UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA D EPIRACICABA

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ASSOCIAÇÃO DE POLIMORFISMOS GENÉTICOS NOS GENES *GLT6D1*, *IL-10* E *ANRIL* COM PERIODONTITE AGRESSIVA EM UMA POPULAÇÃO BRASILEIRA

VALIDATION OF REPORTED *GLT6D1* (rs1537415), *IL10* (rs6667202), AND *ANRIL* (rs1333048) SINGLE NUCLEOTIDE POLYMORPHISMS FOR AGGRESSIVE PERIODONTITIS IN A BRAZILIAN POPULATION

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Trabalho de Conclusão de Curso apresentado à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Cirurgiã Dentista.

Udergraduate final work presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Dental Surgeon.

Orientador: Prof. Dr. Renato Corrêa Viana Casarin
Coorientador: Dr. Tiago Taiete

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DO TRABALHO DE CONCLUSÃO DE CURSO APRESENTADO PELA ALUNA CAMILA SCHMIDT STOLF E ORIENTADA PELO PROFESSOR DOUTOR RENATO CORRÊA VIANA CASARIN

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DEDICATÓRIA

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RESUMO

A periodontite agressiva (AgP) é influenciada por fatores genéticos. Recentemente, os polimorfismos de nucleotídeo único (SNPs) rs1537415 (*GLT6D1*), rs6667202 (*IL10*) e rs1333048 (*ANRIL*) foram associados a AgP em diferentes populações europeias. No entanto, esses SNPs específicos ainda não haviam sido determinados em brasileiros. Portanto, este estudo investigou se esses SNPs previamente associados à AgP poderiam ser replicados entre os brasileiros. Os SNPs rs1537415, rs6667202 e rs1333048 foram genotipados usando ensaio de discriminação alélica com 5 '-nuclease em AgP (n = 200), periodontite crônica (CP), (n = 190) e pacientes saudáveis (H), (n = 196). As diferenças nas frequências alélicas e genotípicas foram analisadas usando testes qui-quadrado e regressão logística. O alelo raro C do SNP rs6667202 foi menos frequentemente detectado em pacientes com AgP quando comparado aos grupos não-AgP, tornando o SNP protetor contra a ocorrência de AgP. Além disso, o modelo logístico final para o diagnóstico do AgP incluiu o gênero (p = 0,001) e o SNP rs6667202 (p <0,001) como variáveis significativas. Os SNPs rs1537415 e rs1333048 não mostraram associações com AgP. Apenas o SNP rs6667202 foi associado com a AgP na população brasileira, sendo o alelo raro C menor protetor contra AgP. Além disso, os SNPs rs1333048 e rs1537415, anteriormente associados a AgP em outras populações, não foram validados para a população brasileira.

PALAVRAS CHAVE: Alelos. Marcadores genéticos. Variação genética. Genótipo. Doença periodontal.

ABSTRACT

Aggressive periodontitis (AgP) is influenced by genetic factors. Recently, the single nucleotide polymorphisms (SNPs) rs1537415 (*GLT6D1*), rs6667202 (*IL10*), and rs1333048 (*ANRIL*) were associated with AgP in different European populations. However, these specific SNPs have not yet been determined in Brazilians. Therefore, this study investigated whether these SNPs previously associated with AgP could be replicated among Brazilians. The SNPs rs1537415, rs6667202, and rs1333048 were genotyped using 5′ - nuclease allelic discrimination assay in AgP (n = 200), chronic periodontitis (CP), (n = 190), and healthy patients (H), (n = 196). Differences in allele and genotype frequencies were analyzed using chi-square tests and stepwise logistic regression. The minor C allele of rs6667202 was less frequently detected in AgP patients when compared to non-AgP groups, making the SNP protective against AgP occurrence. Moreover, the final logistic model for AgP diagnosis included gender ($p = 0.001$) and the SNP rs6667202 ($p < 0.001$) as significant variables. The SNPs rs1537415 and rs1333048 did not show associations with AgP. Only the SNP rs6667202 was associated with AgP in a Brazilian population, being the minor C allele protective against AgP. Moreover, SNPs rs1333048 and rs1537415, previously associated with AgP in other population, was not validated to Brazilian population.

KEYWORDS: Alleles. Genetic markers. Genetic variation. Genotype. Periodontal diseases.

SUMÁRIO

1 INTRODUÇÃO

A periodontite agressiva (AgP) é uma doença periodontal grave, caracterizada pelo seu início precoce, alta taxa de progressão da doença, acometimento de múltiplos dentes e ausência de doenças sistêmicas que comprometem a resposta do hospedeiro à infecção (Armitage, 1999; Albandar, 2014). Essa doença mostra diferenças claras em seu desenvolvimento e progressão quando comparados à forma mais comum de periodontite, a periodontite crônica (CP) (Tonetti, 1999). Entre os pacientes com AgP, a exposição a fatores etiológicos locais geralmente não pode explicar a perda óssea alveolar significativa, sugerindo que fatores do hospedeiro estão envolvidos na determinação da suscetibilidade à doença (Meng, 2000). Embora diferenças na microbiota periodontal ou em outros fatores ambientais possam ter uma influência na ocorrência de AgP, o background genético individual é um fator crucial que influencia o risco sistêmico ou relacionado à resposta do hospedeiro (Meng, 2000; Kinane, 2000; Vieira, 2000).

Fatores genéticos regulam o sistema imunológico, e variações genéticas específicas podem tornar o sistema imunológico defeituoso e incapaz de evitar ataques por periodontopatógenos (Vieira, 2000; Yang, 2009). A etiologia da AgP pode ser explicada por fatores genéticos quando comparada com a CP, que têm um contribuição importante de fatores ambientais e de estilo de vida (Vieira, 2000; Loos, 2015). No entanto, embora alguns estudos tenham sido realizados para identificar polimorfismos de nucleotídeo único (SNPs) associados a AgP, poucos fatores de risco genéticos que contribuem para sua patogênese têm sido consistentemente determinados (Loos, 2015; Laine 2014). Vários estudos relataram tais esforços, mas mostraram resultados variados, se não contraditórios (Loos, 2015; Laine, 2014; Zhang, 2000). Até o momento, poucos genes foram adequadamente identificados como suscetíveis. Alguns estudos relataram uma associação significativa ou sugestiva entre os SNPs em *GLT6D1, ANRIL* e *IL10* e a AgP (Loos, 2015; Schaefer, 2009, 2010, 2011, 2013; Hashim, 2015).

Os estudos que mostraram evidência estatística ou sugestão de associações entre AgP e SNPs nos genes *GLT6D1* (rs1537415), *IL10* (rs6667202) e *ANRIL* (rs1333048) foram realizados e replicados em diferentes populações europeias (Schaefer, 2009, 2010, 2011, 2013; Ernst, 2010). A associação entre rs1537415 (*GLT6D1*) e a AgP também foram observados em uma população sudanesa (Hashim, 2015). No entanto, essas associações

não foram significativas em algumas populações que também eram geograficamente próximas. Embora o SNP rs6667202 para o gene *IL10* tenha sido identificado em uma população alemã e confirmado em uma pequena população alemã-austríaca, essa associação não foi significativa com a AgP em uma população holandesa (Schaefer, 2013). Da mesma forma, o SNP rs1333048 para o gene *ANRIL* foi primeiro associada à AgP em uma população alemã no estudo de Schaefer et al. (Schaefer, 2009) e também observada no estudo de Ernst et al. (Ernst, 2010), que avaliaram pacientes alemães e irlandeses do norte.

Embora esses diferentes resultados possam ser causados por questões metodológicas, diferentes perfis genéticos encontrados entre indivíduos de diferentes populações também podem ser um fator relevante nesses resultados contraditórios (Hashim, 2015; Abecasis, 2012). Como o genótipo e as frequências alélicas podem variar entre diferentes populações étnicas, um fator de risco genético para a suscetibilidade à doença em uma população pode não ser um fator de risco em outra população (Meng, 2000; Yang, 2009; Hashim, 2015). Nesse contexto, os achados sobre os SNPs significativos relacionados a uma doença devem ser amplamente replicados e validados em populações independentes.

Portanto, este estudo se concentrou em investigar se associações anteriormente relatadas de rs1537415 (*GLT6D1*), rs6667202 (*IL10*) e rs1333048 (*ANRIL*) com AgP em populações europeias poderiam ser replicadas em uma população do Brasil. Esse estudo é importante porque a população brasileira tem uma prevalência maior de AgP do que a população europeia (5,5% versus 0,2%) (Susin, 2000, 2005) e uma grande miscigenação étnica, o que pode influenciar significativamente os antecedentes genéticos individuais e determinar diferentes fatores genéticos de risco. Além disso, os resultados da AgP também foram comparados com o CP, investigando se essas associações foram atribuídas unicamente a AgP ou ao histórico genético comum de destruição periodontal.

ARTIGO: VALIDATION OF REPORTED *GLT6D1* (rs1537415), *IL10* (rs6667202), AND *ANRIL* (rs1333048) SINGLE NUCLEOTIDE POLYMORPHISMS FOR AGGRESSIVE PERIODONTITIS IN A BRAZILIAN POPULATION[1]

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ABSTRACT

Background: Aggressive periodontitis (AgP) is influenced by genetic factors. Recently, the single nucleotide polymorphisms (SNPs) rs1537415 (GLT6D1), rs6667202 (IL10), and rs1333048 (ANRIL) were associated with AgP in different European populations. However, these specific SNPs have not yet been determined in Brazilians. Therefore, this study investigated whether these SNPs previously associated with AgP could be replicated among Brazilians.

Methods: The SNPs rs1537415, rs6667202, and rs1333048 were genotyped using 5′ - nuclease allelic discrimination assay in AgP (n = 200), chronic periodontitis (CP, n = 190), and healthy patients (H, n = 196). Differences in allele and genotype frequencies were analyzed using chi-square tests and stepwise logistic regression.

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Results: The minor C allele of rs6667202 was less frequently detected in AgP patients (23.5%) when compared to non-AgP groups (H = 34.2% and CP = 30.3%; $p < 0.01$), making the SNP protective against AgP occurrence. Moreover, the final logistic model for AgP diagnosis included gender ($p = 0.001$) and the SNP rs6667202 ($p < 0.001$) as significant variables. The SNPs rs1537415 and rs1333048 did not show associations with AgP.
Conclusion: Only the SNP rs6667202 was associated with AgP in a Brazilian population, being the minor C allele protective against AgP. Moreover, SNPs rs1333048 and rs1537415, previously associated with AgP in other population, was not validated to Brazilian population.

KEYWORDS alleles, genetic markers, genetic variation, genotype, periodontal diseases

Aggressive periodontitis (AgP) is a severe periodontal disease characterized by early age of onset, high rate of disease progression, affecting multiple teeth, and the absence of systemic diseases that compromise the host response to infection.[1,2] This disease shows clear differences in the development and progression when compared to the most common form of periodontitis, the chronic periodontitis (CP).[3] Among patients with AgP, exposure to local etiologic factors usually cannot account for the significant alveolar bone loss, suggesting that host factors are involved in determining susceptibility to the disease.4 Although differences in the periodontal microbiota or in other environmental factors may have an influence on AgP occurrence, the individual genetic background is a crucial factor that influences systemic or host response-related risk.[4–6]

Genetic factors regulate the immune system, and specific genetic variations may render an immune system defective and unable to successfully fend off assaults by periodontopathogens.[6,7] AgP etiology can be explained to a greater extent by genetic factors when compared to CP, which have a major contribution from environmental and lifestyle factors.[6,8] However, although some studies have been performed to identify single nucleotide polymorphisms (SNPs) associated with AgP, few genetic risk factors that contribute to its pathogenesis have been consistently determined.[8,9] Several studies have reported such efforts but shown varying, if not contradictory results.[8–10] Up to date, few genes have been properly identified as susceptibility ones. Some studies had reported a

significant or a suggestive association between SNPs in *GLT6D1, ANRIL*, and *IL10* and AgP.[8,11–15]

The studies that showed statistical evidence or suggestion of associations between AgP and SNPs in *GLT6D1* (rs1537415), *IL10* (rs6667202), and ANRIL (rs1333048) genes were carried out and replicated in different European populations.[11–14,16] The association between rs1537415 (*GLT6D1*) and AgP was also observed in a Sudanese population.[15] However, these associations were not significant in some populations that were also geographically close. Although *IL10* SNP rs6667202 was identified in a German population and confirmed in a smaller German-Austrian cohort, this association was not significant in AgP from a Dutch cohort.[14] Similarly, rs1333048 in *ANRIL* was first associated with AgP in a German cohort in the study of Schaefer et al.,[11] and also observed in the study of Ernst et al.,[16] which evaluated German and Northern Irish AgP patients. These associations were also suggested in a small Turkish cohort, but not significant in a small Italian cohort of AgP patients.[14]

Although these different results may be because of methodological issues, different genetic profiles found between individual from different populations may also be a relevant factor in these contradictory results.[15,17] Because genotype and allele frequencies can vary between different ethnic populations, a genetic risk factor for disease susceptibility in one population may not be a risk factor in another population.[4,7,15] In this context, findings on significant SNPs being related to a disease should be extensively replicated and validated in independent cohorts.

Therefore, this study focused on investigating whether previously reported associations of rs1537415 (*GLT6D1*), rs6667202 (*IL10*), and rs1333048 (*ANRIL*) with AgP in European populations could be replicated in a population from Brazil. This sounds important because the Brazilian population has a higher prevalence of AgP than the European population (5.5% versus 0.2%)[18,19] and a great ethnicity miscegenation, which can significantly influence individual genetic backgrounds and determine different genetic risk factors. In addition, the results of AgP were also compared with CP, investigating if these associations were solely attributed to AgP or common genetic background of periodontal destruction.

1 MATERIAL AND METHODS

1.1 Study population

The study was approved by the Ethics Committee of the University of Campinas (58679416.4.0000.5418), and it was designed in accordance with the STROBE Statement for observational studies. Informed written consent was granted by each patient after explanations were provided. A total of 586 patients (200 with AgP, 190 with chronic periodontitis – CP, and 196 H) from the Southeastern region of Brazil were recruited from patients referred to the Graduate Clinic of Piracicaba Dental School, University of Campinas, Piracicaba, Brazil, Periodontology Clinic of UNESP, State University of São Paulo, São José dos Campos, Brazil, and Periodontology Clinic of Paulista University, São Paulo, Brazil, between March 2016 and September 2017.

The following clinical parameters were assessed in all patients: full-mouth plaque index (FMPI),[20] full-mouth bleeding score (FMBS),[21] probing pocket depth (PPD), and clinical attachment level (CAL) at six point around each tooth using a manual periodontal probe (PCPUNC 15®, HuFriedy, Chicago, IL). All examinations were performed by experienced periodontists (RCVC, MGC and MPS), one in each center. Tooth mobility, radiographic examination, and complete medical and dental questionnaires were also obtained.

AgP and CP patients were identified on the basis of 1999 International Workshop for the Classification of Periodontal Diseases and Conditions.[1] Most of AgP patients were treated in previous studies[22–25] or in one of the three Graduate Clinics. Further, these patients needed at the first examination the presence of at least 20 teeth, at least 8 teeth with a PD ≥ 5 mm with bleeding on probing (having at least 2 with PPD ≥ 7 mm), < 35 years of age for AgP patients, and > 35 years for CP. Healthy patients did not show interproximal attachment loss and probing pocket depths of > 4 mm.[26,27] Additional exclusion criteria were as follows: smoking or previous history of smoking, as established via a questionnaire, diabetes, hepatitis, HIV infection, other systemic diseases (e.g. cardiovascular disease, systemic lupus erythematosus, etc.), use of immunosuppressive drugs, prolonged use of anti-inflammatory drugs, use of orthodontic appliances, and diseases of the oral hard and soft tissues (except caries and periodontitis).[28]

TABLE 1 Characteristics of the single-nucleotide polymorphisms (SNPs) tested in the current study

SNP	Gene	Chromosome	Position	Alleles	Original Study
rs1537415	*GLT6D1*	9	135637876	C/G	Schaefer et al. 2010
rs6667202	*IL10*	1	206783747	A/C	Schaefer et al. 2013
rs1333048	*ANRIL*	9	22125348	A/C	Schaefer et al. 2009; 2013

1.2 Isolation of genomic DNA

Genomic DNA (gDNA) was isolated from buccal epithelial cells using a salting-out protocol.[29] Briefly, the patients under-took a mouthwash containing 5 mL 3% dextrose solution for60 seconds. Three mL of TNE solution [17 mM Tris/HCl (pH8.0), 50 mM NaCl, and 7 mM EDTA] in 66% ethanol was added to the sample tube collection. Samples were centrifuged (3000 rpm for 10 min), the supernatant was discarded, and the pellet resuspended in 500 μL of extraction buffer [10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.5% SDS] and 10 μL of pro-teinase K (Sigma Chemical Co. St. Louis, MO) (20 mg/ml) was added. DNA was dissolved in nuclease-free water, and its quantity was evaluated spectrophotometrically using a spec-trophotometer (Nanodrop 2000 device, Thermo Scientific, Wilmington, DE).

1.3 Genotyping

PCR-based genotyping of rs1537415 (GLT6D1), rs6667202(IL10), and rs1333048 (ANRIL) (Table 1) was performed on the thermocycler (LightCycler 480, Roche Diagnostics, GmbH, GR) using predesigned 5′-nuclease allelic discrimination assays (Thermo Scientific). PCR were carried out in a total of 10 μl, containing 20 ng of gDNA, 5 μl of a genotyping master mix (Thermo Scientific), 0.5 μl probes assay mix 20 × (Thermo Scientific). PCRs were performed under the following conditions: 10 minutes at 95◦C, 40 × (15 seconds at 95◦C, 1 minute at 60◦C). Reactions were randomly repeated in 10%of the samples for each SNP, for quality control purposes, and the concordance rate was 100%.[30] All samples were success-fully genotyped, with a genotype call rate of 100%.

1.4 Statistical analysis

Demographic and clinical data including age, PPD, CAL, FMPI, and FMBS were initially evaluated by the Shapiro-Wilk test (for normality). Those presenting a Shapiro-Wilk p-value > 0.05 were analyzed by one-way ANOVA followed by Tukey's HSD test. Those presenting a Shapiro-Wilk p-value ≤ 0.05 were analyzed by the Kruskal-Wallis/Dunn test. Comparison among groups with respect to gender was performed by Fisher's Exact test. For demographic and clinical data the p-value < 0.05 was considered statistically significant.

To test deviations of Hardy-Weinberg equilibrium, genotype distribution of each polymorphism in each group was evaluated by a statistical software (BioEstat 5.4, Belém, PA, BR). Association analysis between rs1537415 (GLT6D1), rs6667202 (IL10), rs1333048 (ANRIL), and AgP was per-formed using chi-square test (x^2). The odds ratio associated with the 95% confidence interval was also calculated. A Stepwise logistic regression was used to determine the best model to AgP diagnosis (dependent variable), considering gender, ethnic group (Caucasoid and African American), and all SNPs (allele and genotype associated to disease) as independent variables using a specific statistical software (SIG-MAstat 4.0, Systat, CA). A Bonferroni adjusted p-value of $\alpha \leq 0.017$ (0.05/3) was considered statistically significant for the genotyping analysis. Power calculations were performed using a specific software (Quanto 1.2.4 software, University of Southern California, CA), assuming a prevalence of AgP in Brazil of 0.0549,[18] a *p* value < 0.05 and using the most conservative odds ratios reported in the original studies.

2 RESULTS

2.1 Study population

The demographic and clinical characteristics of the patients enrolled in this study at the moment of patient recruitment and sampling of the buccal epithelial cells are shown in Table 2.The mean age of the AgP patients was 34.0 ± 4.6 years, which was statistically younger than that of the CP (50.0 ± 7.2 years, $p < 0.05$). However, there was no difference in age between the AgP and healthy groups (30.7 ± 5.8), but the healthy patients were significantly younger than the CP ($p < 0.05$). A larger proportion of females was observed

in AgP group compared with CP and H groups. No significant differences were observed between the groups regarding ethnicity and periodontal parameters at the moment of patient recruitment and sampling of buccal epithelial cells.

TABLE 2 Demographic and clinical characteristics

Characteristics	AgP	CP	H
Age (years)	34.0 ± 4.6 A	50.0 ± 7.2 B	30.5 ± 5.8 A
Gender (M/F)	21 / 79 A	34.9 / 65.1 B	35.0 / 65.0 B
Ethnicity (Af /C)	16.5 / 83.5 A	14.1 / 85.9 A	13.8 / 86.2 A
FMPI (%)	23.1±6.5 A	17.05 ± 8.4 A	19.1 ± 5.4 A
FMBS (%)	24.8±9.0 A	23.53 ± 7.7 A	19.2 ± 2.3 A
PPD (mm)	2.35±0.0 A	2.28 ± 0.3 A	2.1 ± 0.2 A
CAL (mm)	5.44±1.0 A	5.44 ± 0.9 A	4.2 ± 0.7 A

Different letters indicate statistical difference. Kruskal Wallis/Dunn test analyzed age. Fisher's Exact Test analyzed gender and ethnicity group. Comparison among groups with respect to FMPI, FMBS, PPD, and CAL was performed by one-way ANOVA/Tukey test.
Gender (M/F) – Gender (percentage of Male/Female); Ethnic (Af / C) – Ethnic (percentage of African American and Caucasoid).

2.2 Genotyping

2.2.1 Hardy-Weinberg equilibrium and power analysis

The genotype frequencies observed for the three SNPs in the three groups were not statistically significantly different from those expected under the Hardy-Weinberg equilibrium, with the exception of rs1333048 (ANRIL), which showed significant deviation from Hardy-Weinberg equilibrium in AgP group (Table 3 and 4). Power analysis showed good statistical power to detect associations with the current sample size for the polymorphisms rs6667202 (90.12% for AgP versus non-AgP; 80.99% for AgP versus H; and 80.41% for AgP versus CP), rs1333048 (99.16% for AgP versus non-AgP; 96.35% for AgP versus H; and 96.18% for AgP versus CP) and rs1537415 (93.89% for AgP versus non-AgP; 85.57% for AgP versus H; and 85.41% for AgP versus CP).

TABLE 3 Allele and genotype frequency of polymorphisms rs1537415, rs6667202, and rs1333048 of AgP and non-AgP group

SNP		AgP	Non-AgP	χ^2 *p value*	AgP × Non-AgP OR (95% CI) *p*-value
rs1537415 (*GLT6D1*)					
HWE *p*-value		0.8264	0.926		
Alleles (C / G)	G	65.4	65.2	*0.9989*	n.s
	C	34.6	34.8		
Genotypes (CC / CG / GG)	CC	11.6	12.2	*0.9718*	*Reference*
	CG	45.9	45.1		n.s
	CC	42.5	42.7		n.s
rs6667202 (*IL10*)					
HWE *p*-value		0.4475	0.7789		
Alleles (A / C)	C	76.5	67.7		*0.64 (0.48 – 0.86) p = 0.0034*
	A	23.5	32.3	*0.0027*	
Genotypes (AA / AC / CC)	AA	57.5	46.2	*0.0092*	*Reference*
	AC	37.8	43		0.71 (0.49 – 1.03) p = 0.0870
	CC	4.7	10.8		*0.34 (0.15 –0.73) p = 0.0081*
rs1333048 (*ANRIL*)					
HWE *p*-value		0.0203	0.8928		
Alleles (A / C)	A	50.2	44.7	*0.1045*	n.s
	C	49.8	55.3		
Genotypes (AA / AC / CC)	AA	29.4	19.8	*0.0421*	*Reference*
	AC	41.6	49.8		n.s
	CC	29	30.4		n.s

HWE – Hardy-Weinberg equilibrium; OR (95% CI): odds ratio values with the respective 95% confidence intervals. Alleles and genotypes frequencies are presented as percentage.

TABLE 4 Allele and genotype frequency of Polymorphisms rs1537415, rs6667202, and rs1333048 of AgP, H and CP groups

SNP		AgP	CP	H	*p value*	AgP × H OR (95% CI) *χ2 p value*	AgP × CP OR (95% CI) *p*-value	CP × H OR (95% CI) *p*-value
rs1537415 (*GLT6D1*)								
HWE *p*-value		0.8264	0.4385	0.5205				
Aleles (C / G)	G	65.4	64.6	65.8	*0.9418*	n.s	n.s	n.s.
	C	34.6	35.4	34.2				
Genotype (CC / CG / GG)	CC	11.6	13	11.4	*0.8222*	*Reference*	*Reference*	*Reference*
	CG	45.9	44.7	48.1		n.s.	n.s.	n.s.
	CC	42.5	42.3	40.5		n.s.	n.s.	n.s.
rs6667202 (*IL10*)								
HWE *p*-value		0.4475	0.2961	0.519				
Aleles (A / C)	C	23.5	30.3	34.2	*0.0063*	*0.59 (0.42 – 0.82) p = 0.0023*	*0.71 (0.50 – 0.98) p = 0.05*	n.s.
	A	76.5	69.7	65.8				
Genotype (AA / AC / CC)	AA	57.5	50.3	42.1	*0.0067*	*Reference*	*Reference*	*Reference*
	AC	37.8	38.7	47.4		0.59 (0.38 – 0.92) p = 0.0076	0.85 (0.55 – 1.33) p = 0.51	n.s.
	CC	4.7	11	10.5		*0.31 (0.13 – 0.75) p = 0.0044*	*0.36 (0.15 – 0.85) p = 0.012*	n.s.
rs1333048 (*ANRIL*)								
HWE *p*-value		0.0203	0.7645	0.9919				
Aleles (A / C)	A	50.2	41.4	49.3	*0.02*	n.s	*n.s*	n.s
	C	49.8	58.6	50.7				
Genotype (AA / AC / CC)	AA	29.4	16.5	23.5	*0.018*	*Reference*	*Reference*	*Reference*
	AC	41.6	49.7	50		n.s	n.s	n.s
	CC	29	33.8	26.5		n.s	n.s	n.s

HWE – Hardy-Weinberg equilibrium; OR (95% CI): odds ratio values with the respective 95% confidence intervals. Alleles and genotypes frequencies are presented as percentage.

2.2.2 AgP versus Non-AgP

First, the analysis was performed with the chronic periodontitis and healthy patients pooled together in the non-AgP group. SNP rs6667202 (*IL10*) showed an association with AgP in this Brazilian cohort when compared to non-AgP group. In this group, the genotype frequencies were 57.5% AA, 37.8%AC, and 4.7% CC, resulting in a frequency of 23.5% of the allele C and a carrier frequency of at least one C allele of 42.5%. In the non-AgP group, the frequencies of AA, AC, and CC were 46.2%, 43%, and 10.8%, respectively, resulting n a C allele frequency of 32.3% and carrier frequency of lea stone C allele of 53.8%. The C minor allele associated with SNP rs6667202 (IL10) was protective of AgP occurrence, presenting odds ratio of 0.64 (95% CI, 0.48–0.86, p = 0.0034) in relation to non-AgP group. The genotype CC presented an odds ratio of 0.34 (95% CI, 0.15–0.73, p

= 0.0081) when comparing AgP and the non-AgP group (Table 3). Allele and genotype frequencies regarding rs1537415 inGLT6D1 and rs1333048 in ANRIL were not significant for AgP diagnosis in this Brazilian cohort after Bonferroni correction.

2.2.3 AgP versus CP or Healthy

AgP group was subsequently compared to healthy and CP group separately. In the healthy group, the frequencies of AA, AC, and CC were 42.1%, 47.4%, and 10.5%, respectively, resulting in a C allele frequency of 34.2%, and carrier frequency of least one C allele of 57.9% (Table 4). The CP group showed genotype frequencies of 50.3% AA, 38.7% AC and 11.0% CC, resulting in a frequency of 30.4%of the allele C and carrier frequency of at least one C allele of 49.7% (Table 4). The C minor allele of SNP rs6667202 (*IL10*) remained protective of AgP occurrence, presenting odds ratios of 0.59 (95% CI, 0.42–0.82, p = 0.0023) and 0.71 (95% CI, 0.50–0.98, p = 0.05) in relation to the healthy and chronic periodontitis groups, respectively. However, the association in relation to CP group was no longer significant after application of a Bonferroni correction for multiple testing. The AC genotype was associated with AgP in comparison to healthy group (OR 0.59, 95% CI, 0.38–0.92, *p* = 0.0076). The presence of CC genotype represented decreased risk for AgP of 0.31 (95% CI, 0.13–0.75, *p* = 0.0044) and 0.36 (95% CI, 0.15 – 0.85, p = 0.012) when compared to healthy and CP groups respectively.

TABLE 5 Final Stepwise Regression model for the diagnosis of aggressive periodontitis considering genotype and allele

	R	R^2	*p*
Genotype			
Gender	0.346	0.120	0.001
GLT6D1	–	–	n.s
IL-10	0.346	0.107	<0.001
ANRIL	–	–	n.s
Allele			
Gender	0.317	0.101	0.001
GLT6D1	–	–	n.s
IL-10	0.340	0.116	<0.001
ANRIL	–	–	n.s

Allele and genotype frequencies regarding rs1537415 in GLT6D1 and SNP rs1333048 in ANRIL were not significant for AgP diagnosis in this Brazilian cohort after Bonferroni correction.

2.2.4 Stepwise logistic regression

Stepwise logistic regression was used to construct a model to indicate AgP diagnosis. Considering the genotype distribution, AgP diagnosis was associated with gender ($p < 0.005$), and SNP rs6667202 (IL10) ($p < 0.001$). Regarding allele distribution, AgP diagnosis was associated with age ($p < 0.001$), gender ($p < 0.003$), and SNP rs6667202 (IL10) ($p < 0.001$) (Table 5).

3 DISCUSSION

The main finding of the present study was the association of rs6667202 in *IL10*, but not rs1537415 in *GLT6D1* and rs1333048 in *ANRIL*, with AgP in the Brazilian population. The C minor allele and the CC genotype of rs6667202 were protective to AgP when compared to H, CP and when both populations were pooled (non-AgP group). The AC genotype was statistically significant only when compared with AgP and H. To the best of our knowledge, this is the first association found between AgP and the SNP rs6667202 in *IL10* in a Brazilian population.

The SNP rs6667202 is present in the upstream region of *IL10* and it was first evaluated in the study of Schaefer et al.,[14] who showed an association of this SNP and AgP in the evaluated German cohort, but not in the Dutch cohort. Corroborating Schaefer et al.'s trial,[14] in the present study, the minor C allele was less frequently detected in AgP patients compared to H patients. Moreover, the present study demonstrated that the C allele was also less frequently detected in AgP when compared to CP. This is an important and first described result, because CP and AgP are phenotypically different diseases and, based on our findings, could present different genetic backgrounds. Moreover, this finding could represent a way to differentiate the risk for these diseases, which, clinically, could help the future development of individualized treatments and preventive protocols.

IL10 encodes the cytokine interleukin-10 (IL-10), an anti-inflammatory cytokine that can regulate the expression of pro inflammatory cytokines. This cytokine is usually expressed in inflamed periodontal tissues and is associated with lower disease severity, once it promotes the suppression of pro inflammatory cytokines.[32,33] It can be speculated that the minor C allele of rs6667202, which is enriched in the healthy patients, results in increased production of IL-10,because previous studies have shown lower IL-10 levels in the gingival crevicular fluid of AgP patients.[34,35] Future studies will be important to highlight the impact of this SNP on IL-10 production and on AgP pathogenesis.

Moreover, the results of the present study did not confirm the associations between rs1537415 in GLT6D1 andrs1333048 in *ANRIL* with AgP in the Brazilian population. SNP rs1333048 is in a regulatory region and not a protein-coding region of *ANRIL*, and it was identified as being associated with generalized and localized AgP in a large German cohort,[11] and replicated in a German-Northern Irish population.[16] The *GLT6D1* SNP rs1537415 was discovered and replicated in 2 independent German cohorts, and also replicated in a Sudanese population.[12,15] Interestingly, Schaefer et al.[12] reported a statistical significant enrichment of the minor allele of 9.4% to 12%, similar to that reported by Hashim et al.[15] — about 9%. In the present study, no differences between the minor allele frequencies between the groups evaluated were noted. These differences emphasize the role of ethnicity in individual genetic background, which in turner in forces the importance of replication studies in different populations to understand the genetic basis of AgP.

The lack of associations between AgP and rs1537415 (*GLT6D1*) and rs1333048 (*ANRIL*) in the present study could be because of some factors. AgP is considered a complex and multifactorial disease; therefore, genetic risk factors can have different effects in different populations.[15, 36, 37] For example, three SNPs in the *IL10* gene (rs1800896, rs1800871, and rs1800872) previously associated to CP were not associated with AgP in a Northeastern Brazilian population.[38]

On the other hand, in the present study, the SNP rs6667202 of *IL10* gene was associated for a Southeastern Brazilian population with AgP. This phenomenon could indicate that other polymorphisms, in another region of the loci, may be associated with the disease in different populations.[8] Further, the present study has some limitations. The main limitation are the modest sample size when compared to expected to

periodontal genetic studies.[39] Therefore, modest association of these SNPs and AgP may have been missed. However, power calculations indicated that the sample size provided more than 80% statistical power to detect an association with rs1537415 (*GLT6D1*), rs1333048 (*ANRIL*), andrs6667202 (*IL10*). Moreover, in the present study AgP group consisted of patients suffering or with history of severe form of generalized AgP, as described in the inclusion criteria (early age of onset and higher level of severity at diagnosis). The inclusion of severe disease phenotype are very likely to improve the chance of detecting a disease associated variant.[39] Also, efforts were made to minimize of environ-mental and life-style factor, which can act as sources of phenotypic heterogeneity.[39]

4 CONCLUSION

In conclusion, the SNP rs6667202 in *IL10*, but not the SNPs rs1537415 in *GLT6D1* and rs1333048 in *ANRIL*, was associated with AgP in the Brazilian population. The minor C allele of rs6667202 was less frequently detected in AgP patients than in healthy patients, suggesting that this SNP may have a protective effect in this Brazilian cohort.

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3 CONCLUSÃO

O principal achado desse estudo foi a associação do SNP rs6667202 para o gene *IL10*, mas não dos SNPs rs1537415 para o gene *GLT6D1* e rs1333048 para o gene *ANRIL*, com a AgP na população brasileira. O alelo raro C e o genótipo CC para o SNP rs6667202 foram protetores à ocorrência da AgP quando comparado aos grupos H e CP, e quando ambas as populações foram agrupadas (grupo não-AgP). O genótipo AC foi estatisticamente significativo apenas quando comparado aos grupos AgP e H. Até onde sabemos, esta é a primeira associação encontrada entre o AgP e o SNP rs6667202 para o gene *IL10* em uma população brasileira.

Concluindo, o SNP rs6667202 do gene *IL10*, foi associado a AgP na população brasileira. O alelo raro C para o SNP rs6667202 foi menos frequentemente detectado em pacientes com AgP do que em pacientes saudáveis, sugerindo que este SNP pode ter um efeito protetor nessa população brasileira estudada.

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ANEXOS

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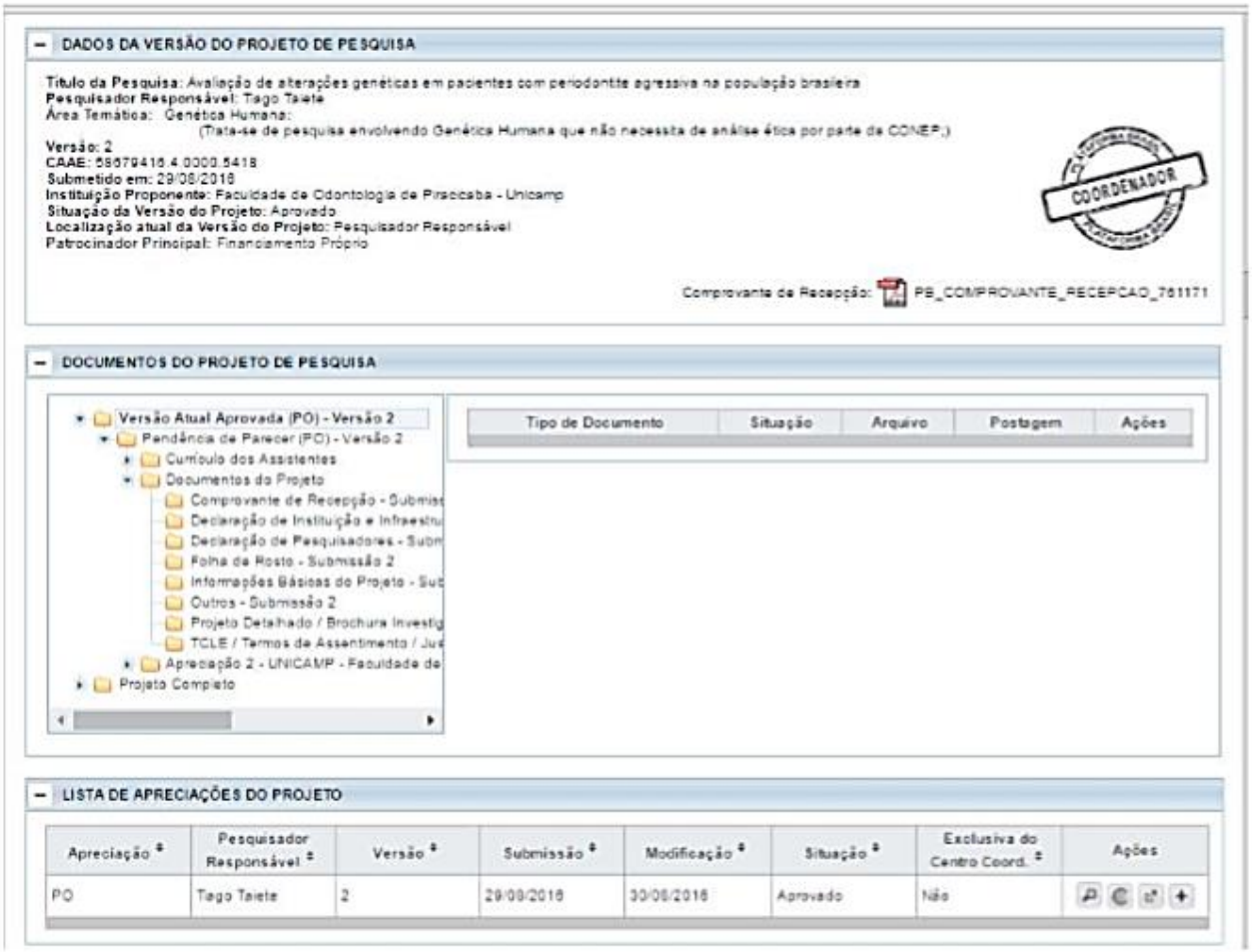

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Submetido em: 29/08/2016
Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp
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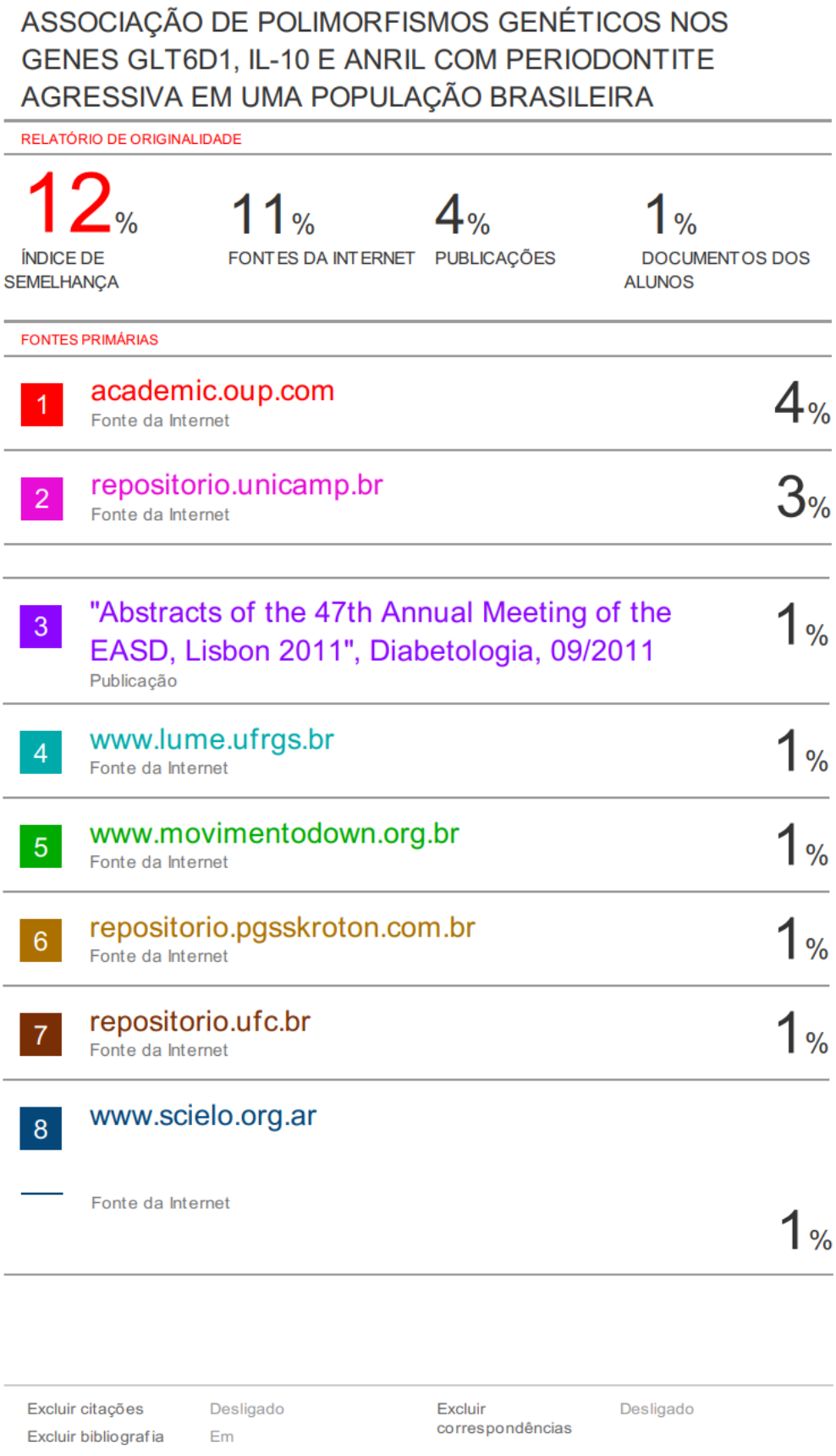

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