando a transcrição de *IL10*. Fatores de transcrição importantes, como o S-MARCA4-201, ligam-se a esta sequência onde rs6667202 está localizado, resultando na transcrição de *IL10*. Porém, a presença de um alelo alterado nessa região (por exemplo o alelo alterado A para este SNP) pode modular a ligação de fatores de transcrição, alterando a expressão gênica e, consequentemente, alterando a produção de proteínas (IL-10, por exemplo).

Essa possibilidade é indiretamente apoiada por estudos prévios, que demonstraram que os pacientes com Perio4C apresentavam níveis reduzidos de IL-10 no fluido crevicular gengival (FCG) e no soro quando comparados a outros perfis periodontais (Duarte et al., 2010; Casarin et al., 2010; Teles et al., 2010; Mattuella et al., 2013). Casarin et al., 2010 demonstraram uma produção menor de IL-10 no FGC em bolsas moderadas e profundas de pacientes com diagnóstico de Perio4C quando comparados com locais semelhantes de indivíduos com periodontite crônica (Casarin et al., 2010).

Contudo, a justificativa biológica para essa redução nos níveis dessa citocina se baseia em uma possível relação com alterações genéticas, o que pode ser confirmado apenas com estudos que buscam entender a funcionalidade das variantes genéticas, ou seja, de que forma essas variações podem influenciar no funcionamento celular. Avaliando SNPs no gene *IL10* (-592 C>A), Claudino et al., 2008 coletaram tecido gengival de indivíduos com periodontite crônica e determinou expressão gênica de *IL10*, metaloproteinases (TIMPs) e osteoprotegerina

(OPG), observando uma redução de *IL10* (mRNA), bem como (e possivelmente em consequência deste), uma redução de TIMP-3 e OPG (Claudino et al., 2008). Em 2012, Sugita et al. avaliaram que o SNP FcγRIIB-nt645+25 (A>G), no gene FcγRIIb (um receptor de imunoglobulina G (IgG) que inibe a ativação do linfócito B) estava associado a periodontite crônica e, por meio de células sanguíneas estimuladas por proteínas de *P. gingivalis*, mostrou que esse SNP promove um aumento na expressão de FcyRIIb e reduz níveis de IgG contra P. gingivalis (Sugita et al., 2012).

Um estudo preliminar *in vivo* realizado também pelo nosso grupo indicou o potencial papel funcional do SNP rs6667202, uma vez que este foi associado a menores concentrações de IL-10 em bolsas profundas de indivíduos portadores de Perio4C (Stolf et al., 2021). Esse achado pode ser explicado pela ação da Interleucina-10 (IL-10), uma vez que esta citocina apresenta propriedades regulatórias e anti-inflamatórias, reduzindo a expressão de citocinas pró-inflamatórias, como a interleucina 1β (IL-1β) e fator de necrose tumoral α (TNF- α) (Garlet et al., 2006; Garlet, 2010). No entanto, até o momento, nenhuma análise funcional celular do SNP rs6667202 foi realizada. Assim, este estudo teve como objetivo determinar o impacto funcional do SNP rs6667202 na cultura primária de fibroblastos gengivais de pacientes periodontalmente saudáveis e de pacientes com Perio4C, após o desafio celular com extrato proteico do *Aggregatibacter actinomycetencomitans* (*Aa*).

2. ARTIGO

IL10 PROMOTER rs6667202 POLYMORPHISM IS FUNCTIONAL IN HEALTH BUT NOT IN GRADE C PERIODONTITIS PATIENTS

Functionality of IL10 rs6667202 SNP

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ABSTRACT

Aims: Evaluate the functionality of *IL10* rs6667202 (C > A) in gingival fibroblasts (GFs) of individuals with Grade C periodontitis (Perio4C) and with periodontal health (PH) stimulated with *Aggregatibacter actinomycetemcomitans* protein extract (*Aa*PE).

Methods: Nine patients with PH and eight with Perio4C were segregated according to their genotype (AA, AC, or CC), and a biopsy was performed to establish the primary culture of the GFs. After GFs exposure to *Aa*PE at 5 µg/ml for 1.5 hours, total RNA was extracted to analyze *IL10* expression by qPCR. Aliquots of the cell's supernatant were subjected to immunoenzymatic analysis (MAGpix) to detect IL-10.

Results: In PH, the genotypes AA and AC are related to a lower expression of *IL10* ($p = 0.027$ and $p < 0.0001$) and lower production of IL-10 ($p = 0.002$ and $p = 0.001$), when compared to CC. In Perio4C there was no statistical difference between the genotypes ($p > 0.05$), although a lower IL-10 expression and release compared to PH CC was seen ($p = 0.033$ and $p < 0.001$).

Conclusion: This SNP has a functional character in PH, as it decreases the expression and production of IL-10. In Perio4C, other factors may be masking the SNP's action by altering the IL-10's response.

Keywords: Single nucleotide polymorphism. Cytokines. Interleukin-10. Aggressive periodontitis. Gene expression.

CLINICAL RELEVANCE

Scientific rationale for study: The functional impact of *IL10* rs6667202 (C > A) SNP, previously associated with Periodontitis Grade C (Perio4C), on IL-10 expression is still unexplored.

Principal findings: In periodontal health participants, the altered A allele (AA and AC genotypes) is related to lower levels of *IL10* mRNA and consequent decreased production of this cytokine compared to the CC genotype. No influence of this SNP occurred in Perio4C, although lower IL-10 release was seen compared to PH CC.

Practical implications: Once *IL10* rs6667202 (C > A) SNP appears to be functional, we can stipulate individual risk to developing periodontal disease, allowing its early diagnosis and preventive treatment. However, Perio4C still need clarification to understand its aberrant host-response.

INTRODUCTION

Periodontitis is a disease triggered by the host's immune-inflammatory response to periodontopathogens present in subgingival biofilm. Grade C, Stage 3 or 4 periodontitis in young people (Perio4C), previously named as aggressive periodontitis (1, 2), could be considered one of the most severe forms, as it presents rapid progression, inadequate response to therapeutic approaches, and aggregation of cases (3-5). Individuals' susceptibility to this disease is mainly associated with genetic factors, such as the presence of Single Nucleotide Polymorphisms (SNPs) and microRNAs (6, 7).

Up to date, a florid number of genetic alterations have been associated with periodontitis, in particular to Perio4C. Previous studies demonstrated an association between the rs6667202 SNP (C > A), located in the promoter region of *IL10* gene, and the Perio4C in a Brazilian and a pooled German-Austrian sample (8, 9). This SNP, A allele, was detected in a higher frequency in Perio4C patients when compared to individuals with periodontal health and chronic periodontitis (8, 9).

Meanwhile, the presence of an SNP is not, *per se*, determinant for the development of Perio4C; To provide any biological risk, the genetic variation must be functional, and when it does, it influences both the host's immune response to the microbial challenge (10-12), the periodontal clinical parameters, and the presence of specific subgingival microorganisms (13, 14). This way, studies have focused on evaluating the functionality of periodontitis-associated SNPs. Claudino et al., 2008 studied the functionality of an also *IL10* promoter SNP (-592 C > A) by collecting gingival tissue of patients with periodontitis. They determined the gene expression of *IL10*, observing a reduction in its mRNA levels on affected patients (15).

Regarding the rs6667202 SNP, a recent *in vivo* preliminary study indicated its potential functional role, with lower interleukin 10 (IL-10) production in the crevicular fluid (16). Once IL-10 presents regulatory and anti-inflammatory properties, reducing the expression of pro-inflammatory cytokines, such as interleukin 1β (IL-1β) and tumoral necrosis factor α (TNF- α) (17, 18), the higher frequency of rs6667202 SNP in Perio4C population can be explained. However, no cellular functional analysis of this variation has been done up to date. Thus, this study aimed to determine the functional impact of rs6667202 SNP on gingival fibroblasts from periodontal healthy (PH), and Perio4C patients challenged with protein extract from *Aggregatibacter actinomycetencomitans* (*Aa*).

MATERIAL AND METHODS

1. Patient Selection

The study design was approved by FOP-UNICAMP Research Ethics Committee (14822719.8.0000.5418). Based on our previous study (8), in which 200 health and 200 Perio4C (previously aggressive periodontitis) patients were screened for rs6667202 variation, nine patients with periodontal health (PH) and eight with Perio4C, with different *IL10* genotyping, were selected for this study, according to inclusion criteria.

For inclusion, patients would perform oral surgeries (for example, aesthetic smile correction, open-flap debridement, and implant insertion), which allowed us to collect a small biopsy of gingival tissue for subsequent primary culture of human gingival fibroblasts (HGFs). All participants were genotyped again for this SNP according to the methodology applied by Taiete et al., 2018 (8) and provided written informed consent for participation in this study.

The patients were allocated into six groups, based on SNP and periodontal status, as follow:

I. *PH AA* (n=3): Health individuals with rs6667202 variation (A allele) in homozygosis.
II. *PH AC* (n=3): Health individuals with rs6667202 variation (A allele) in heterozygosis.
III. *PH CC* (n=3): Health individuals with the absence of rs6667202 variation.
IV. *Perio4C AA* (n=3): Perio4C individuals with rs6667202 variation (A allele) in homozygosis.
V. *Perio4C AC* (n=3): Perio4C individuals with rs6667202 variation (A allele) in heterozygosis.
VI. *Perio4C CC group* (n=2): Perio4C individuals with the absence of rs6667202 variation.

2. HGFs Culture

To obtain the primary culture of HGFs, the protocol described by Silvério et al., 2010 was adopted (19). The gingival tissues were placed in Falcon tubes (BD - Labware) containing biopsy medium composed of Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 250 µg/ml gentamicin sulfate, 5 µg/ml amphotericin B, 100 µg/ml Streptomycin (S) and 100 U/ml Penicillin (P) (Gibco BRL, life technologies, Rockville, MD, USA). After

digesting the tissue with 3 mg/mL of collagenase type 1 and 4 mg/ml of dispase (Gibco BRL, life technologies, Rockville, MD, USA), the cells were filtered through 100 µm Cell Strainer (BD - Labware). The suspension was centrifuged, and the cell pellet resuspended in standard culture medium, composed of DMEM, 10% FBS, 100 µg/ml S, and 100 U/ml P (Gibco BRL, life technologies, Rockville, MD, USA), sown in 100x20mm cell culture plates and incubated at 37°C in an atmosphere saturated with 5% CO_2 and 98% humidity. Cells between the third and fifth passages were used for all experiments performed.

3. *A. actinomycetencomitans* total protein extract (*Aa*PE)

JP2 strain of the *A. actinomycetencomitans*, ATCC 29522, provided by FIOCRUZ/Manguinhos, were used. The strains were reactivated at 37°C in an atmosphere saturated with 5% CO_2 and 98% humidity on agar plates containing Brain and Heart Infusion medium with Hemin and Vitamin K1 (Sigma, St. Louis, MO, USA). After 24 hours of bacterial growth, the total protein was extracted as proposed by Albiero et al., 2017 (20). Briefly, after the centrifugation of isolated colonies from independent cultures of *Aa* in 0.9% NaCl, the sediment was supplemented with 700 µl of ultrapure water and ≈ 0.16 g of 0.1 mm diameter zirconia beads (BioSpec Products, Inc., Bartlesville, OK, USA) and agitated in a Mini-BeadBeater (BioSpec) device and then centrifuged to obtain a beads-free supernatant. Aliquots of this supernatant were prepared and stored at -80°C. The Bradford method determined the total protein concentration (Bradford kit, Bio-Rad, São Paulo, Brazil).

4. Cell Viability

Cell viability of HGFs incubated with *Aa*PE was determined using one population of Perio4C AA group (most susceptible to *Aa* aggression) and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were plated in 96-well plates in a concentration of $0.5x10^4$ cells/well in a standard medium. After 24 hours, medium was replaced to medium containing 1 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml, and 25 µg/ml of *Aa*PE. The HGFs were cultured for 7 days. At days 3 and 7, MTT reagent was added to each well and incubated for 4 hours at 37°C in a humidified 5% CO_2 incubator. At the end of the incubation period, the medium was removed, and the converted dye was solubilized with 100% ethanol. The absorbance of the converted dye was measured at a wavelength of 570 nm.

5. Time course and dose-response pilot study

5.1 *HGFs exposure to Aa*PE

A time-course and dose-response experiment was performed to evaluate which concentration of *Aa*PE, and which period of exposure promotes maximum *IL10* expression by the HGFs. After analyzing the *Aa*PE concentrations that did not compromise cell viability in a maximum period of 3 days, the *IL10* expression was evaluated for two different extract concentrations, 5 and 20 μg/ml. HGFs from a single population of PH AA and Perio4C AA groups were seeded in 6-well cell culture plates at a concentration of 15 x 10^4 cells per well and grown in a standard culture medium for 24 hours in a 37°C, 5% CO_2, and 98% humidity incubator. After this period, the medium was replaced with medium containing *Aa*PE at the predetermined concentrations and with medium containing the combination of 12-myristate 13-phorbol acetate (Invitrogen) and ionomycin - calcium salt (Sigma-Aldrich) (PMA), a potent inducer of inflammation, at concentrations of 1 ng/ml and 100 ng/ml, respectively (21). The cells were maintained in these culture conditions for 1.5, 3, 6, 12, and 24 hours. Negative controls were represented by unstimulated cells.

5.2 *IL10 Expression*

At the end of each period, the total RNA of the cells was collected using the TRIzol reagent (Invitrogen), extracted according to the manufacturer's specific protocol, and treated with deoxyribonuclease (DNase) (DNA-free ™, Ambion Inc., Austin, TX, USA). The RNA concentration and quality were measured using a spectrophotometer (Nanodrop 2000, 10 Thermo Scientific), and a 10μL aliquot of the sample was used for the synthesis of complementary single-stranded DNA (cDNA) (Roche Diagnostic Co., Indianapolis) in a final volume of 20 μl. Quantitative Polymerase Chain Reaction (qPCR) was performed using the cDNA in a concentration of 25 ng/ml, the kit LightCycler 480 SYBR Green I Master (Roche Diagnostic Co.), and a pair of predefined human primers: 1) *18S:* 3'-CGGACAGGATTGACAGATTGATAGC-5' (F), 5'-TGCCAGAGTCTCGTTCGTTATCG-3' (R), 2) *IL10:* 5´-GCCTACATGACAATGAAGATACGA-3' (F), 3'-CTATAAGAGAGGTACAATAAGGTTTCTCAA-5' (R). Each experiment was performed in triplicate, using the water as a negative control. The relative gene expression

level was determined using the cycle threshold (Ct) method and normalized to housekeeping gene *18S*. δCt formula were used to calculate the expression of the target gene.

6. HGFs *IL10* expression

HGFs from all the six groups, *PH AA* (n=3), *PH AC* (n=3), *PH CC* (n=3), *Perio4C AA* (n=3), *Perio4C AC* (n=3), *Perio4C CC* (n=2) were seeded in 100 x 20mm cell culture plates, at a concentration of 90 x 10^4 cells per plate and grown in standard medium for 24 hours in a 37°C and 5% CO_2 environment. Then, the cells were challenged with *Aa*PE at 5 µg/ml, except on control plates, for 1.5 hours. After this period, total RNA was collected, extracted, and treated as reported above. For the synthesis of cDNA in a final concentration of 25 ng/ml, a pre-calculated aliquot of the RNA sample from each HGFs population within each group was used. The primers for *IL10* and *18S* (housekeeping gene), also described above, were used for the qPCR and gene expression analysis.

7. HGFs IL-10 secretion

The HGFs from all the six groups were seeded in 6-well cell culture plates at a concentration of 15 x 10^4 cells per well and grown in standard culture medium for 24 hours in a 37°C, 5% CO_2, and 98% humidity incubator. Then, the standard medium was replaced for medium containing *Aa*PE at 5 µg/ml, except on control plates. After the exposure period, 1.5 hours, the medium was replaced by the standard medium again. After another 24 hours – time previously demonstrated as ideal for maximum secretion of IL-10 (data not shown) - the supernatant was collected and stored at -80^{o}C.

The supernatant aliquots were subjected to Luminex/MAGPIX analysis to IL-10 detection. These were carried out in 96-well plates, with sensitive panels' aid (Millipore Corporation, Billerica, MA, USA), following the manufacturer's instructions. In summary, microspheres conjugated to monoclonal antibodies against the analytes were added to the wells and incubated for 2 hours at room temperature. The wells were washed, and a mixture of secondary antibodies was added. After incubation for 1 hour, the final detection was made using a fluorescent marker, Streptavidin-Phycoerythrin, which binds to the detection antibody.

8. Statistical Analyses

Demographical and clinical data were compared between groups by Chi-square and Student’s t test. Data of mRNA levels and cytokine production were firstly assessed for normal

distribution using the Shapiro-Wilk test. If the results were normal, the comparison between groups was performed using the two-way ANOVA/Tukey statistical test. If not, they were compared using Friedman's statistical test. A significance level of 5% was considered for all analyses.

RESULTS

Table 1 shows the clinical and demographic characteristics of the participants. In the comparison between the periodontal status (PH and Perio4C), there is a statistical difference between age (p=0.008) and the clinical parameters evaluated: plaque index (%) (p=0.0025), bleeding on probing (%) (p<0.001), probing depth (PD) (%) (p<0.001) and percentage of sites presenting PD ≥ 5mm (%) (p<0.0017).

Table 1. Clinical (mean ± standard deviation - SD) and demographic data of the study participants.

	PH (n=9)	**Peiro4C (n=8)**
Age (years)	26.0 ± 5.7	35.0 ± 6.9*
Gender (female/male)	7/2	6/2
Ethnicity (caucasian/African)	9/0	6/2
Plaque Index (%)	4.0 ± 4.6	25.0 ± 16.6*
Bleeding on Probing (%)	3.2 ± 1.1	33.0 ± 15.9*
Probing Depth (PD) (%)	1.6 ± 0.3	3.2 ± 0.8*
Sites presenting PD ≥ 5mm (%)	0	18.0 ± 14.2*

*Indicates statistical difference between groups.

1. HGFs viability and maximum *IL0* expression pilot study

An MTT assay was performed to determine the survival rate of HGFs to prolonged exposure to *Aa*PE **(Fig. 1).** After 3 days, HGFs maintained a cell survival rate greater than 80% for all *Aa*PE concentrations. After 7 days, cell viability was lost at all concentrations, except 25 μg/ml. As all experiments would be performed within a maximum period of 24 hours, the initial working *Aa*PE concentrations chosen were 5 and 20 μg/ml.

In the pilot study, a single population of PH AA and Perio4C AA groups were exposed to the two predetermined concentrations of *Aa*PE and PMA, a neutral stimulus, during 1.5, 3,

6, 12, and 24 hours. At the end of each period, cellular RNA was collected, and *IL10* mRNA levels were determined for PH **(Fig. 2A)** and Perio4C **(Fig. 2B)**. For the PH population, the exposure with 5 µg/ml of *Aa*PE for 1.5 hours increased the expression of *IL10*. For the Perio4C, 5 µg/ml of *Aa*PE promoted *IL10* expression after 1.5 and also 3 hours of stimulation. 20 µg/ml of *Aa*PE did not seem to influence the levels of *IL10* mRNA in health or disease conditions; it increased *IL10* expression only after 6 hours of stimulation.

The PMA promotes a differentiated cellular response in relation to *Aa*PE, proving the bacterial stimulus's specificity. Thus, it was decided to follow up on the following experiments by stimulating the cells with 5 µg/ml of *Aa*PE for 1.5 hours to assess the functionality of rs6667202 SNP after *Aa*PE stimulus.

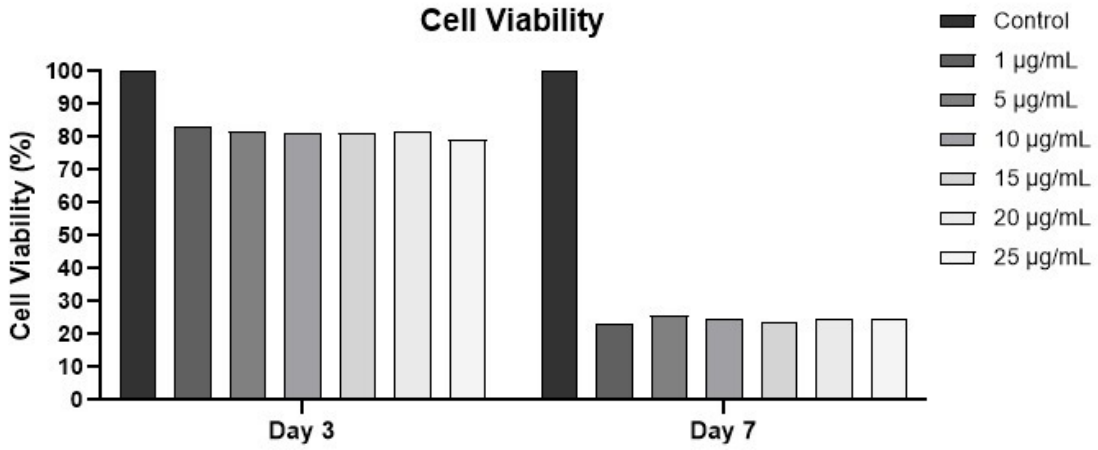

Figure 1. Effect of *Aa*PE on cell viability. HGFs were challenged with 0 (control) to 25 µg/ml of *Aa*PE, and the MTT assay for assessing cell metabolism and viability was performed on days 3 and 7. The control represents 100% of viability. Data were analyzed using GraphPad Prism software (version 9; GraphPad Software, Inc.).

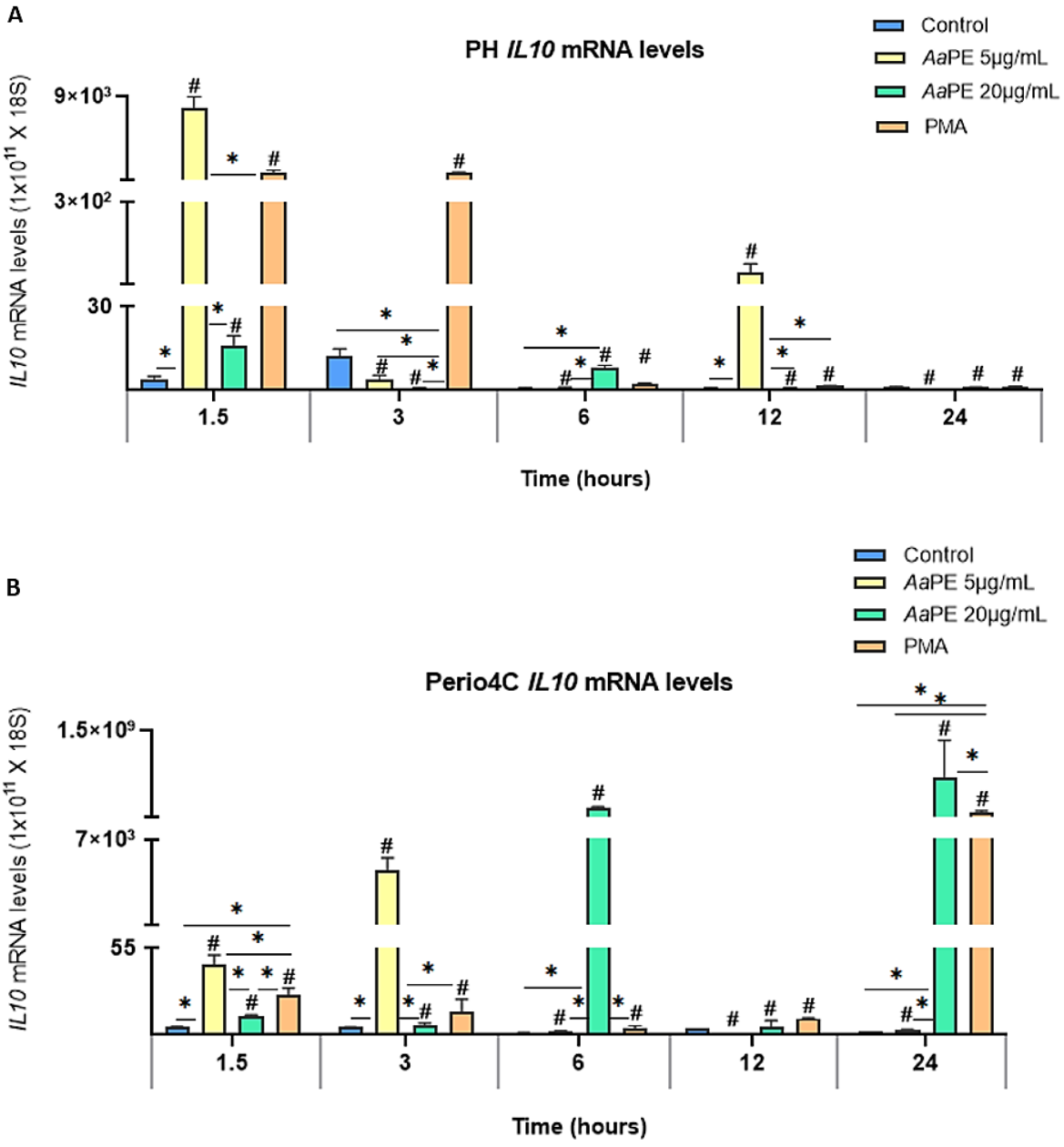

Figure 2. HGFs expression of *IL10* mRNA on PH **(A)** and Perio4C **(B)** at 0 (control), 1.5, 3, 6, 12, and 24 hours after cell exposure with 5 and 20 µg/ml. Data were analyzed using GraphPad Prism software (version 9; GraphPad Software, Inc.). Bars indicate mean ± SD from the experimental triplicate. *Indicates the statistical difference between stimulus at the same time ($p \leq 0.05$). #Indicates the statistical difference between exposure periods for the same stimuli ($p \leq 0.05$). PH (periodontal health); Perio4C (periodontitis Grade C, stage 3 or 4); *Aa*PE (*Aggregatibacter actinomycetencomitans* protein extract).

2. rs6667202 modulates HGFs IL-10 response after *Aa*PE stimulation.

Decreased levels of *IL10* mRNA were seen for the PH AA and AC groups (presence of rs66667202 (C>A); A allele) after being exposed to 5 µg/ml of *Aa*PE for 1.5 hours, when compared to the CC genotype ($p = 0.027$ and $p < 0.0001$, respectively) **(Fig. 3)**. Moreover, HGFs from PH AA and AC groups also secreted less IL-10 than the CC genotype ($p = 0.002$ and $p = 0.001$, respectively) when exposed to *Aa*PE **(Fig. 4)**.

For patients with Perio4C, the A allele's presence did not alter *IL10* expression or release ($p > 0.05$). However, there is a decrease in *IL10* mRNA levels and cytokine production in the CC genotype compared to PH ($p = 0.033$ and $p < 0.001$, respectively).

Comparing all PH patients with all those on Perio4C groups, we observe that Perio4C had lower levels of *IL10* mRNA after the bacterial stimuli ($p = 0.024$) **(Fig. 3).** Regarding the IL-10 secretion, in addition, to produce lower baseline levels of IL-10 ($p = 0.0007$), patients with Perio4C also had decreased IL-10 production after *Aa*PE stimuli ($p = 0.0022$) **(Fig. 4).**

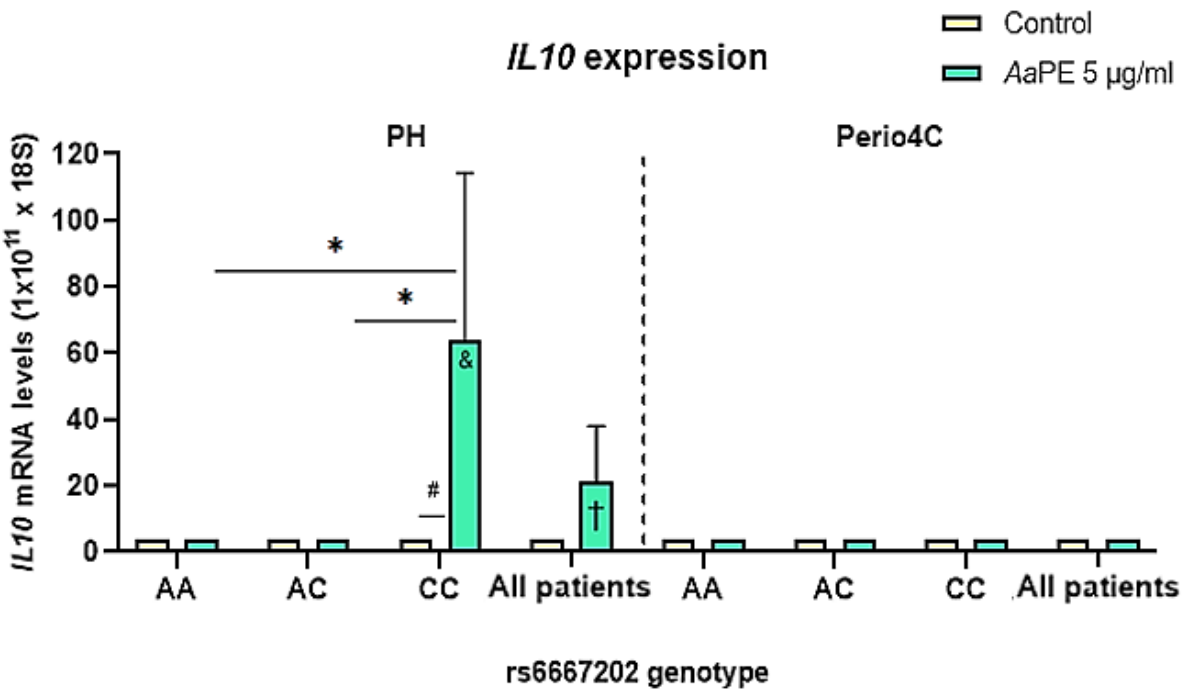

Figure 3. HGFs expression of *IL10* mRNA from PH and Perio4C patients with AA, AC, or CC genotypes for rs6667202 variation, after being exposed to 5 µg/ml of *Aa*PE for 1.5 hours. Bars indicate mean ± SD from the experimental triplicate. Data were analyzed using GraphPad Prism software (version 9; GraphPad Software, Inc.). *Indicates the statistical difference between genotypes ($p \leq 0.05$) # Indicates the statistical difference between control and *Aa*PE exposure

(p ≤ 0 .05). &Indicates statistical difference between PH and Perio4C (p ≤ 0.05). †Indicates statistical difference between all PH and all Perio4C patients (p ≤ 0.05). PH (periodontal health); Perio4C (periodontitis Grade C, stage 3 or 4); *Aa*PE (*Aggregatibacter actinomycetencomitans* protein extract).

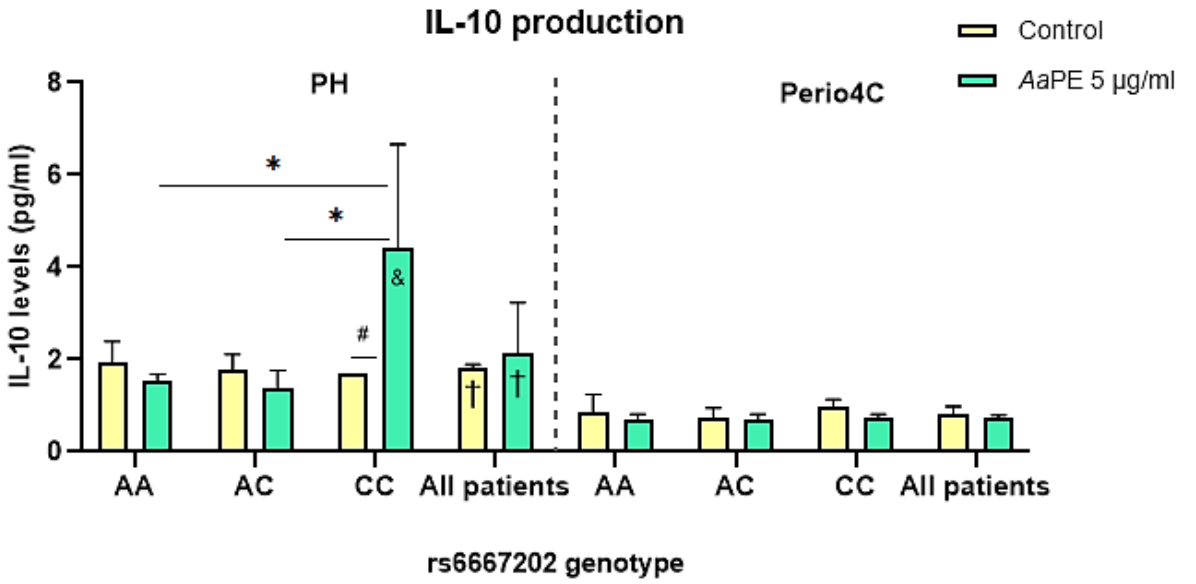

Figure 4. IL-10 production, expressed in pg/ml, of HGFs from PH and Perio4C patients with AA, AC, or CC genotypes for rs6667202 variation, after being exposed to 5 µg/mL of *Aa*PE for 1.5 hours. Bars indicate mean ± SD from the patients' response. Data were analyzed using GraphPad Prism software (version 9; GraphPad Software, Inc.). *Indicates the statistical difference between genotypes (p ≤ 0.05) # Indicates the statistical difference between control and *Aa*PE exposure (p ≤ 0 .05). &Indicates statistical difference between PH and Perio4C (p ≤ 0.05). †Indicates statistical difference between all PH and all Perio4C patients (p ≤ 0.05). PH (periodontal health); Perio4C (periodontitis Grade C, stage 3 or 4); *Aa*PE (*Aggregatibacter actinomycetencomitans* protein extract).

DISCUSSION

Once rs6667202 (C > A) SNP has been associated with Perio4C, determine its functionality is essential to better address its role on periodontal etiopathogenesis. Our results show that the AA and AC genotypes decrease *IL10* mRNA levels and, consequently, IL-10 production in PH HGFs triggered with *Aa*PE compared to the CC genotype. Genetic research has identified SNPs as risk factors for developing periodontitis (22, 23) once they can alter the

host's immunological response by impacting the structure, function, or production of cytokines. This way, they are responsible for approximately 50% of the variability in the clinical periodontal parameters and may determine susceptibility profiles to Perio4C development and response to therapy (5, 12).

By analyzing *the IL10* promoter region and the genic position of rs6667202, we can understand how it may impact the *IL10* mRNA levels. This SNP is located in an upstream genic region (1.3 kb region), in a distal enhancer DNA sequence that acts regulating the *IL10* transcription. Important transcription factors, like S-MARCA4-201, binds to this enhancer sequence where rs6667202 is located, resulting in *IL10* transcription stimulation. However, given the present results, it is possible to suggest that the presence of an altered allele (A allele for this SNP) may prevent the bind of transcription factors, altering the gene expression and, consequently, altering protein production.

SNPs in a similar genic region have already shown the potential to alter protein production. Zhang et al., 1999 reported the functionality of -1562 SNP (C>T) in a promoter region of the metalloproteinase-9 (MMP-9) gene encoding gelatinase. Transient transfection experiments and DNA-protein interaction assays were used to verify the functionality of this SNP, and, as an example of how variations in the promoter genic region can trigger changes in its expression, these tests indicated that the T allele had a higher promoter activity than the C allele (24).

Interestingly, different from the PH group, rs6667202 did not affect IL-10 production in Perio4C patients, although the comparison between CC groups has shown a lower IL-10 release in the Perio4C group. Although rs6667202 was previously related to Perio4C in populational studies, several factors may be behind this lack of response. In our research, we opted to use cells from primary cultures. These contain the individual's complete genetic information; that is, other SNPs can act together, leading to other changes in the immune response, counter-balancing the rs6667202 effect in this group. Other *IL10* promoter SNPs, like rs1800871 and rs1800872, have also been associated with Perio4C in Latin American populations (25). Thus, some other methodological options may be considered to isolate some other SNPs' influence.

Recently, an interesting study assessed the effect of IL-8 haplotype previously related to periodontitis occurrence, using the CRISPR/Cas9 technique, which could isolate the impact of the genetic alteration. Using this technique, Benakanakere et al., 2016, demonstrated that cells with the ATC/TTC haplotype in the *IL8* gene, by themselves, significantly increased

neutrophil transmigration in inflammatory lesions, confirming its functional role (26). Even though this approach isolates the SNP's effect and could be an alternative for accessing IL-10 response on Perio4C, it does not represent the etiopathogenic context in which the patient is inserted.

It should be considered that the HGFs harvested from affected subjects could also carry epigenetic changes. Periodontal tissues are susceptible to epigenetic modifications (hypomethylation or hypermethylation) when in contact with periodontopathogen toxins. However, the methylation consequent effects on gene regulation depend on cell type and function after interaction with the periodontopathogen (27). Martins et al., 2015 demonstrated that infection with *P. gingivalis* and *F. nucleatum* could induce acetylation of histones in oral epithelial cells by activating the Toll-like receptors 1, 2, and 4 (28). Altogether, this cell memory could represent a possibility to lastly alter its behavior under following stimulation, leading to the altered cytokine profile, as seen in our study. This is important when some microbiological assays have shown a more specific microbial community in Perio4C (29, 30), along with altered cytokines in gingival fluid, as IL-10 (31).

IL-10 is a Treg representant and plays a role in regulating and attenuating the immune response, acting on the pro-inflammatory cytokines of the Th1 response and inducing the expression of osteoprotegerin and tissue inhibitors of metalloproteinases (17). Sakai et al., 2019 investigated the importance of IL-10 protection character in systemic inflammatory diseases, where IL-10 knockout mice increased mRNA expression of the pro-inflammatory cytokines IL-1β, IL-6, and IL-18. At the same time, when recombinant IL-10 was added, *in vitro*, to the epithelial cells of these mice, the expression of pro-inflammatory cytokines was suppressed (32).

Comparing PH and Perio4C patients, despite the genotype, we observe that Perio4C had lower levels of *IL10* mRNA and decreased IL-10 release after the bacterial stimuli. Previous studies have also shown that patients with Perio4C had reduced levels of IL-10 in the gingival crevicular fluid (GCF) and serum compared to other periodontal profiles (10, 33, 34). Zein Elabdeen et al., 2017 analyzed the profile of plasma and gingival crevicular fluid (GCF) cytokines of patients with Perio4C and PH also using Luminex/MAGpix technology. In this study, Perio4C patients had lower levels of IFN-γ and Th1:Th2 ratios in GCF and lower anti-inflammatory cytokines IL-10, IL-13, and IL-1Ra in plasma (35). These studies indicate that IL-10 may represent an essential player in the multifactorial etiology of this specific phenotype.

Multiple factors, as SNPs and a specific dysbiotic microbiota, usually act together, altering the host's inflammatory response (5, 30, 36, 37). Integrating different factors that participate in the etiopathogenesis of Perio4C, Casarin et al., 2010 showed a lower level of IL-10 in the GCF of individuals with Perio4C while detected a higher concentration of *Aa* and reduced concentration of Immunoglobulin G (IgG) against this pathogen (38). More recently, a preliminary *in vivo* study has shown that rs6667202 SNP leads to a decrease in IL-10 production only in deep pockets of Perio4C patients, where a mature and dysbiotic biofilm is instigating the host response (16). Teles et al., 2010 correlated bacterial species of the red complex with a higher expression of biomarkers (IL-1β, IL-8, and MMP-8) in GCF, while species of the yellow and purple complex were associated only with MMP-8. In addition, even the healthy sites of patients with periodontitis had higher levels of IL-1β and IL-8 and periodontal pathogens than the PH group, showing distinct associations between the host's immune response and microbial complexes (39).

Considering type-specific microbiota, *Aggregatibacter actinomycetencomitans*, an oral gram-negative pathogen, has been isolated from sites with active periodontal disease in young people for decades and represents an increased risk for the development of rapidly progressive periodontitis (40-42). Although there are six serotypes of *Aa* (a-g), serotype b, represented by the JP2 genotype, is the one that is associated with the greater virulence, therefore, with the greater risk of periodontal insertion loss (43, 44). In our study, the total protein of these more cytotoxic strains was used, and several virulent aspects were given to cells.

When in contact with *Aa* exotoxins, the oral epithelium and the adjacent connective tissue form the first defense barrier against this pathogen (45). Briefly, *Aa* can easily migrate from the gingival sulcus to the internal epithelium through its ability to adhere to epithelial cells (46, 47). After penetrating the subepithelial connective tissue, its exotoxins stimulate gingival fibroblasts to secrete pro-inflammatory cytokines, which will start the defense process against *Aa*, confirming the importance of evaluating the response of fibroblasts against bacterial stimulation (48).

The frequency of rs6667202 SNP is considerably high in the Brazilian population and even higher among patients with Perio4C (frequency of 76,5% for A allele) (8), which led to difficulties in finding patients with this disease who presented the C allele in homozygosis. Thus, new studies with a higher number of subjects and using different technologies should be necessary to elucidate this SNP role in Perio4C patients. Despite that, our findings demonstrate

an important step in understanding how genetic variations can influence the immune regulation after cellular challenge with periodontopathogens.

CONCLUSION

In conclusion, in the presence of rs6667202 SNP, HGFs of PH patients had lower expression of *IL10* and consequently lower production of this cytokine compared to the CC genotype, proving its functionality in these patients. In Perio4C, other factors may be masking the SNP's action by altering the IL-10's response.

REFERENCES

1. Armitage, G. C. (1999) Development of a classification system for periodontal diseases and conditions. Ann Periodontol 4, 1-6. doi:10.1902/annals.1999.4.1.1.

2. Caton JG, Armitage G, Berglundh T, Chapple ILC, Jepsen S, Kornman KS, Mealey BL, Papapanou PN, Sanz M, Tonetti MS. A new classification scheme for periodontal and peri-implant diseases and conditions - Introduction and key changes from the 1999 classification. J Clin Periodontol. 2018 Jun;45 Suppl 20:S1-S8. doi: 10.1111/jcpe.12935.

3. Albandar, J. M. (2014a) Aggressive and acute periodontal diseases. Periodontol 2000 65, 7-12. doi:10.1111/prd.12013.

4. Albandar, J. M. (2014b) Aggressive periodontitis: case definition and diagnostic criteria. Periodontol 2000 65, 13-26. doi:10.1111/prd.12014.

5. Deas DE, Mealey BL. Response of chronic and aggressive periodontitis to treatment. Periodontol 2000. 2010 Jun; 53:154-66. doi:10.1111/j.1600-0757.2009.00334.x. Review.

6. Loos BG, Papantonopoulos G, Jepsen S, Laine ML. What is the Contribution of Genetics to Periodontal Risk? Dent Clin North Am. 2015 Oct;59(4):761-80. doi: 10.1016/j.cden.2015.06.005. Epub 2015 Aug 1. Review.

7. Stoecklin-Wasmer C1, Guarnieri P, Celenti R, Demmer RT, Kebschull M, Papapanou PN. MicroRNAs and their target genes in gingival tissues. J Dent Res. 2012 Oct;91(10):934-40. Epub 2012 Aug 9.

8. Taiete T, Casati MZ, Stolf CS, Corrêa MG, Santamaria MP, Andere NMRB, et al. Validation of reported GLT6D1 (rs1537415), IL10 (rs6667202), and ANRIL (rs1333048)

single nucleotide polymorphisms for aggressive periodontitis in a Brazilian population. J Periodontol. 2019 Jan;90(1):44-51. doi: 10.1002/JPER.18-0071.

9. Schaefer AS, Bochenek G, Manke T, Nothnagel M, Graetz C, Thien A, et al. Validation of reported genetic risk factors for periodontitis in a large-scale replication study. J Clin Periodontol. 2013 Jun;40(6):563-72. doi: 10.1111/jcpe.12092.

10. Duarte, P. M., Bastos, M. F., Fermiano, D., Rabelo, C. C., Perez-Chaparro, P. J., Figueiredo, L. C., Faveri, M. & Feres, M. (2015) Do subjects with aggressive and chronic periodontitis exhibit a different cytokine/chemokine profile in the gingival crevicular fluid? A systematic review. J Periodontal Res 50, 18-27. doi:10.1111/jre.12180.

11. Kebschull, M., Guarnieri, P., Demmer, R. T., Boulesteix, A. L., Pavlidis, P. & Papapanou, P. N. (2013) Molecular differences between chronic and aggressive periodontitis. J Dent Res 92, 1081-1088. doi:10.1177/0022034513506011.

12. Vieira, A. R. & Albandar, J. M. (2014) Role of genetic factors in the pathogenesis of aggressive periodontitis. Periodontol 2000 65, 92-106. doi:10.1111/prd.12021.

13. Nibali L, Ready DR, Parkar M, Brett PM, Wilson M, Tonetti MS, Griffiths GS. Gene polymorphisms and the prevalence of key periodontal pathogens. J Dent Res. 2007 May;86(5):416-20. doi: 10.1177/154405910708600505.

14. Nibali L, Pelekos G, D'Aiuto F, Chaudhary N, Habeeb R, Ready D, Parkar M, Donos N. Influence of IL-6 haplotypes on clinical and inflammatory response in aggressive periodontitis. Clin Oral Investig. 2013 May;17(4):1235-42. doi: 10.1007/s00784-012-0804-3. Epub 2012 Aug 24.

15. Claudino M, Trombone AP, Cardoso CR, Ferreira SB Jr, Martins W Jr, Assis GF, Santos CF, Trevilatto PC, Campanelli AP, Silva JS, Garlet GP. The broad effects of the functional IL-10 promoter-592 polymorphism: modulation of IL-10, TIMP-3, and OPG expression and their association with periodontal disease outcome. J Leukoc Biol. 2008 Dec;84(6):1565-73. doi: 10.1189/jlb.0308184. Epub 2008 Aug 25.

16. Stolf CS, Taiete T, Zaffalon Casati M, Sallum EA, Nociti Júnior FH, Ruiz KGS, Casarin RV. Influence of rs6667202 SNP on Interleukin-10 levels in the gingival fluid of patients with periodontitis grade C. Braz. J. Oral Sci. [Internet]. 2021Feb.8 [cited 2021Mar.24];20(00):e211654.

17. Garlet GP. Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. J Dent Res. 2010 Dec;89(12):1349-63. doi: 10.1177/0022034510376402.

18. Garlet GP, Cardoso CR, Campanelli AP, Martins W Jr, Silva JS. Expression of suppressors of cytokine signaling in diseased periodontal tissues: a stop signal for disease progression? J Periodontal Res. 2006 Dec;41(6):580-4. doi: 10.1111/j.1600-0765.2006.00908.x.

19. Silvério KG, Rodrigues TL, Coletta RD, Benevides L, Da Silva JS, Casati MZ, Sallum EA, Nociti FH Jr. Mesenchymal stem cell properties of periodontal ligament cells from deciduous and permanent teeth. J Periodontol. 2010 Aug;81(8):1207-15. doi: 10.1902/jop.2010.090729.

20. Albiero ML, Stipp RN, Saito MT, et al. Viability and osteogenic differentiation of human periodontal ligament progenitor cells are maintained after incubation with Porphyromonas gingivalis protein extract. J Periodontol. 2017;88:e188-e199.

21. Ehrnhöfer-Ressler MM, Fricke K, Pignitter M, Walker JM, Walker J, Rychlik M, Somoza V. Identification of 1,8-cineole, borneol, camphor, and thujone as anti-inflammatory compounds in a Salvia officinalis L. infusion using human gingival fibroblasts. J Agric Food Chem. 2013 Apr 10;61(14):3451-9. doi: 10.1021/jf305472t. Epub 2013 Mar 26.

22. Laine ML, Moustakis V, Koumakis L, Potamias G, Loos BG. Modeling susceptibility to periodontitis. J Dent Res. 2013 Jan;92(1):45-50. doi: 10.1177/0022034512465435. Epub 2012 Oct 25.

23. Schaefer, A. S., Richter, G. M., Nothnagel, M., Manke, T., Dommisch, H., Jacobs, G., Arlt, A., Rosenstiel, P., Noack, B., Groessner-Schreiber, B., Jepsen, S., Loos, B. G. & Schreiber, S. (2010) A genome-wide association study identifies GLT6D1 as a susceptibility locus for periodontitis. Hum Mol Genet 19, 553-562. doi:10.1093/hmg/ddp508.

24. Zhang B, Ye S, Herrmann SM, Eriksson P, de Maat M, Evans A, Arveiler D, Luc G, Cambien F, Hamsten A, Watkins H, Henney AM. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. Circulation. 1999 Apr 13;99(14):1788-94. doi: 10.1161/01.cir.99.14.1788.

25. Yang SL, Huang SJ. Interleukin-10 polymorphisms (rs1800871, rs1800872 and rs1800896) and periodontitis risk: A meta-analysis. Arch Oral Biol. 2019 Jan;97:59-66. doi: 10.1016/j.archoralbio.2018.10.012. Epub 2018 Oct 12.

26. Benakanakere MR, Finoti LS, Tanaka U, Grant GR, Scarel-Caminaga RM, Kinane DF. Investigation of the functional role of human Interleukin-8 gene haplotypes by CRISPR/Cas9 mediated genome editing. Sci Rep. 2016 Aug 8;6:31180. doi: 10.1038/srep31180.

27. Barros SP, Hefni E, Nepomuceno R, Offenbacher S, North K. Targeting epigenetic mechanisms in periodontal diseases. Periodontol 2000. 2018 Oct;78(1):174-184. doi: 10.1111/prd.12231.

28. Martins MD, Jiao Y, Larsson L, Almeida LO, Garaicoa-Pazmino C, Le JM, Squarize CH, Inohara N, Giannobile WV, Castilho RM. Epigenetic Modifications of Histones in Periodontal Disease. J Dent Res. 2016 Feb;95(2):215-22. doi: 10.1177/0022034515611876. Epub 2015 Oct 23.

29. Monteiro MF, Altabtbaei K, Kumar PS, Casati MZ, Ruiz KGS, Sallum EA, Nociti-Junior FH, Casarin RCV. Parents with periodontitis impact the subgingival colonization of their offspring. Sci Rep. 2021 Jan 14;11(1):1357. doi: 10.1038/s41598-020-80372-4.

30. Childers NK, Grenett H, Morrow C, Kumar R, Jezewski PA. Potential Risk for Localized Aggressive Periodontitis in African American Preadolescent Children. Pediatr Dent. 2017 Jul 15;39(4):294-298.

31. Taiete T, Monteiro MF, Casati MZ, do Vale HF, Ambosano GMB, Nociti FH, Sallum EA, Casarin RCV. Local IL-10 level as a predictive factor in generalized aggressive periodontitis treatment response. Scand J Immunol. 2019 Dec;90(6):e12816. doi: 10.1111/sji.12816. Epub 2019 Oct 3.

32. Sakai K, Nozaki Y, Murao Y, Yano T, Ri J, Niki K, Kinoshita K, Funauchi M, Matsumura I. Protective effect and mechanism of IL-10 on renal ischemia-reperfusion injury. Lab Invest. 2019 May;99(5):671-683. doi: 10.1038/s41374-018-0162-0. Epub 2019 Jan 30.

33. Mattuella LG, Campagnaro MB, Vargas AE, Xavier LL, Oppermann RV, Chies JA, et al. Plasma cytokines levels in aggressive and chronic periodontitis. Acta Odontol Scand. 2013 May-Jul;71(3-4):683-8. doi: 10.3109/00016357.2012.715191.

34. Teles RP, Gursky LC, Faveri M, Rosa EA, Teles FR, Feres M, Socransky SS, Haffajee AD. Relationships between subgingival microbiota and GCF biomarkers in generalized aggressive periodontitis. J Clin Periodontol. 2010 Apr;37(4):313-23. doi: 10.1111/j.1600-051X.2010.01534.x.

35. Zein Elabdeen HR, Mustafa M, Ali R, Bolstad AI. Cytokine profile in gingival crevicular fluid and plasma of patients with aggressive periodontitis. Acta Odontol Scand. 2017 Nov;75(8):616-622. doi: 10.1080/00016357.2017.1372623. Epub 2017 Sep 3.

36. Kornman KS. Mapping the pathogenesis of periodontitis: a new look. J Periodontol. 2008 Aug;79(8 Suppl):1560-8. doi: 10.1902/jop.2008.080213.

37. Borilova Linhartova P, Danek Z, Deissova T, Hromcik F, Lipovy B, Szaraz D, Janos J, Fassmann A, Bartova J, Drizhal I, Izakovicova Holla L. Interleukin Gene Variability and Periodontal Bacteria in Patients with Generalized Aggressive Form of Periodontitis. Int J Mol Sci. 2020 Jul 2;21(13):4728. doi: 10.3390/ijms21134728.

38. Casarin, R. C., Ribeiro Edel, P., Mariano, F. S., Nociti, F. H., Jr., Casati, M. Z. & Goncalves, R. B. (2010) Levels of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, inflammatory cytokines and species-specific immunoglobulin G in generalized aggressive and chronic periodontitis. J Periodontal Res 45, 635-642. doi:10.1111/j.1600-0765.2010.01278.x.

39. Teles R, Sakellari D, Teles F, Konstantinidis A, Kent R, Socransky S, Haffajee A. Relationships among gingival crevicular fluid biomarkers, clinical parameters of periodontal disease, and the subgingival microbiota. J Periodontol. 2010 Jan;81(1):89-98. doi: 10.1902/jop.2009.090397.

40. Slots J, Reynolds HS, Genco RJ. Actinobacillus actinomycetemcomitans in human periodontal disease: a cross-sectional microbiological investigation. Infect Immun. 1980 Sep;29(3):1013-20.

41. Fine DH, Patil AG, Velusamy SK. Aggregatibacter actinomycetemcomitans (Aa) Under the Radar: Myths and Misunderstandings of Aa and Its Role in Aggressive Periodontitis. Front Immunol. 2019 Apr 16;10:728. doi: 10.3389/fimmu.2019.00728.

42. Fine DH, Markowitz K, Furgang D, Fairlie K, Ferrandiz J, Nasri C, McKiernan M, Gunsolley J. Aggregatibacter actinomycetemcomitans and its relationship to initiation of localized aggressive periodontitis: longitudinal cohort study of initially healthy adolescents. J Clin Microbiol. 2007 Dec;45(12):3859-69. doi: 10.1128/JCM.00653-07. Epub 2007 Oct 17.

43. Haubek D, Ennibi OK, Poulsen K, Vaeth M, Poulsen S, Kilian M. Risk of aggressive periodontitis in adolescent carriers of the JP2 clone of Aggregatibacter (Actinobacillus) actinomycetemcomitans in Morocco: a prospective longitudinal cohort study. Lancet. 2008 Jan 19;371(9608):237-42. doi: 10.1016/S0140-6736(08)60135-X.

44. Höglund Åberg C, Kwamin F, Claesson R, Dahlén G, Johansson A, Haubek D. Progression of attachment loss is strongly associated with presence of the JP2 genotype of Aggregatibacter actinomycetemcomitans: a prospective cohort study of a young adolescent population. J Clin Periodontol. 2014 Mar;41(3):232-41. doi: 10.1111/jcpe.12209. Epub 2014 Jan 16.

45. Haraszthy VI, Hariharan G, Tinoco EM, Cortelli JR, Lally ET, Davis E, Zambon JJ. Evidence for the role of highly leukotoxic Actinobacillus actinomycetemcomitans in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. J Periodontol. 2000 Jun;71(6):912-22. doi: 10.1902/jop.2000.71.6.912.

46. Fives-Taylor PM, Meyer DH, Mintz KP, Brissette C. Virulence factors of Actinobacillus actinomycetemcomitans. Periodontol 2000. 1999 Jun;20:136-67. doi: 10.1111/j.1600-0757.1999.tb00161.x.

47. Meyer DH, Fives-Taylor PM. Characteristics of adherence of Actinobacillus actinomycetemcomitans to epithelial cells. Infect Immun. 1994 Mar;62(3):928-35. doi: 10.1128/IAI.62.3.928-935.1994.

48. Belibasakis GN, Johansson A, Wang Y, Chen C, Lagergård T, Kalfas S, Lerner UH. Cytokine responses of human gingival fibroblasts to Actinobacillus actinomycetemcomitans cytolethal distending toxin. Cytokine. 2005 Apr 21;30(2):56-63. doi: 10.1016/j.cyto.2004.11.008.

3. CONCLUSÃO

Em conclusão, na presença do SNP rs6667202, os fibroblastos gengivais de pacientes com saúde periodontal apresentaram menor expressão de *IL10* e, consequentemente, menor produção dessa citocina, quando comparados ao genótipo CC, sendo um indicador de risco biológico e comprovando sua funcionalidade nesses pacientes. Em Perio4C, outros fatores podem estar mascarando a ação do SNP, alterando a resposta da IL-10.

REFERÊNCIAS*

Albandar, J. M. (2014) Aggressive periodontitis: case definition and diagnostic criteria. Periodontol 2000 65, 13-26. doi:10.1111/prd.12014.

Armitage GC. 1999. Development of a classification system for periodontal diseases and conditions. Ann Periodontol. 4(1):1-6.

Casarin RC, Ribeiro Edel P, Mariano FS, Nociti FH Jr, Casati MZ, Gonçalves RB. Levels of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, inflammatory cytokines and species-specific immunoglobulin G in generalized aggressive and chronic periodontitis. J Periodontal Res. 2010 Oct;45(5):635-42. doi: 10.1111/j.1600-0765.2010.01278.x.

Caton JG, Armitage G, Berglundh T, Chapple ILC, Jepsen S, Kornman KS, Mealey BL, Papapanou PN, Sanz M, Tonetti MS. A new classification scheme for periodontal and peri-implant diseases and conditions - Introduction and key changes from the 1999 classification. J Periodontol. 2018

Claudino M, Trombone AP, Cardoso CR, Ferreira SB Jr, Martins W Jr, Assis GF, et al. (2008). The broad effects of the functional IL-10 promoter-592 polymorphism: modulation of IL-10, TIMP-3, and OPG expression and their association with periodontal disease outcome. J Leukoc Biol 84:1565-1573.

De Souza AP, Planello AC, Marques MR, De Carvalho DD, Line SR. High-throughput DNA analysis shows the importance of methylation in the control of immune inflammatory gene transcription in chronic periodontitis. Clin Epigenetics. 2014 Aug 12;6(1):15. doi: 10.1186/1868-7083-6-15. eCollection 2014.

Deas DE, Mealey BL. Response of chronic and aggressive periodontitis to treatment. Periodontol 2000. 2010 Jun;53:154-66. doi:10.1111/j.1600-0757.2009.00334.x. Review.

Duarte PM, da Rocha M, Sampaio E, Mestnik MJ, Feres M, Figueiredo LC, et al. Serum levels of cytokines in subjects with generalized chronic and aggressive periodontitis before and after non-surgical periodontal therapy: a pilot study. J Periodontol. 2010 Jul;81(7):1056-63. doi: 10.1902/jop.2010.090732.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

Duarte, P. M., Bastos, M. F., Fermiano, D., Rabelo, C. C., Perez-Chaparro, P. J., Figueiredo, L. C., Faveri, M. & Feres, M. (2015) Do subjects with aggressive and chronic periodontitis exhibit a different cytokine/chemokine profile in the gingival crevicular fluid? A systematic review. J Periodontal Res 50, 18-27. doi:10.1111/jre.12180.

Garlet GP, Cardoso CR, Campanelli AP, Martins W Jr, Silva JS. Expression of suppressors of cytokine signaling in diseased periodontal tissues: a stop signal for disease progression? J Periodontal Res. 2006 Dec;41(6):580-4.

Garlet GP. Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. J Dent Res. 2010 Dec;89(12):1349-63. doi: 10.1177/0022034510376402.

Kebschull, M., Guarnieri, P., Demmer, R. T., Boulesteix, A. L., Pavlidis, P. & Papapanou, P. N. (2013) Molecular differences between chronic and aggressive periodontitis. J Dent Res 92, 1081-1088. doi:10.1177/0022034513506011.

Laine ML, Crielaard W, Loos BG. Genetic susceptibility to periodontitis. Periodontol 2000. 2012 Feb;58(1):37-68. doi: 10.1111/j.1600-0757.2011.00415.x.

Loos BG, Papantonopoulos G, Jepsen S, Laine ML. What is the Contribution of Genetics to Periodontal Risk? Dent Clin North Am. 2015 Oct;59(4):761-80. doi: 10.1016/j.cden.2015.06.005. Epub 2015 Aug 1. Review.

Mattuella LG, Campagnaro MB, Vargas AE, Xavier LL, Oppermann RV, Chies JA, et al. Plasma cytokines levels in aggressive and chronic periodontitis. Acta Odontol Scand. 2013 May-Jul;71(3-4):683-8. doi: 10.3109/00016357.2012.715191.

Stoecklin-Wasmer C1, Guarnieri P, Celenti R, Demmer RT, Kebschull M, Papapanou PN. MicroRNAs and their target genes in gingival tissues. J Dent Res. 2012 Oct;91(10):934-40. Epub 2012 Aug 9.

Stolf CS, Taiete T, Zaffalon Casati M, Sallum EA, Nociti Júnior FH, Ruiz KGS, Casarin RV. Influence of rs6667202 SNP on Interleukin-10 levels in the gingival fluid of patients with periodontitis grade C. Braz. J. Oral Sci. [Internet]. 2021Feb.8 [cited 2021Mar.24];20(00):e211654.

Sugita N, Iwanaga R, Kobayashi T, Yoshie H. Association of the FcγRIIB-nt645+25A/G polymorphism with the expression level of the FcγRIIb receptor, the antibody response to Porphyromonas gingivalis and the severity of periodontitis. J Periodontal Res. 2012 Feb;47(1):105-13. doi: 10.1111/j.1600-0765.2011.01411.x. Epub 2011 Sep 12.

Susin, C., Haas, A. N. & Albandar, J. M. (2014) Epidemiology and demographics of aggressive periodontitis. Periodontol 2000 65, 27-45. doi:10.1111/prd.12019.

Taiete T, Casati MZ, Stolf CS, Corrêa MG, Santamaria MP, Andere NMRB, et al. Validation of reported GLT6D1 (rs1537415), *IL10* (rs6667202), and ANRIL (rs1333048) single nucleotide polymorphisms for aggressive periodontitis in a Brazilian population. J Periodontol. 2019 Jan;90(1):44-51. doi: 10.1002/JPER.18-0071.

Teles RP, Gursky LC, Faveri M, Rosa EA, Teles FR, Feres M, Socransky SS, Haffajee AD. Relationships between subgingival microbiota and GCF biomarkers in generalized aggressive periodontitis. J Clin Periodontol. 2010 Apr;37(4):313-23. doi: 10.1111/j.1600-051X.2010.01534.x.

Vieira, A. R. & Albandar, J. M. (2014) Role of genetic factors in the pathogenesis of aggressive periodontitis. Periodontol 2000 65, 92-106. doi:10.1111/prd.12021.

ANEXO 1 – Parecer Consubstanciado do Comitê de Ética em Pesquisa

UNICAMP - FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNIVERSIDADE DE CAMPINAS - FOP/UNICAMP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Funcionalidade do polimorfismo rs6667202 no gene IL10 em pacientes portadores de periodontite estágio 4 grau C: estudos in vitro e in vivo

Pesquisador: Camila Schmidt Stolf

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 3

CAAE: 14822719.8.0000.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.456.327

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PIRACICABA, 04 de Agosto de 2019

Assinado por:
Fernanda Miori Pascon
(Coordenador(a))

ANEXO 2 – Verificação de Originalidade e Prevenção de Plágio

FUNCIONALIDADE DO IL10 SNP rs6667202 EM FIBROBLASTOS GENGIVAIS DE PACIENTES COM PERIODONTITE GRAU C

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ANEXO 3 – Comprovante de Submissão do Artigo

Journal of Clinical Periodontology

EFP European Federation of Periodontology

Official scientific Journal of the European Federation of Periodontology and its member National Societies

IL10 PROMOTER rs6667202 POLYMORPHISM IS FUNCTIONAL IN HEALTH BUT NOT IN GRADE C PERIODONTITIS PATIENTS

Journal:	*Journal of Clinical Periodontology*
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Topic:	Aetiology
Main Methodology:	In Vitro Research
Keywords:	Single nucleotide polymorphism, Cytokines, Interleukin-10, Aggressive periodontitis, Gene expression