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Periodontite agressiva: abordagens diagnósticas, terapêuticas e preventivas.

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"A verdade só é encontrada quando os homens são livres para a perseguir."
Franklin Delano Roosevelt

"Ganhamos força, coragem e confiança a cada experiência em que verdadeiramente paramos para enfrentar o medo."
Eleanor Roosevelt

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SUMÁRIO

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RESUMO

A periodontite agressiva (PA) tem grande impacto na qualidade de vida dos indivíduos acometidos por esta patologia. Possui início precoce, rápida e severa progressão, pobre resposta as abordagens terapêuticas e é associada a altas taxas de perda dentária. De patogênese complexa, o início e progressão da doença e a resposta ao tratamento são determinadas pela microbiota, resposta imune-inflamatória do hospedeiro e pelas diferentes abordagens terapêuticas adotadas. Contudo, nenhum desses fatores está totalmente elucidado na literatura. Dessa forma, foram compilados 9 estudos que focaram entender fatores etiológicos, terapêuticos e preventivos dessa doença. No primeiro estudo, foram avaliados 200 indivíduos brasileiros portadores de PA quanto a presença de polimorfismos genéticos dos genes rs1537415 (GLT6D1), rs6667202 (IL10) e rs1333048 (ANRIL), polimorfismos já descritos em outras populações também afetadas pela PA. Foi observado que o alelo raro C do gene da IL-10 apresentou menor frequência (23,5%) na população PA comparado com sujeitos com periodontite crônica (n=190; 30,3%) e saudáveis (n=196; 34,2%), indicando um caráter protetor desse polimorfismo para a ocorrência da PA. Contudo, abordagens mais amplas poderiam indicar novos polimorfismos relacionados a essa doença. No segundo capítulo foi realizado uma análise exômica com um protocolo de filtragem familiar, confirmado em estudo populacional, em núcleos familiares com PA. Após a análise do exoma e validação por PCR-real time, análise funcional e *in silico*, foi possível identificar o indel no gene GPRC6A (p.Tyr775LeufsTer776), com a PA, o qual apresentou maior frequência na população afetada, indicando um novo foco para avaliar a ocorrência da mesma. Entretanto, alterações genéticas podem afetar o funcionamento dos sistemas de proteção e homeostase, levando a ocorrência da doença. Assim, no intuito de identificar alterações constitutivas dos tecidos gengivais de indivíduos com PA, foi realizado um terceiro estudo no qual avaliou-se o transcriptoma de tecidos saudáveis, sem contato com biofilme bacteriano, de sujeitos PA, comparando-os à periodontite crônica (PC) e saúde

periodontal. Observou-se diversos genes e vias diferencialmente expressos entre as condições, dentre as quais a maior expressão de receptores de células Natural Killers e genes relacionados ao sistema imune (KIR2DL4, IL6 e SELE) estavam super-expressos na PA comparado à saúde e PC, indicando alterações constitutivas nessa população. De certa forma, essas avaliações poderiam indicar o caminho pelo qual a PA ocorre, embora não considerem o fator etiológico primário – biofilme. Assim, no quarto capítulo, foi comparado não apenas características imunológicas como também microbiológicas entre 40 indivíduos PA e 28 indivíduos PC. Na comparação das concentrações subgengivais de *Aggregatibacter actinomycetemcomitans* (Aa) e *Porphyromonas gingivalis* (Pg), foi observado maior quantidade e frequência de Aa em PA, associado à menor concentração de IgG contra essa espécie e menor IL-10 subgengival, indicando uma possível combinação de fatores associados à essa doença. Interessantemente, os níveis de citocinas no fluido gengival, embora pareçam estar ligados à origem, foram discretamente modificados pelos tratamentos, como observados nos capítulos 5 a 7. Tanto na avaliação da associação do debridamento periodontal ultrasônico de boca toda em conjunto com a administração sistêmica de amoxicilina 375mg + metronidazol 250mg (capítulos 5 e 6), como na irrigação subgengival com iodo-povidine (capítulo 7), não houve modificação significativa nos níveis de citocinas no fluido gengival, embora clinicamente, a associação antibiótica promova benefícios adicionais na redução de profundidade de sondagem (PS) de sítios profundos (PS>7mm) e sítios residuais (PS>5mm com sangramento à sondagem). Soma-se a esse benefício clínico, a maior redução de Aa nas bolsas periodontais obtida com a associação de antimicrobianos e irrigação com iodo-povidine. Essa redução de Aa é um fator considerado importante na PA em vista de sua relação intíma com a doença. Assim, no intuito de identificar o início dessa colonização em uma população de risco, isto é, descendentes de sujeitos portadores de PA, os capítulos 8 e 9 avaliaram a presença de Aa, bem como de Pg, na saliva e no ambiente subgengival de crianças de 6-12 anos com pais portadores de PA comparado a crianças de pais saudáveis periodontalmente. Pode-se notar uma maior prevalência e concentração de Aa na saliva de crianças filhos de PA, com 16x maior chance de infecção na infância quando um dos pais é portador dessa espécie. No ambiente subgengival, pode-se notar também maior concentração desta, associado à maior acúmulo de biofilme e sangramento gengival. Essa informação é essencial para abordagens preventivas precoces, uma vez que há uma clara relação familiar na ocorrência da PA. Em conjunto, os capítulos apresentados trazem informações relevantes quanto a etiologia, permitindo avanços na busca por protocolos mais objetivos e previsíveis de

terapia e, em especial, baseando-se nos achados das crianças, protocolos preventivos podem ser criados e planejados futuramente.

Palavras-chave: Periodontite agressiva, etiologia, biofilme, antibacterianos, genética.

ABSTRACT

Aggressive periodontitis (AgP) has a great impact on the quality of life of the individuals affected by this pathology. It has early onset, rapid and severe progression, poor response to therapeutic approaches and is associated with high rates of tooth loss. AgP presents a complex pathogenesis - the onset and progression of the disease, as well as the response to treatment, are determined by the microbiota, the host's immune-inflammatory pattern and the therapeutic approaches adopted. However, none of these factors is fully elucidated in the literature. Thus, 9 studies were compiled focusing on understanding etiological, therapeutic and preventive factors of this disease. In the first study, 200 Brazilian individuals with AgP were evaluated for the presence of genetic polymorphisms of the rs1537415 (GLT6D1), rs6667202 (IL10) and rs1333048 (ANRIL). These polymorphisms have already been described in other populations also affected by AgP. It was observed that the rare C allele of the IL-10 gene presented a lower frequency (23.5%) in the AgP population compared to subjects with chronic (n=190, 30.3%) and healthy periodontitis (n=196; 34.2%), indicating a protective character of this polymorphism for the occurrence of AgP. However, high-throughput approaches could indicate new polymorphisms related to this disease. In the second chapter, exomic analysis was performed using a familiar filtering approach, confirmed in a population-based study, in familiar nuclei with AgP. After analysis of the exome and validation by real-time PCR, functional and *in silico* analysis, it was possible to identify the indel in the GPRC6A gene (p.Tyr775LeufsTer776) associated with AgP, presenting higher frequency in the affected population, indicating a new target to understand its occurrence. However, genetic alterations can affect the functioning of the protection systems and homeostasis, leading to the occurrence of the disease. Thus, in order to identify constitutive changes in the gingival tissues of individuals with AgP, a third study was conducted in which the transcriptome of healthy, free-of-biofilm tissues from AgP subjects were evaluated, comparing them to chronic periodontitis (ChP) and periodontal health subjects. Several genes and pathways were differentially expressed between the conditions, among which the greater expression of Natural Killers cell receptors and genes related to the immune system (KIR2DL4, IL6 and SELE) were over-expressed in AgP compared to health and ChP, Indicating constitutive changes in this population. In this vein, these evaluations could indicate the path by which AgP occurs, although they do not consider the primary etiological factor - biofilm. Thus, in the fourth chapter, it was compared not only immunological but also microbiological characteristics among 40 individuals with AgP and 28 with ChP individuals. In the comparison of the subgingival concentrations of

Aggregatibacter actinomycetemcomitans (Aa) and *Porphyromonas gingivalis* (Pg), a greater amount and frequency of Aa in AgP was observed, associated to the lower concentration of IgG against this specie and lower subgingival IL-10 level, indicating a possible combination of factors associated with this disease. Interestingly, cytokine levels in the gingival fluid, although they appear to be linked to the origin, were discretely modified by the treatments, as observed in Chapters 5 to 7. Both in the evaluation of the association of full-mouth ultrasonic periodontal debridement together with the systemic administration of Amoxicillin 375mg + metronidazole 250mg (chapters 5 and 6), as well as in subgingival irrigation with iodine-povidone (Chapter 7), there was no significant change in cytokine levels in gingival fluid, although clinically the antibiotic combination promoted additional benefits in reducing depth (PS> 7 mm) and residual sites (PS> 5 mm with probing bleeding). In addition to this clinical benefit, the greatest Aa reduction in the periodontal pockets obtained with the combination of antimicrobials and iodine-povidine irrigation. This reduction of Aa is a factor considered important in AgP in view of its relation to the disease. Thus, in order to identify the beginning of this colonization in a population at risk, that is, descendants of subjects with AgP, chapters 8 and 9 evaluated the presence of Aa, as well as Pg, in the saliva and in the subgingival environment of children of 6-12 years whose parents are AgP, compared to children of periodontally healthy parents. It can be noticed a higher prevalence and concentration of Aa in the saliva of children from AgP families, with a 16x higher chance of infection in childhood when one of the parents is a carrier of this specie. In the subgingival environment, it is possible to notice a higher concentration of this, associated to the greater accumulation of biofilm and gingival bleeding. This information is essential for early preventive approaches, since there is a clear family relationship in the occurrence of AgP. Together, the chapters presented present relevant information regarding the etiology, allowing advances in the search for more objective and predictable protocols of therapy and, especially, based on the findings of the children, preventive protocols can be created and planned in the future.

Keywords: Aggressive periodontitis, etiology, biofilm, antibacterials, genetic.

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 dos dentes. Existem duas formas principais de periodontite: a periodontite crônica (PC) e a periodontite agressiva (PA). Enquanto a PC tem prevalência maior, progressão lenta, afetando geralmente adultos, e apresenta boa resposta à descontaminação mecânica, a PA é uma forma particularmente grave de doença periodontal, acomete indivíduos sistemicamente saudáveis, e é caracterizada por início precoce, rápida progressão, agregação familiar dos casos e pobre resposta as abordagens terapêuticas (Albandar 2014a; 2014b; Armitage 1999; Deas and Mealey 2010).

Estudos epidemiológicos apontam que a prevalência da PA pode variar de 0,3% a 5,5% dos indivíduos jovens na população brasileira (Susin et al. 2014), enquanto que na população mundial a prevalência varia de 1 a 15% (Demmer and Papapanou 2010). Embora a prevalência seja relativamente baixa, esta patologia possui grande impacto na qualidade de vida dos pacientes, uma vez que por apresentar rápida e severa destruição dos tecidos periodontais, frequentemente leva à múltiplas perdas dentais de maneira precoce (Albandar 2014b). A deterioração funcional e estética pode levar a alterações comportamentais, como o desenvolvimento de depressão, agravando ainda mais o impacto na qualidade de vida dos indivíduos afetados pela PA (Priyadarshini et al. 2014).

A susceptibilidade dos indivíduos à periodontite é determinada por uma complexa interação entre microbiota, sistema imune e fatores comportamentais, e é principalmente 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 a susceptibilidade dos indivíduos as doenças periodontais (De Souza et al. 2014; Loos et al. 2015; Stoecklin-Wasmer et al. 2012). Acredita-se que cada um desses fatores contribui em diferentes intensidades entre os indivíduos afetados pela periodontite agressiva e crônica (Loos et al. 2015).

Embora alguns estudos demonstraram que pacientes com PA apresentam diferenças específicas nas características microbiológicas, imunológicas e de expressão gênica, seus fatores

etiopatogênicos ainda não estão completamente estabelecidos. Da mesma forma, pouco se sabe sobre o perfil genético associado a PA, e sobre marcadores que influenciem a resposta do hospedeiro ao desafio microbiano, e o resultado do tratamento periodontal (Duarte et al. 2015; Kebschull et al. 2013; Vieira and Albandar 2014). Além disso, raramente foi avaliada a inter-relação dessas características, com a resposta frente as diferentes etapas do tratamento do paciente com PA, portanto, atualmente não é possível individualizar o tratamento desses pacientes de acordo com suas características etiopatogênicas.

Outro fator neglicenciado pela literatura é a prevenção dessa doença. Uma vez que estudo apontem a forte agregação familiar, indivíduos descendentes de sujeitos afetados pela doença representam uma população de maior risco. Essa maior susceptibilidade poderia estar associada a características microbiológicas, inflamatórias e/ou comportamentais. Estudos de Haubek et al, 2008 e Aberg et al, 2012 já mostraram que indivíduos jovens que portadores de Aa, em especial o subtipo JP2, apresentam maior perda de inserção ao longo do tempo, independente de seu histórico familiar. Assim, avaliar esses indivíduos permitiria a avaliação de fatores precoces diagnósticos que poderiam guiar terapias preventivas.

Soma-se a esses fatores etiopatogênicos e preventivos, a maior dificuldade de tratamento de pacientes portadores de periodontite agressiva. O tratamento da PA foca o controle do fator etiológico primário, o biofilme, por meio da terapia mecânica. Contudo a mesmo apresenta uma menor efetividade comparada a pacientes com periodontite crônica (Scharf et al, 2014). Contudo, embora a terapia periodontal promova a redução da profundidade de sondagem e ganho de inserção após a terapia, bem como a redução de patógenos e grupos de micro-organismos associados a doença, ela não é capaz de reestabelecer completamente a saúde periodontal. Estudos recentes reportaram a ocorrência de bolsas residuais (Mestnik et al. 2012), alto risco de recorrência da doença e de perda dental após a fase ativa da terapia periodontal (Teughels et al., 2014). Recentemente, Dopico et al. (2016) mostraram que pacientes de PA apresentam uma significante taxa de recorrência e perda dental após o tratamento e que, a presença de bolsas residuais profundas eleva de maneira significativa a taxa anual de perda dental. Também existe evidência para suportar que diferentes pacientes apresentarão diferentes reposta a terapia, taxa de progressão da doença e perda dental (Teughels et al., 2014, Meyer-Baumer et al., 2012, Baumer et al., 2011). Assim, a utilização de antibióticos e/ou antimicrobianos tornou-se uma terapia utilizada clinicamente, embora com um número ainda reduzido de estudos clínicos randomizados (Teughels et al., 2014, Mestnik et al., 2012).

Entretanto, no intuito de permitir uma completa ação do antimicrobiano, a desestruturação do biofilme é necessária, uma vez que o mesmo é protegido contra sua ação quando organizado (Marsh et al, 2005). Herrera et al, 2008, indicou que a utilização de terapias que desorganizassem o biofilme em tempo reduzido, durante o período de administração do antimicrobiano, seria um protocolo favorável. Dessa forma, a associação de antimicrobianos e as terapias de desinfecção de boca toda (proposta inicialmente por Quyrinen et al, 1995 e modificada por Wennstron et al, 2005), seria uma abordagem clinicamente importante de ser avaliada.

Assim, diante do exposto, fica clara a necessidade de estudos focados em avaliar diversos fatores associadas a este tipo específico de periodontite. A partir desse melhor entendimento da doença, formas mais efetivas de diagnóstico, decorrentes da identificação de fatores de risco, possibilitarão a implementação de abordagens de tratamento baseados na individualização do tratamento de acordo com as necessidades de cada paciente.

2. PROPOSIÇÃO

O objetivo da presente tese foi avaliar diferentes aspectos diagnósticos, terapêuticos e preventivos da periodontite agressiva. Dessa forma, foi estruturada uma tese em capítulos, correspondentes a artigos publicados e/ou enviados para publicação, seguindo uma sequência lógica de objetivos principais como segue:

1. Validation of Reported GLT6D1 (rs1537415), IL10 (rs6667202), and ANRIL (rs1333048) Single Nucleotide Polymorphisms for Aggressive Periodontitis in a Brazilian Population. Journal of Clinical Periodontology. 2017 (submitted) QUALIS A1 e JCR Impact Factor 3,477.

2. Whole-exome sequencing analysis in generalized aggressive periodontitis: a familial screening approach. Human Genetics 2017 (submitted) QUALIS A1 e JCR Impact Factor 4,637.

3. Transcriptome of healthy gingival tissue from edentulous sites in patients with a history of aggressive periodontitis Journal of Periodontology 2017 (accepted) QUALIS A1 e JCR Impact Factor 3,03.

4. Levels of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, inflammatory cytokines and species-specific immunoglobulin G in generalized aggressive and chronic periodontitis. Journal of Periodontal Reserach 2010 Oct;45(5):635-42. doi: 10.1111/j.1600-0765.2010.01278.x. QUALIS A2 e JCR Impact Facto 2,662

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treatment. Journal of Periodontology 2012 Aug;83(8):988-98. doi: 10.1902/jop.2012.110513. QUALIS A1 e JCR Impact Factor 3,03.

7. Amoxicillin/metronidazole associated with nonsurgical therapy did not promote additional benefits in immunologic parameters in generalized aggressive periodontitis: A randomized controlled clinical trial. Quintessence International. 2016 Apr;47(4):281-92. doi: 10.3290/j.qi.a34723. QUALIS B1 e JCR Impact Factor 0,995

8. Salivary carriage of periodontal pathogens in generalized aggressive periodontitis families. International Journal of Paediatric Dentistry 2014 Mar;24(2):113-21. doi: 10.1111/ipd.12035. QUALIS A1 e JCR Impact Factor 1,532

9. Periodontal clinical and microbiological characteristics in healthy versus generalized aggressive periodontitis families. Journal of Clinical Periodontology 2015 Oct;42(10):914-21. doi: 10.1111/jcpe.12459. QUALIS A1 e JCR Impact Facto 3,477

3.ARTIGOS

3.1 Validation of Reported GLT6D1 (rs1537415), IL10 (rs6667202), and ANRIL (rs1333048) Single Nucleotide Polymorphisms for Aggressive Periodontitis in a Brazilian Population*

ABSTRACT

Aim: 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.

Materials and Methods: The SNPs rs1537415, rs6667202, and rs1333048 were genotyped using TaqMan assays 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.

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: The SNP rs6667202 was associated with AgP in a Brazilian population. The minor C allele was less frequently detected in AgP subjects than in other groups; therefore, this SNP is protective against AgP.

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Validation of Reported GLT6D1 (rs1537415), IL10 (rs6667202), and ANRIL (rs1333048) Single Nucleotide Polymorphisms for Aggressive Periodontitis in a Brazilian Population

Short title: SNPs and AgP in Brazilians

Key words: periodontal diseases, genetic variation, genotype, alleles, genetic markers.

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Abstract

Aim: 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.

Materials and Methods: The SNPs rs1537415, rs6667202, and rs1333048 were genotyped using TaqMan assays 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.

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: The SNP rs6667202 was associated with AgP in a Brazilian population. The minor C allele was less frequently detected in AgP subjects than in other groups; therefore, this SNP is protective against AgP.

Clinical Relevance

Scientific Rationale for the Study: Recent studies have shown evidence of an association between AgP and SNPs in GLT6D1 (rs1537415), IL10 (rs6667202), and ANRIL (rs1333048) in different European populations. However, these specific SNPs have not been validated in AgP subjects in Brazil, which has a relatively high prevalence of AgP.

Principal findings: The SNP rs6667202 (IL10), but not the SNPs in GLT6D1 and ANRIL, was associated with AgP when compared to CP and healthy subjects in a Brazilian population.

Practical implications: Recognizing the genetic factors associated with AgP is important to understanding its pathogenesis and could help in developing new diagnostic tools and therapy strategies for prevention and treatment of the disease.

Introduction

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 (Armitage, 1999, Albandar, 2014). This disease shows clear differences in the development and progression when compared to the most common form of periodontitis, the chronic periodontitis (CP) (Tonetti & Mombelli, 1999). Among patients with AgP, exposure to local etiological factors usually cannot account for the significant alveolar bone loss, suggesting that host factors are involved in determining susceptibility to the disease (Meng et al., 2011). Although differences in the microbial flora 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 (Kinane et al., 2005, Meng et al., 2011, Vieira & Albandar, 2014).

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 (Yang et al., 2009, Vieira & Albandar, 2014). 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 (Vieira & Albandar, 2014, Loos et al., 2015). 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 (Laine et al., 2014, Loos et al., 2015). Several studies have reported such efforts but shown varying, if not contradictory results (Zhang et al., 2011, Laine et al., 2012, Loos et al., 2015). Up to date, few genes have been properly identified as susceptibility ones. Recently, some recent studies reported a significant or a suggestive association between SNPs in GLT6D1, ANRIL, and IL10 and AgP (Schaefer et al., 2009, Schaefer et al., 2010, Schaefer et al., 2011, Schaefer et al., 2013, Hashim et al., 2015, Loos et al., 2015).

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 (Schaefer et al., 2009, Schaefer et al., 2010, Ernst

et al., 2010, Schaefer et al., 2011, Schaefer et al., 2013). The association between rs1537415 (GLT6D1) and AgP was also observed in a Sudanese population (Hashim et al., 2015). 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 (Schaefer et al., 2013). Similarly, rs1333048 in ANRIL was first associated with AgP in a German cohort in the study of Schaefer et al. (2009), and also observed in the study of Ernst et al. (2013), 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 (Schaefer et al., 2013).

Although these different results may be due to methodological issues, different genetic profiles found between individual from different populations may also be a relevant factor in these contradictory results (Abecasis et al., 2012, Hashim et al., 2015). Since 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 (Loos et al., 2005, Meng et al., 2007, Hashim et al., 2015). 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 once Brazilian population has a higher prevalence of AgP than the European population (5.5% vs 0.2%) (Susin & Albandar, 2005, Susin et al., 2014) 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 to CP, investigating if these associations were solely attributed to AgP or common genetic background of periodontal destruction.

Material and methods

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 subject after explanations

were provided. A total of 586 subjects (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) (Ainamo & Bay, 1975), full-mouth bleeding score (FMBS) (Muhlemann & Son, 1971), probing pocket depth (PPD) and clinical attachment level (CAL) at six point around each tooth using a manual probe (PCPUNC 15®, HuFriedy, Chigaco, IL, USA). Tooth mobility, radiographic examination, and complete medical and dental questionnaires were also obtained.

AgP and CP patients were identified on the basis of American Academy of Periodontology criteria (Armitage, 1999). Most of AgP patients were treated in previous studies (Casarin et al., 2012, Taiete et al., 2016, do Vale et al., 2016, Rebelatto Bechara Andere et al., 2017) or in one of the three Graduate Clinics. Furthermore, these patients needed at the first examination the presence of at least 20 teeth, at least 8 teeth with a PD ≥ 5mm with bleeding on probing (having at least 2 with PPD ≥ 7mm), < 35 years of age for AgP patients, and > 35 years for CP. Healthy subjects did not show interproximal attachment loss and probing pocket depths of >4mm (Sorensen et al., 2008, Jonsson et al., 2011). 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) (Andia et al., 2013).

Isolation of genomic DNA

Genomic DNA (gDNA) was isolated from buccal epithelial cells using a salting-out protocol (Aidar & Line, 2007). Briefly, the subjects undertook a mouthwash containing 5 mL 3% dextrose solution for 60 seconds. Three mL of TNE solution [17 mM Tris/HCl (pH 8.0), 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 proteinase K (Sigma

Chemical Co., St. Louis, MO, USA) (20 mg/ml) was added. DNA was dissolved in nuclease-free water, and its quantity was evaluated spectrophotometrically using a Nanodrop 2000 device (Thermo Scientific, Wilmington, DE, USA).

Genotyping

PCR-based genotyping of rs1537415 (GLT6D1), rs6667202 (IL10), and rs1333048 (ANRIL) (Table 1) was performed on the LightCycler 480 (Roche Diagnostics GmbH) using TaqMan 5'-exonuclease allelic discrimination assays (Thermo Scientific). PCR were carried out in a total of 10 µl, containing 20 ng of gDNA, 5 µl TaqMan Genotyping Master Mix, 0.5 µl TaqMan genotyping assay mix 20X. PCRs were performed under the following conditions: 10 min at 95 oC, 40 x (15 sec at 95 oC, 1 min at 60 oC). Reactions were randomly repeated in 10% of the samples for each SNP, for quality control purposes, and the concordance rate was 100% (Machado et al., 2016). All samples were successfully genotyped, with a genotype call rate of 100%.

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.

To test deviations of Hardy-Weinberg equilibrium, genotype distribution of each polymorphism in each group was evaluated (BioEstat 5.4, Belém, PA, Brasil). Association analysis between rs1537415 (GLT6D1), rs6667202 (IL10), rs1333048 (ANRIL) and AgP was performed using chi-square test. 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 (SIGMAstat 4.0, Systat, CA, USA). A Bonferroni-adjusted p-value of $\alpha \leq 0.017$ (0.05/3) was considered statistically significant. Power calculations were performed using Quanto 1.2.4 software (University of Southern California), assuming a prevalence of AgP in Brazil of 0.0549 (Susin & Albandar, 2005), a p value < 0.05 and using the most conservative odds ratios reported in the original studies.

Results

Study Population

The demographic and clinical characteristics of the subjects 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 subjects 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.

Genotyping

Hardy-Weinberg equilibrium and power analysis

The genotype frequencies observed for the three SNPs in the three groups were not statistically significant 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 vs non-AgP; 80.99% for AgP vs H; and 80.41% for AgP vs CP), rs1333048 (99.16% for AgP vs non-AgP; 96.35% for AgP vs H; and 96.18% for AgP vs CP) and rs1537415 (93.89% for AgP vs non-AgP; 85.57% for AgP vs H; and 85.41% for AgP vs CP).

AgP vs Non-AgP

Firstly, the analysis was performed with the chronic periodontitis and healthy subjects pooled together in the non-AgP group. SNP rs6667202 (IL10) showed an association with AgP in this Brazilian case control 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 in a C allele frequency of 32.3% and carrier frequency of least one 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 in GLT6D1 and rs1333048 in ANRIL were not significant for AgP diagnosis in this Brazilian cohort after Bonferroni correction.

AgP vs 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.

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.

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).

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 population were pooled (non-AgP group). The AC genotype was statistically significant only when compared 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 (2013), 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 (2013), 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 firstly described result, since 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.

Interestingly, the final logistic model included IL10 SNP rs6667202 (allele or genotype) and gender as diagnostic factors to AgP. This result indicates that women who do not present the C allele of rs6667202 in the IL10 gene could represent the high-risk population for AgP. However, some caution should be taken when considering this result. In our population, there was a higher prevalence of females in the AgP cohort. Although several studies have neglected the association with gender (Laine et al., 2012, Vieira & Albandar, 2014), this result should be considered in future studies.

IL10 encodes the cytokine interleukin-10 (IL-10), an anti-inflammatory cytokine that can regulate the expression of proinflammatory cytokines. This cytokine is usually expressed in inflamed periodontal tissues and is associated with lower disease severity, once it promotes the suppression of proinflammatory cytokines (Garlet et al., 2006, Garlet, 2010). It can be speculated that the minor C allele of rs6667202, which is enriched in the healthy subjects, results in increased production of IL-10, since previous studies have shown lower IL-10 levels in the gingival crevicular fluid of AgP patients (Casarin et al., 2010, Teles et al., 2010). 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 and rs1333048 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 (Schaefer et al., 2009), and replicated in a German-Northern Irish population (Ernst et al., 2010). The GLT6D1 SNP rs1537415 was discovered and replicated in 2 independent German cohorts, and also replicated in a Sudanese population (Schaefer et al., 2010, Hashim et al., 2015). Interestingly, Schaefer et al. (2010) reported a statistical significant enrichment of the minor allele of 9.4% to 12%, similar to that reported by Hashim et al. (2015) - about 9%. In the present study, no differences between the minor allele frequency between the groups evaluated were noted. These differences emphasize the role of ethnicity in individual genetic background, which in turn reinforces 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 due to some factors. AgP is considered a complex and multifactorial disease; therefore, genetic risk factors can have different effects in different populations (Loos et al., 2005, Meng et al., 2007, Hashim et al., 2015). 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 (Silveira et al., 2016). 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 (Loos et al., 2015). Furthermore, the present study has some limitations. The main limitation are the modest sample size when compared to expected to periodontal genetic studies (Schafer et al., 2011). 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) and rs6667202 (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 the moment of the diagnosis). The inclusion of severe disease phenotype are very likely to improve the chance of detecting a disease-associated variant (Schafer et al., 2011). Also, efforts were made to minimize of environmental and life-style factor, which can act as sources of phenotypic heterogeneity (Schafer et al., 2011).

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 subjects, suggesting that this SNP may have a protective effect in this Brazilian cohort.

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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

Table 2. Demographic and clinical characteristics.

Characteristics	AgP	CP	H
Age	34.0 ± 4.6 A	50.0 ± 7.2 B	30.5 ± 5.8 A
Gender	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	2.35±0.0 A	2.28 ± 0.3 A	2.1 ± 0.2 A
CAL	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.
AgP – Aggressive periodontitis; CP – chronic periodontitis; H – healthy patients; Age (years - mean ± standard deviation); Gender (M/F) – Gender (percentage of Male / Female); Ethnic (Af / C) – Ethnic (percentage of African American and Caucasoid); FMPI – Full Mouth Plaque Index (mean ± standard deviation); FMBS – Full Mouth Bleeding Index (mean ± standard deviation); PPD – Probing Pocket Depth (mean ± standard deviation); CAL – Clinical Attachment Level (mean ± standard deviation).

Table 3. Allele and genotype frequency of Polymorphisms rs1537415, rs6667202, and rs1333048 of AgP and non-AgP group.

SNP		AgP	Non-AgP	*p value*	AgP x 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	*0.0027*	*0.64 (0.48 - 0.86) p = 0.0034*
	A	23.5	32.3		
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

AgP – Aggressive periodontitis; Non-AgP (chronic periodontitis and healthy patients pooled together); 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 x H OR (95% CI) *p*-value	AgP x CP OR (95% CI) *p*-value	CP x 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

AgP – Aggressive periodontitis; CP - chronic periodontitis; H - healthy patients; 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 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

3.2 Whole-exome sequencing analysis in aggressive periodontitis: a familial screening approach*

Abstract

Aim: Aggressive periodontitis (AgP) is a severe form of periodontitis, which presents early onset, rapid progression, and familial aggregation. Although genetic factors are involved in determining susceptibility to AgP, they have been identified to a limited extent.

Methods: Thus, aim of the present study was to identify genetic variants associated with AgP via whole-exome sequencing (WES) through a familial screening approach. WES was performed in 2 nuclear families, each one with four subjects, including a proband affected by AgP, a healthy sibling, an affected parent, and an unaffected parent. Firstly, each family were analyzed separately. Common variants between the affected parent and the unaffected parent were excluded, as well as common variants from the unaffected sibling. After, only the common variants between this data set and the proband were selected. Finally, only the common variants between the two families were considered associated with AgP. Candidate genes were further sequenced by Sanger technique to confirm the identified variants sequences. To explore functional molecular correlations among genes with the identified genetic variants, a protein-protein interaction network (PPI) was constructed. Moreover, in silico analysis were done to identify deleterious impact of each variant on protein structure.

Results: Three missense single nucleotide variations (SNVs) in exomic region, rs142548867 in EEFSEC [c.668C>T (p.Pro223.Leu)], rs574301770 in ZNF136 [c.466C>G (p.Arg156Gly)], and rs72821893 in KRT25 [c.800G>A (p.Arg267His)], and 2 indels with frameshift, rs37146475 in GPRC6A [c.2323- 2324insT (p.Tyr775LeufsTer776)], and in ELN gene [c.1366_1372insGGAGCAG (p.Ala457fs or p.Ala457SrfsTer37)] were associated with AgP and presented functional impact in SIFT and Polyphen database. Moreover, PPI analysis showed highly interconnected network, linking SNVs to central nodes, as ubiquitin C. Meanwhile, in silico analysis indicate that a SNV on EEFSEC (p.Pro223.Leu) and GPRC6A (p.Tyr775LeufsTer776) genes, generate altered proteins, especially in GPRC6A where a loss in C-terminal tail were seen.

Conclusion: It can be concluded that using a familial filtering approach, missense SNVs in EEFSEC (p.Pro223.Leu), ZNF136 (p.Arg156Gly) and KRT25 (p.Arg267His) genes, and indels in GPRC6A

(p.Tyr775LeufsTer776), and ELN ((p.Ala457fs or p.Ala457SrfsTer37) genes were identified and associated to AgP occurrence.

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Whole-exome sequencing analysis in aggressive periodontitis: a familial screening approach

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Abstract

Aggressive periodontitis (AgP) is a severe form of periodontitis, which presents early onset, rapid progression, and familial aggregation. Although genetic factors are involved in determining susceptibility to AgP, they have been identified to a limited extent. Thus, aim of the present study was to identify genetic variants associated with AgP via whole-exome sequencing (WES) through a familial screening approach. WES was performed in 2 nuclear families, each one with four subjects, including a proband affected by AgP, a healthy sibling, an affected parent, and an unaffected parent. Firstly, each family were analyzed separately. Common variants between the affected parent and the unaffected parent were excluded, as well as common variants from the unaffected sibling. After, only the common variants between this data set and the proband were selected. Finally, only the common variants between the two families were considered associated with AgP. Candidate genes were further sequenced by Sanger technique to confirm the identified variants sequences. To explore functional molecular correlations among genes with the identified genetic variants, a protein-protein interaction network (PPI) was constructed. Moreover, *in* silico analysis were done to identify deleterious impact of each variant on protein structure. Three missense single nucleotide variations (SNVs) in exomic region, rs142548867 in *EEFSEC* [c.668C>T (p.Pro223.Leu)], rs574301770 in *ZNF136* [c.466C>G (p.Arg156Gly)], and rs72821893 in *KRT25* [c.800G>A (p.Arg267His)], and 2 indels with frameshift, rs37146475 in

GPRC6A [c.2323-2324insT (p.Tyr775LeufsTer776)], and in *ELN* gene [c.1366_1372insGGAGCAG (p.Ala457fs or p.Ala457SrfsTer37)] were associated with AgP and presented functional impact in SIFT and Polyphen database. Moreover, PPI analysis showed highly interconnected network, linking SNVs to central nodes, as ubiquitin C. Meanwhile, *in silico* analysis indicate that a SNV on *EEFSEC* (p.Pro223.Leu) and *GPRC6A* (p.Tyr775LeufsTer776) genes, generate altered proteins, especially in *GPRC6A* where a loss in C-terminal tail were seen. It can be concluded that using a familial filtering approach, missense SNVs in *EEFSEC* (p.Pro223.Leu), *ZNF136* (p.Arg156Gly) and *KRT25* (p.Arg267His) genes, and indels in *GPRC6A* (p.Tyr775LeufsTer776), and *ELN* ((p.Ala457fs or p.Ala457SrfsTer37) genes were identified and associated to AgP occurrence.

Keywords: genetic association studies, periodontal disease, genetic variations, genetic markers

Introduction

Aggressive periodontitis (AgP) is characterized by an early age of onset, rapid progression in otherwise healthy individuals, and familial aggregation, which, if left untreated, can lead to early edentulism [1]. Its prevalence ranges from 0.1% to 6.5% in different populations around the world, while in Brazilian population the prevalence reported is 5.5% [2, 3]. Although affecting a relatively small proportion of the population compared with chronic periodontitis (CP) (prevalence of 14% to > 50%), the rapidity of progression and high risk of tooth loss at a young age make AgP a unique and relevant disease [4-6]. This marked periodontal destruction cannot be explained only by the exposure to local irritants (i.e., subgingival biofilm and calculus), suggesting that host factors are involved in determining susceptibility to AgP [7].

There is evidence that individual's genetic make-up is a crucial factor influencing host susceptibility to AgP [7-10], which is highlighted by the familial aggregation, that is one of the primary features of AgP [11]. It is noteworthy that the prevalence of AgP is disproportionately high among certain families, with some studies reporting that the percentage of affected siblings may reach 40-50% or even higher [7]. However, although many studies have been carried out to investigate the association between genetic factors and AgP, varying and often contradictory results were found [11-13]. This recognition of genetic factors those contribute to disease susceptibility in AgP is important to understand its pathogenesis, and could help develop new diagnostics tools and therapy strategies for prevention and treatment of the disease [10, 11, 13, 14].

Currently, specific genetic factors associated with to AgP have been identified to a limited extent [11, 12]. Previous studies evaluated specific single nucleotide polymorphisms (SNPs) in only a few genes, and have not captured the complete genetic information of a particular region of interest [11, 12]. Because of these limitations, it is essential to carry out unbiased large scale analysis to identify novel disease-associated genetic variants for AgP [14, 15]. The emergence of genome-wide association studies (GWAS) using whole genome sequencing (WGS) or whole exome sequencing (WES) is improving comprehensive, open-ended and hypothesis-free studies. However, regarding AgP disease, scarce information was provided [11, 14].

Schaefer et al. [16] conducted a GWAS in a German population and reported the association between the intronic SNP rs1537415 of the *GLT6D1* gene with the AgP. Recently, Kitagaki et al. [14] conducted a WES in a Japanese population with AgP, and found that this disease could be associated with SNP rs536714306 of *GPR126*. However, no GWAS was performed in Brazilian population. In addition, one of most important characterisc of AgP, the familial aggregation, have never been considered in data analysis. Since that AgP is thought to be a hereditary trait and show a clear familial aggregation, the family-based approach became extremely valuable. Thus, in view of these points, the present study aimed to investigate AgP genetic variations studying familial nuclei via whole-exome sequencing.

Material and Methods

Subjects

The study was designed in accordance with the STROBE Statement for observational studies, and it was approved by the Ethics Committee of the University of Campinas (58679416.4.0000.5418). Subjects from 2 nuclear families, each one with four subjects, from the Southeastern region of Brazil were recruited from the patients referred to the Graduate Clinic of Piracicaba Dental School, University of Campinas, Brazil, between September 2014 and November 2014. Informed written consent was granted by each subject after explanations were provided.

All patients received complete intraoral and periodontal examination, including full-mouth plaque index (FMPI), according to Ainamo and Bay [17], and full-mouth bleeding score (FMBS), according to Mühlemann and Son [18], tooth mobility, radiographic examination, and complete medical and dental questionnaires. Additionally, for the parents, periodontal records

were recovered from dental records. The diagnosis of AgP and CP was based on the American Academy of Periodontology criteria [19]. The study was accomplished with two probands affected by AgP, their parents (one with a history of AgP, one without a history of AgP) and their sibling (healthy subject – without AgP), as described below and in Fig1:

AgP Probands – Subjects presented periodontal pockets with clinical attachment loss (CAL) and radiographic bone loss in at least three teeth different from 1st molars and incisors, at least eight teeth with a probing pocket depth (PPD) ≥ 5 mm and bleeding on probing. At least two of the eight qualifying teeth must have PPD ≥ 7 mm; age inferior to 35 years.

Non-AgP siblings – Healthy subjects, who did not show any sites with CAL and PPD ≥ 3 mm and presented FMBS < 25%; tooth mobility was not found; age inferior to 35 years.

AgP affected parents – the progenitors who presented the same periodontal characteristics as AgP probands, but had experienced multiple tooth loss due to disease progress; also, they were older than 35 years. The diagnosis was obtained after careful intra-oral examination and evaluation of past radiographic and dental records. Periodontal characteristics of AgP parents were recovered from their dental records.

Non-AgP parents – the progenitors who did not presented any characteristic of AgP, but they had slight or moderate CP.

Additionally, the exclusion criteria were: smoking, systemic diseases (e.g., diabetes, hepatitis or HIV infection), diseases of the oral hard or soft tissues (except caries and periodontitis), use of orthodontic appliances and immunosuppressive chemotherapy [20].

Isolation of genomic DNA

The sampling of buccal epithelial cells was performed as described by Trevilatto & Line [21]. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation, dissolved in nuclease-free water, and its concentration was evaluated spectrophotometrically using a Nanodrop 2000 device (Thermo Scientific, Wilmington, DE, USA). DNA integrity was evaluated using 1% agarose gel electrophoresis.

Exome Analysis

Whole-exome sequencing (WES) was performed using the Illumina's next-generation sequencing technology. Library preparation was performed according to the Nextera Rapid-Capture Exome kit (Illumina, San Diego, CA, USA), and the sequencing was performed in Illumina

HiSeq 2500 system (Illumina Inc). The samples were sequenced using the Illumina HiqSeq 2500 platform (Illumina, San Diego, CA, USA), using TrueSeq SBS kit V3 cBot-HS with 200 cycles [22, 23].

FASTAQ files were generated using a Consensus assessment of sequence and variation (Casava, illumina, version 1.8.2). The quality of FASTQ reads was analyzed by FastQC software. Trimmomatic software [24] was used to remove adapter sequences and low quality reads, using filters such as Phred quality score ≥ 20 and a fragment size ≥ 65. Quality-trimmed reads from each individual were aligned against human genome reference (hg19/GRCh38.p2) using the Burrows-Wheeler Aligner software [25]. After, the data were converted SAM to BAM file, removing duplicates, using PICARD software (http://broadinstitute.github.io/picard). The Genome Analysis Toolkit (GATK) [26] was used to find SNVs and small insertions and deletions (Indels). The frequencies of each SNV/Indel were calculated from the GATK output files (VCF–Variant Caller Format) based on Bayesian method (McKenna et al., 2010). Functional effects and pathogenic potential of SNVs and indels were predicted with the Variant Effect Predictor [27]. Perl scripts were used to exclude variants with minor allele frequency (MAF) ≥ 1% in the population and retain rare variants (MAF <1%) using a catalog generated by the 1000 Genomes Project [28] and International HapMap Project [29].

In order to identify the genetic variations in WES associated with AgP, the candidate genes were selected from a series of filters. Firstly, each family were analyzed separately. Common variants between the affected parent and the unaffected parent were excluded, as well as common variants from the unaffected sibling. After, only the common variants between this data set and the affected proband were selected. And finally, only the common variants between the two families were considered as associated with AgP. In order to reduce the potential number of disease-associated variants, only variants in protein coding regions, including missense (non-synonymous) and non-sense (stop-gain, stop-lost) variants, and indels (frameshift variant). In order to establish the candidate variants and predict the possible impact of amino acid substitutions on the stability and function of proteins, it was used the Sorting Intolerant From Tolerant (SIFT) [30] and PolyPhen-2 [31] prediction programs via VEP to confirm the identified variants sequences in the present study.

PCR amplification and Sanger Sequencing

Sanger sequencing was performed using *EEFSEC* (eukaryotic elongation factor, selenocysteine-tRNA specific)*, ZNF136* (zinc finger protein 136)*, KRT25* (keratin 25)*, GPRC6A* (G protein-coupled receptor class C group 6 member A) and *ELN* (elastin)-specific primers (Supplementary Table 1) ase chain reaction (PCR) was performed in a final volume of 25 µL with 400 ng of DNA, 50 µM forward and reverse primers, 0.2 mM dNTP mix, 0.75 U Taq Polymerase, and 1.25 mM $MgCl_2$ (Invitrogen, Life Technologies, Carlsbad, CA, USA). Cycle conditions, annealing temperature, and primer specificity were optimized for each primer pair (Supplementary Table 1).

PCR products were purified from agarose gel using PureLink™ Quick Gel Extraction Kit (Thermo Fisher Scientific, Graiciuno, Vilnius, Lithuania), according to the manufactures' instructions. DNA sequence analysis was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit and migrated on capillary 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequence data were analyzed and compared with the human reference genome (hg19/GRCh38.p2).

Protein-protein interaction network analysis

Interactions between candidate genes genes harboring SNVs and indels were mapped using a protein-protein interaction network analysis (PPI), a set of protein-protein interactions (PPI) whose occurs inside of a cell [32]. A network is a set of elements interacting with each other through pairwise interactions. In the case of biological PPI networks, the components are the coding genes/proteins (nodes) that are connected to each other by links (edges) representing known physical interactions between these components. In order to generate the PPI networks, we used Integrated Interactome System (IIS) [33]. ISS integrated nine public available databases used in system biology analysis: BioGRID [34], Intact [35], DIP [36], MINT [37], HPRD [38], DrugBank [39], HMDB [40], YMDB [41], and ECMDB [42]. Cytoscape version 2.8.2 [43] was used to visualize the PPI network. String [44] was used to predicted the molecular action of networks edges (interactions between nodes).

Residue conservation, structure and contacts analysis

Multiple-species amino acid sequence alignment of *EEFSEC*, *KRT25*, *ZNF136*, *GPRC6A* and *ELN* proteins were performed using Clustal Omega (European Bioinformatics Institute,

EMBL-EBI; http://www.ebi.ac.uk/Tools/msa/clustalo/). Sequence logo for *EEFSEC*, *KRT25*, *ZNF136*, *GPRC6A* and *ELN* sequences were carried out using WebLogo analysis [45].

Three-dimensional (3D) models for native and mutant EEFSEC, KRT25, ZNF136, GPRC6A and ELN proteins were built based on previously determined crystal structure of these protein using an SWISS-MODEL software [46]. These models were aligned, visualized, and analyzed using the PyMOL (PyMOL Molecular Graphics System, Version 1.7.4, Schrödinger, LLC). Stick models, showing the hydrogen bond in native or mutant 3D models, and inter-atom distances were constructed using the modeling tool PdbViewer [47].

Results

Subjects

The demographic and clinical characteristics of the study participants are displayed in Table 1, in which the clinical diagnosis of AgP in affected probands and their affected-parents, healthy status in siblings, and slight or moderate CP in non-affected parents is confirmed.

Exome Analysis

From the eight individuals, there was a total of 480093728 reads with high quality, mapping 97.81% (paired reads) on the human exome (GRCh38p2). All information regarding number of reads, sample coverage and sequencing depth, as well as the data quality, is summarized in the Supplementary Tables 2 and 3.

After filtering the initial variants, 3 SNVs and 2 indels were identified in the patients with AgP (Table 2 and 3). Considering the SNVs, missense variants were found in *EEFSEC* [rs142548867 - c.668C>T (p.Pro223.Leu)], *ZNF136* [rs574301770 - c.466C>G (p.Arg156Gly)] and *KRT25* [rs72821893 - c.800G>A (p.Arg267His)] genes. All three variants were classified as moderate impact on VEP analysis. SIFT predicted these variants as having a deleterious effect on protein function, whereas Polyphen predicted variants in *EEFSEC* and *ZNF136* as possibly damaging and KRT25 variant as benign (Table 5). Indels was observed in *GPRC6A* [rs371464745 - c.2323-2324insT (p.Tyr775LeufsTer776)], and in *ELN* [c.1366_1372instGGAGCAG (p.Ala457fs or p.Ala457SrfsTer37)] genes, which result in frameshit variants, which were classified as high impact on VEP analysis.

Sanger Sequencing

Thus, the regions in *EEFSEC*, *ZNF136, KRT25*, *GPRC6A* and *ELN* genes containing their respective variants were amplified and sequenced using Sanger sequencing in all subjects to confirm their status. Sanger sequencing confirmed the SNVs in *EEFSEC* [c.668C>T (p.Pro223.Leu)], *ZNF136* [c.466C>G (p.Arg156Gly)], *KRT25* [c.800G>A (p.Arg267His)], and the indels *GPRC6A* [c.2323-2324insT (p.Tyr775LeufsTer776)], and in *ELN* [c.1366_1372instGGAGCAG (p.Ala457fs or p.Ala457SrfsTer37)] in all AgP subjects.

Protein-protein interaction network analysis

The 3 SNVs and 2 indels found as asssociated to AgP was used to construct the PPI network (Tables 2 and 3). 4 of the candidate genes (*EEFESC, ZNF136, KRT25* and *ELN*) were connected to other nodes (Fig 2). *GPRC6A* were not present in the PPI databases. In the resulting PPI network, *EEFESC, ZNF136,* and *KRT25* were interconnected with them and other neighboring and non-neighboring nodes, which were not present in the list of genes candidates. Ubiquitin C (*UBC*) node was classified as a broker, having connection with the three missense variants. All three missense variants presented edges networks with *UBC* predicted to had binding action. Also, interaction between *ZNF136* and *UBC* was predicted to had catalytic action. *ELN* had interactions only with neighboring nodes, as MMP7, MMP9, and MMP12, but, it did not shown interactions with other nodes.

Residue conservation, structure and contact analysis

The residue Pro223 affected by missense SNV in *EEFSEC* is phylogenetic conserved among vertebrate species, while the Arg267 residue affected by missense SNV in *KRT25* is conserved only in mammals, and the Arg263 residue affected by missense SNV in *ZNF136* is conserved only in primates (Supplementary figure 1). In addition, the SNV in *KRT25* and *ZNF136* was supposed to not display a relevant impact structural and functional considering their Pfam-based domain annotation and identity of sequence protein (data not showed). Structural analysis for SNV in *EEFSEC* (p.Pro223Leu) showed that the replacement of Pro by Leu residue at 223 protein position was not predicted to promote conformational changes in eEFSec protein structure (Supplementary figure 2).

In regards to *ELN* and *GPRC6A*, the insertion of Thymine (T) nucleotide in 2110-2111 position (c.2110-2111insT) result in a substitution of Try for Leu residue in the 775 protein position of hGPRC6A isoform 1 and insertion of premature stop codon in the 776 position

(p.Tyr775LeufsTer2), causing the loss of all portion downstream, including the final portion of 7tm3 and whole C-terminus intracellular region (Fig 3A and Fig 4). This genetic alteration is supposed to cause the loss of some potential sites required for calcium sensing and calcimimetics action, in three isoforms of hGPRC6A (Fig 3B and 3C). The multiple sequence alignment for hGPRC6A isoforms (corresponding to 775-832 residues from hGPRC6A isoform 1), and other C family G-protein-coupled receptors members, such as a calcium-sensing receptor (CaSR) and Metabotropic glutamate receptor 1 (mGluR-1) revealed the conservation of sequence of part of lost residues in hGPRC6A mutant protein (figure 3B). In addition, the multi-species sequence alignment followed by WebLogo analysis demonstrated the phylogenetic conservation of part of lost residues in hGPRC6A mutant protein (Figure 3C). Indel in ELN was supposed to not display a relevant impact structural and functional considering their Pfam-based domain annotation and identity of sequence protein (data not showed).

Discussion

In the present study, WES approach was chosen to identify novel genetic risk factors for AgP using a family-based association study. Although there is a substantial evidence for a role of genetic factors in increasing the host susceptibility to AgP, to our knowledge, this is the first reported WES study in AgP designed to search genetic variants across all the coding regions of the genome through a familial screening approach. Affected family members are thought to have the same genetic variant, inherited the disease-causing variant from a common ancestor [48]. Also, non-affected family members, i.e., parents, and siblings, have high genetic similarity and can be sequenced to exclude common benign variation [49]. Thus, family-based WES thereby substantially reduces the list of candidate genes and increases the power to identify genetic variants associated to the disease [48, 49]. Using a familial-based association study, the present study indicates the presence of missense SNVs in *EEFSEC* [c.668C>T (p.Pro223.Leu)], *ZNF136* [c.466C>G (p.Arg156Gly)], and *KRT25* [c.800G>A (p.Arg267His)] genes, and indels in *GPRC6A* [rs371464745 - c.2323-2324insT (p.Tyr775LeufsTer776)] and *ELN* [c.1366_1372instGGAGCAG (p.Ala457fs or p.Ala457SrfsTer37)] associated to AgP. Furthermore, the PPI network and *in* silico analyses was combined to generate hypotheses of potentially relevant alterations.

A missense variant found to be associated with AgP occurred in *KRT25* gene [c.800G>A (p.Arg267His)]. This gene encodes a member of type I (acidic) keratin family, which along with actin microfilaments and microtubules, compose the cytoskeleton of epithelial cells.

Keratin is an essential protein in epithelial cells, but this family of proteins was not previously associated with periodontitis. This SNV classified as deleterious by SIFT and benign by Polyphen, was previously associated with non-responsiveness in steroidal therapy childhood asthma [50]. In child presenting this SNV a worsening of pulmonary function, hyperreactive of airways and impair in corticosteroid treatment response were seen. Thus, apparently, this SNV could be associated to immune-inflammatory response, and, consequently, to oral health. Therefore, further investigation is necessary to explain the association between this SNV and AgP.

Another gene that presented missense SNV was the *ZNF136*. It has been estimated that there are 300-700 different ZNF protein genes, and many of them are clustered within the human genome [51, 52]. These proteins are considered transcriptions factors, sequence-specific binding protein, which play a central role in regulating gene expression and therefore coordinate a plethora of biological process, including development, differentiation, metabolism, apoptosis, and autophagy [51]. *ZNF136*, specifically, may be involved in transcriptional regulation as a weak repressor, probably associated with the signaling pathway of apoptotic cell death [53]. Interestingly, a recent study demonstrated that *ZNF-A20* (that presents high homology to *ZNF136*) exerts anti-inflammatory effects by limiting proinflammatory cytokine expression and secretion by periodontal ligament cells exposed to *P. gingivalis* LPS [54]. *ZNF-A20* overexpression decreases expression of several *NF-κβ* target genes, including *E-selectin, ICAM-1, IL-8, VCAM-1, IL-6* and *GM-CSF* [54]. Moreover, Lee et al. [55] found several ZNF proteins genes associated with the diagnosis of peri-implantitis. Altogether, these studies indicate that zinc finger proteins play an important role in periodontal and peri-implant diseases, and therefore, variants may lead to impairment in the immune-inflammatory response against periodontopathogens.

Finally, the third missense variation was in *EEFSEC* gene. It is an important translation factor of selenoproteins and selenoenzymes, who are critical players for the redox potential homeostasis maintenance, regulating inflammatory response and immune cells and protection of the genetic material and cell membrane from oxidative damage [56, 57]. It is enrolled in the formation of the selenoenzymes glutathione peroxidase and thioredoxin reductases, which are antioxidant pathways that protect from reactive oxygen species (ROS) [57]. Interestingly, oxidative stress seems to be one high contributor to AgP pathogenesis, since this disease presents higher oxidative stress markers than other periodontal diseases [58, 59]. The missense SNV observed in *EEFSEC* gene in the present study, classified as probably damage by SIFT and Polyphen, may contribute to decrease or impair the synthesis of these proteins, altering the

control of oxidative state of immune cells, which, in turn, affect inflammatory response of AgP patients [57].

The phenotypic impact of an SNV occurs due to several vias, as an altered gene expression, alteration in protein structure, or due to the combination of some alterations in a regulatory pathway. *In silico* analysis evaluated the 3D protein structure and only *EEFSEC* affected by [c.668C>T (p.Pro223.Leu)] revealed some major impact (in a linker region, preserving proteins domains). In fact, missense variations in *ZNF136* and *KRT25* did not impact on protein structure. However, when a PPI network was constructed, all the three missenses variations were linked in a major node – ubiquitin C (*UBC*). *UBC* was highly connected with non-neighboring genes, suggesting that it may be more relevant for AgP. UBC belongs to biological process of activation of MAPK activity, antigen processing and presentation of exogenous peptide antigen via MHC-I, and apoptotic process, which are well stablished processes in the pathogenesis of periodontitis. A recent study found *UBC*, together with two other genes (Jun proto-oncogene, and matrix metalloproteinase-14) as the most central and important genes for periodontitis, after interactome analysis [60]. Although important, this gene did not show any genetic alteration. Similarly, Chan et al. [61], who evaluated shared genes and molecular pathways for type 2 diabetes mellitus and cardiovascular disease, reported that critical regulators are rarely mutations target and those genome-wide candidate genes are mostly peripheral nodes in PPI networks. This fact support that genetic variants found in WGS and WES may serve as subtle modifiers of disease predisposition [61]. Indeed, genetic variants which have subtle effects are expected in complex disease, which is characterized by involvement of multiple causal components, which interplay with each other simultaneously, and therefore, a multitude of genes are most likely involved. Anyway, this in the first study using a PPI network construct in AgP etiology study, and, added to WES platform, new information could be achieved and investigated in future.

However, the most striking result of the present study was the frameshift indel in *GPRC6A* [c.2323-2324insT (p.Tyr775LeufsTer776)]. *GPRC6A* is a multiligand GPCR that is activated by cations, L-amino acids (L-arginine, L-lysine, and L-ornithine), steroid testosterone and osteocalcin [62]. Although recently discovered, increasing evidence suggest a role of this receptor in the regulation of osteoblast function, inflammation, metabolism, and endocrine functions [62-64]. The insertion of Thymine (T) nucleotide in 2110-2111 position (c.2110-2111insT) result in loss of all intra-cellular domain, as well as part of transmembrane region (due

to a stop codon creation), generating an altered protein structure resembled in 3D modeling. This finding suggests a damage role of this indel and a possible effect of AgP pathogenesis. Once *GPRC6A* is expressed by immune cells (like monocytes, macrophages, and B cells) some studies demonstrated a significant role of this receptor in inflammation [62, 65, 66]. Quandt et al. [66] showed an important function of *GPRC6A* in the regulation of TH_2 cytokine release, such as IL-4, IL-10, and IL-13, and consequently the humoral response after stimulation of B cells with *GPRC6A* agonists. Corroborating this finding, AgP patients present lower gingival crevicular fluid levels of IL-10 and IL-4 when compared to healthy and CP subjects [67, 68]. Further, *GPRC6A* was identified by GWAS as loci associated with circulating C-reactive protein (CRP), which is a general marker of systemic chronic inflammation [69]. Podzimek et al. [70] reported the highest CRP levels in AgP patients when compared to gingivitis or CP patients, and also showed the correlation of increased CRP levels with the severity of the periodontitis and bleeding on probing.

On the other hand, emerging information has determined a strong effect of *GPRC6A* in bone metabolism. Pi et al. [64] found an association between two SNPs in *GPRC6A*, the rs686708 and the rs571296, and reduction in spinal bone mineral density in Caucasians, demonstrating a possible contribution of *GPRC6A* polymorphisms in human osteopenia. Moreover, *GPRC6A* knockout mice (*GPRC6A*$^{-/-}$) also presented reduced bone matrix density. This impact of *GPRC6A* polymorphism/variant on bone was assessed in *in vitro* studies. Osteoblasts isolated from *GPRC6A* knockout mice (*GPRC6A*$^{-/-}$) presented reduced mRNA expression of important bone markers, such as osteocalcin, ALP, osteoprotegerin, and Runx2-II [64]. This impact on cell response could be linked to reduction in ERK-phosphorylation, once bone marrow stromal cells (MC3T3 cells) transfected with siRNA to reduce 50%-97% of *GPRC6A* presented reduced activation of phospho-ERK after stimulation by calcium. ERK (extracellular calcium-stimulated extracellular signal-related kinase) is an internal downstream player after *GPRC6A* activation and, its reduction, could explain the impact on bone-markers production by osteoblastic cells. This pathway agreed our results once all intra-cellular c-terminal tail is lost when indel in *GPRC6A* is present, blocking the signal transduction and cell functioning. Corroborating this idea, Pi et al. [71] inducing different variant in *GPRC6A* of β-cells observed that mutagenesis of Arg662, Phe666, Glu746, and Trp797 (locations close to observed in AgP subjects included in the present study) promoted alteration for binding Ocn, indeed resulted in reduced Ocn receptor activation *in vitro* and some of them leading to complete loss of functional responses to Ocn and Ocn-6aa-C but not L-Arg. Altogether, it is remarkable the role of *GPRC6A*

on bone metabolism and this reduced action seen in variant-cells could represent a new possibility to explain severe and fast bone resorption observed in AgP, resulting in a possible imbalance in apposition-resorption features during disease progression. Therefore, this new information, although well based on pre-clinical previous studies, should be further investigated. Up to date, some information in clinical trials indicated lower levels of osteocalcin in GCF of AgP subjects [72], what corroborate this idea.

Finally, while indel in *GPRC6A* showed an *in silico* impact on protein structure and possible deleterious effect, frameshift indel in ELN was not relevant. ELN is one of the major components of extracellular matrix component (ECM) and is degraded, as well as other components of ECM, by periodontopathogens enzymes and host matrix metalloproteinases (MMPs) during periodontitis progression [73]. However, the absence of a clear impact in 3D modeling, as well as in PPI network, associated to its several isoforms and splicing variants, could resemble its lower importance in disease occurrence.

As cited above, this is the first study assessing genetic variants in AgP through large-scale technique and by a familial approach, what could bring new information to a high non-consensus area in oral diagnosis. However, some points should be further assessed based on these findings. The involvement of specific genetics variants in AgP might be also analyzed through replication studies and functional analysis. Future studies need to validate the SNVs and indels candidates in an independent and large cohort with a candidate gene study approach and investigate the underlying mechanisms of them in AgP. Either way, the results of the present study raise important aspects. AgP is associated with polymorphisms in several genes, therefore it could suggest its polygenic disease status. Using a familial filtering approach and WES, the present study identify three missense SNVs in *EEFSEC* [c.668C>T (p.Pro223.Leu)], *ZNF136* [c.466C>G (p.Arg156Gly)] and *KRT25* [c.800G>A (p.Arg267His)] genes, and two indels in *GPRC6A* [c.2323-2324insT (p.Tyr775LeufsTer776)] and *ELN* [c.1366_1372instGGAGCAG (p.Ala457fs or p.Ala457SrfsTer37)] genes on this periodontitis phenotype. These affected genes are connected to each other, as observed in the PPI analysis, suggesting that different genetic variants in different genes may have similar effects if they affect a common pathway. Moreover, some genetic alterations reported in the present study lead to changes in the structure of some proteins, which participate in the regulation of inflammation, bone homeostasis and tissue injury, which may increase the susceptibility to AgP.

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Table 1 – Demographic and clinical characteristics of the study participants

Subject	Age (years)	Gender	PPD (mm)	CAL (mm)	FMPI (%)	FMBS (%)
F1-1 (AgP parent)	49	F	2.83	5.16	21.5	13.9
F1-2 (Non-AgP parent)	53	M	2.67	6.07	25.3	31.7
F1-3 (AgP proband)	29	F	3.46	5.85	17.3	24.3
F1-4 (Non-AgP sibling)	31	M	2.06	3.14	11.1	20.3
F2-1 (AgP parent)	56	F	3.1	5.49	22.1	25.2
F2-2 (Non-AgP parent)	60	M	2.48	5.60	35.3	17.5
F2-3 (AgP proband)	32	M	3.30	5.05	24.2	17.4
F2-4 (Non-AgP sibling)	28	M	2.04	3.3	19.5	21.4

Table 2. SNVs associated with AgP phenotype and in silico predictions.

Location	Gene	Protein	SNVs	NCBI (gene/protein)	Uniprot	Existing variation	Ensembl	SIFT (score)	PolyPhen (score)	Consequence
3:128264663-128264663	*EEFSEC*	eukaryotic elongation factor, selenocysteine-tRNA specific	c.668C>T (p.Pro223Leu)	NM_021937.4 NP_068756.2	P57772	rs142548867	ENSG00000132394	deleterious (0.01)	Possibly damaging (0.843)	missense
17:40751196-40751196	*KRT25*	keratin 25	c.800G>A (p.Arg267His)	NM_181534.3 NP_853512.1	Q7Z3Z0	rs72821893	ENSG00000204897	deleterious (0.02)	benign (0.329)	missense
19:12186844-12186844	*ZNF136*	zinc finger protein 136	c.466C>G (p.Arg156Gly)	NM_003437.3 NP_003428.1	P52737	rs574301770	ENSG00000196646	deleterious (0.03)	Probably damaging (0.935)	missense

Table 3. Indels (Frameshift variants) associated with AgP phenotype.

Location	Gene	Protein	Indels	NCBI (gene/protein)	Uniprot	Existing variation	Ensembl	Consequence
6:116792599-116792599	*GPRC6A* (isoform 1)		c.2323-2324insT (p.Tyr775LeufsTer776)	NM_148963.3 NP_683766.2	Q5T6X5-1	rs371464745	ENSG00000173612	frameshift_variant
6:116792599-116792599	*GPRC6A* (isoform 2)	G protein-coupled receptor class C group 6 member A	c.1798-1799insT (p.Tyr600LeufsTer600)	NM_001286354.1 NP_001273283.1	Q5T6X5-2	rs371464745	ENSG00000173612	frameshift_variant
6:116792599-116792599	*GPRC6A* (isoform 3)		c.2110-2111insT (p.Tyr704LeufsTer705)	NM_001286355.1 NP_001273284.1	Q5T6X5-3	rs371464745	ENSG00000173612	frameshift_variant
7:74057389-74057396	*ELN-201*	Elastin, transcript variant 13	c.1366_1372insGGAGCAG (p.Ala457fs or (p.Ala457SrfsTer37)	NM_001278939.1 NP_001265868.1 ENST00000358929	F8WAH6	-	ENSG00000049540	frameshift_variant

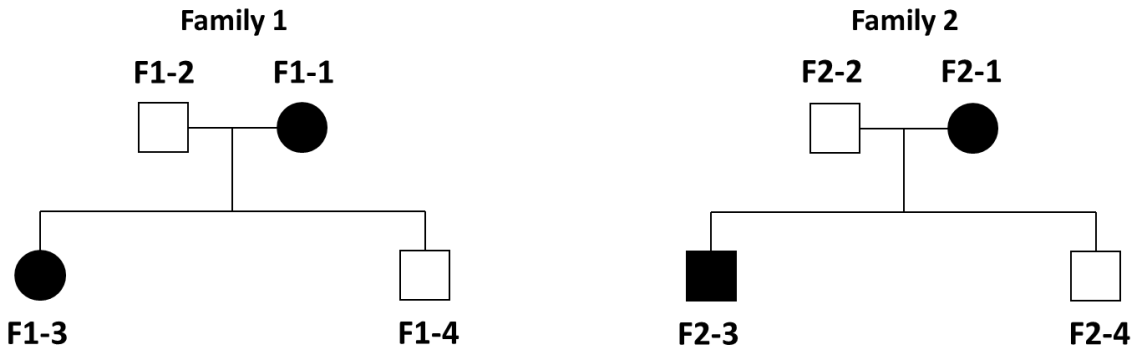


Figure 1 – Heredogram / Pedigree of the Study Families. Squares indicate male subjects, and circles female subjects. Affected individuals are denoted by filled symbols.

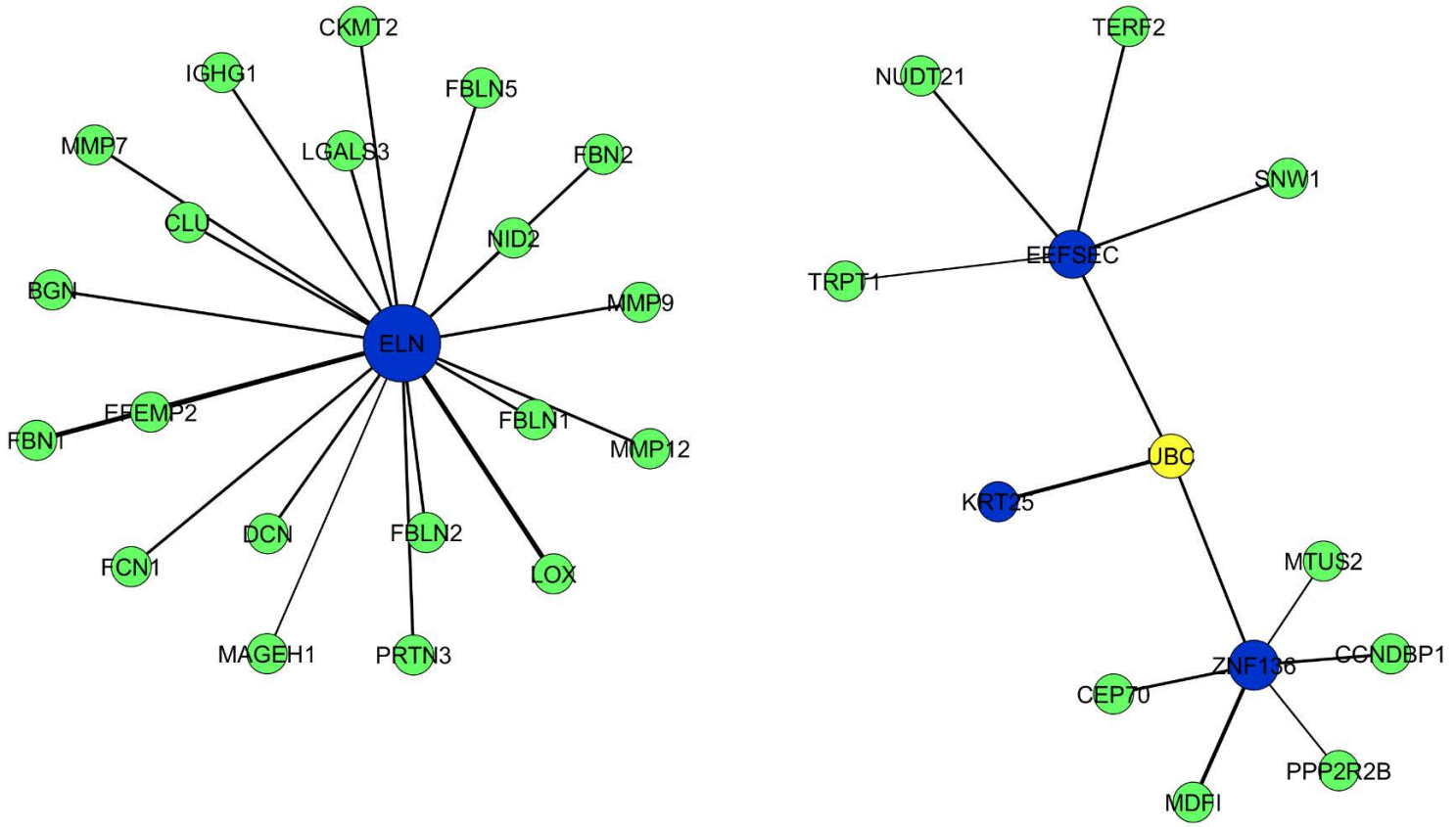


Figure 2 – Protein-protein interaction network including all single nucleotide variations and indels associated to AgP. Genes connecting components of the protein-protein interaction network harbor SNVs or indels among all AgP patients (blue circles, and red circles for the candidate genes discussed in the present study). Gene shaded yellow is bridge, linking well-connected regions of the PPI network.

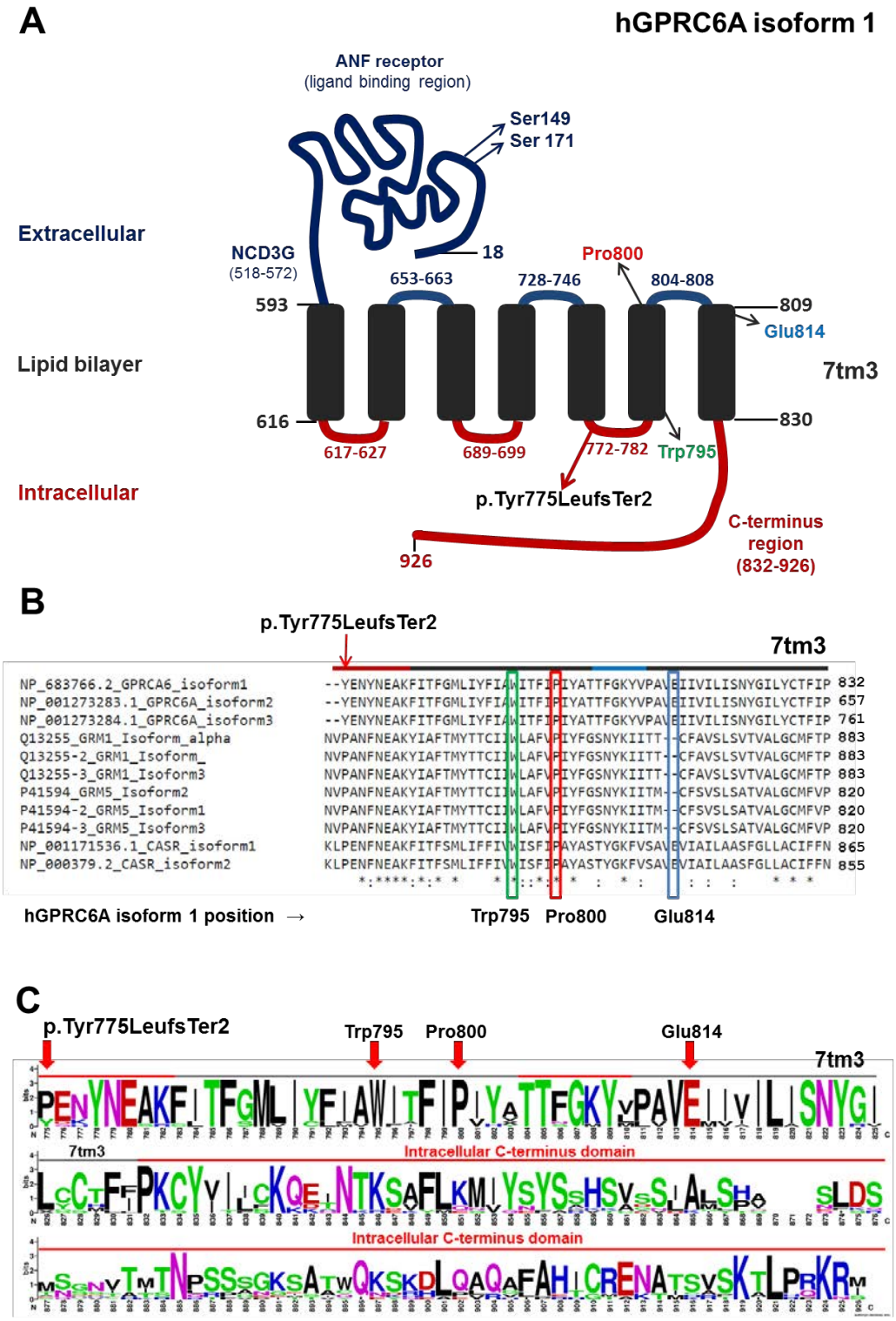


Figure 3 – A) Schematic illustration of the native human G-protein coupled receptor family C group 6 member A (hGPRC6A) encoded by GPRC6A gene (19-926). The domain organization of a GPRC6A protein sequence was based on functional annotation of Pfam domains, and include signal peptide (1-18 residues), ANF receptor (ligand binding region) (72-483 residues), Nine Cysteines Domain of family 3 GPCR (NCD3G, 518-572 residues), 7 transmembrane sweet-taste receptor of 3 GCPR (7tm3, 592-836 residues), low complexity region (758-770 and 852-870 residues). B) Multiple sequence alignment for hGPRC6A (isoforms 1, 2 and 3), CaSR (isoforms 1 and 2), mGluR-1 (isoforms 1, 2 and 3) and mGluR-5 (isoforms 1, 2 and 3)

were generated using Clustal Omega. The sequence comprising 775-783 residues are showed in the figure. A red arrow indicates the first amino acid changed position as a result of frameshift variant in GPRC6A isoforms [p.Tyr775LeufsTer2 (NP_683766.2), p.Tyr600LeufsTer2 (NP_001273283.1) and p.Tyr704LeufsTer2 (NP_001273284.1)]. B) protein sequence conservation analysis in GPRC6A related family members, showing the region subsequent to frameshift variation in hGPRC6A isoform 1 (775-832 residues), and which comprise sites important in calcium sensing by CASR (red boxed residues), binding site for calcimimetics in CASR (blue boxed residues) (Pi et al. J Biol Chem. 2005 December 2; 280(48): 40201–40209.) and site involved in response to Osteocalcin by GPRC6A (https://academic.oup.com/endo/article-lookup/doi/10.1210/en.2015-2010.). C) Graphical representation of an amino acid multi-species sequence alignment using a sequence Logo generator (WebLogo). The graphic represents protein sequence conservation (770-926 residues) of three isoforms of hGPRC6A and other 10 different species of mammalians and vertebrate.

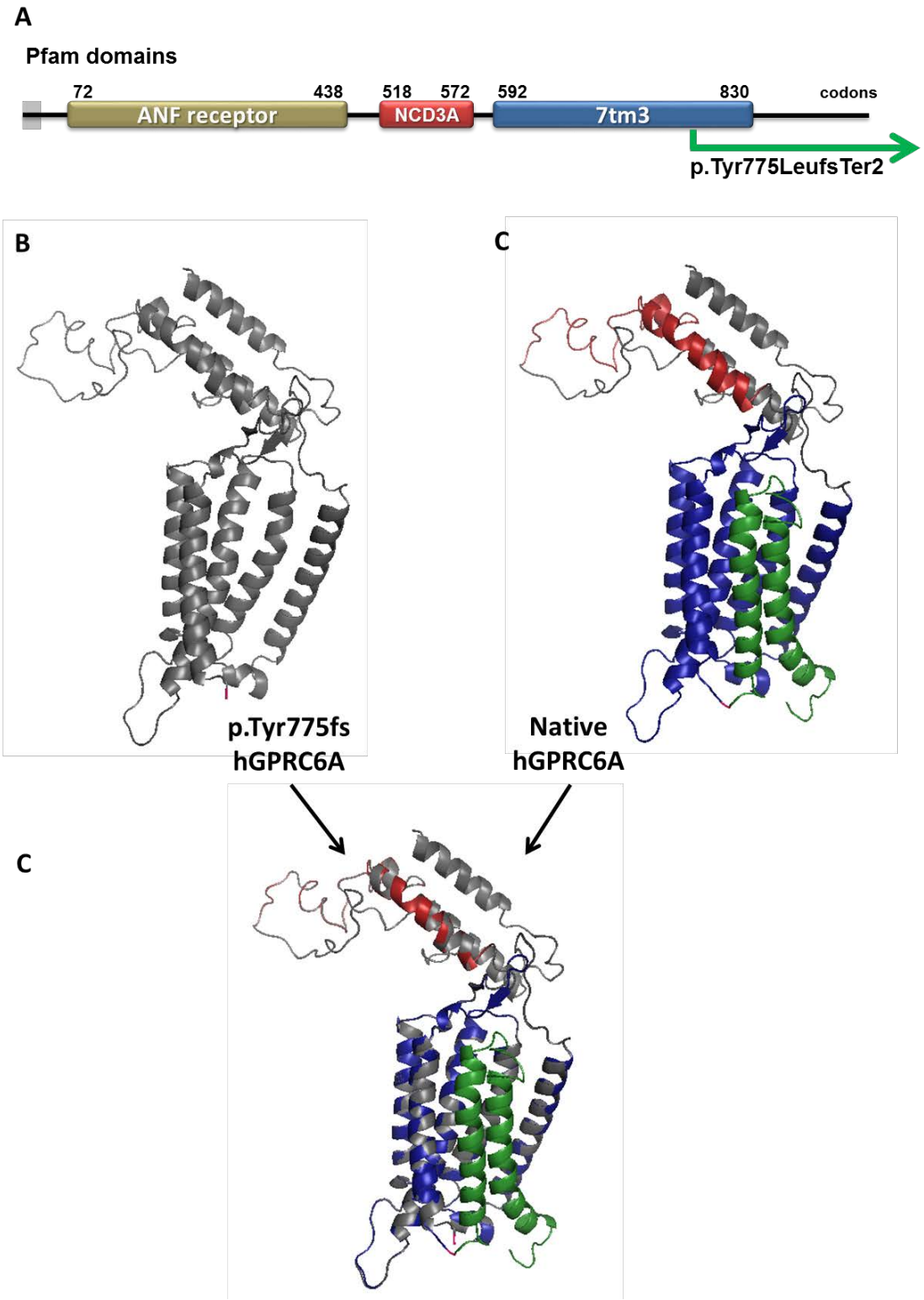


Figure 4 – Domain organization and 3D models analysis for hGPRC6A isoform 1. A) Domain for hGPRC6A based on Pfam functional annotation. B and C) Cartoon representation comprising de 441-841 residues, including the NCD3A domain, 7tm3 and part of C-terminus region of altered hGPRC6A (p.Tyr775LeufsTer2; in B) and native (in C) protein sequence. The ANF receptor domain in native hGPRC6A protein sequence was labeled in green, NCD3A domain is

labeled in red and 7tm3 is labeled in blue, like in figure A. The lost residues in the hGPRC6A mutant protein (p.Tyr775LeufsTer2, B) were highlighted in green in the native 3D model (C). D) Superimposed 3D models for the mutant (p.Tyr775LeufsTer2) and native hGPRC6A protein sequence, evidencing the α-helix and coil structures absent in hGPRC6A mutant (highlighted in green). The models were based on the crystal structure of the glutamate metabotropic receptor 1 (PDB: 4OR2). The models were aligned, visualized, and analyzed using the PyMOL software (PyMOL Molecular Graphics System, Version 1.7.4, Schrödinger, LLC).

Supplementary Table 1 - Primers used for PCR reactions

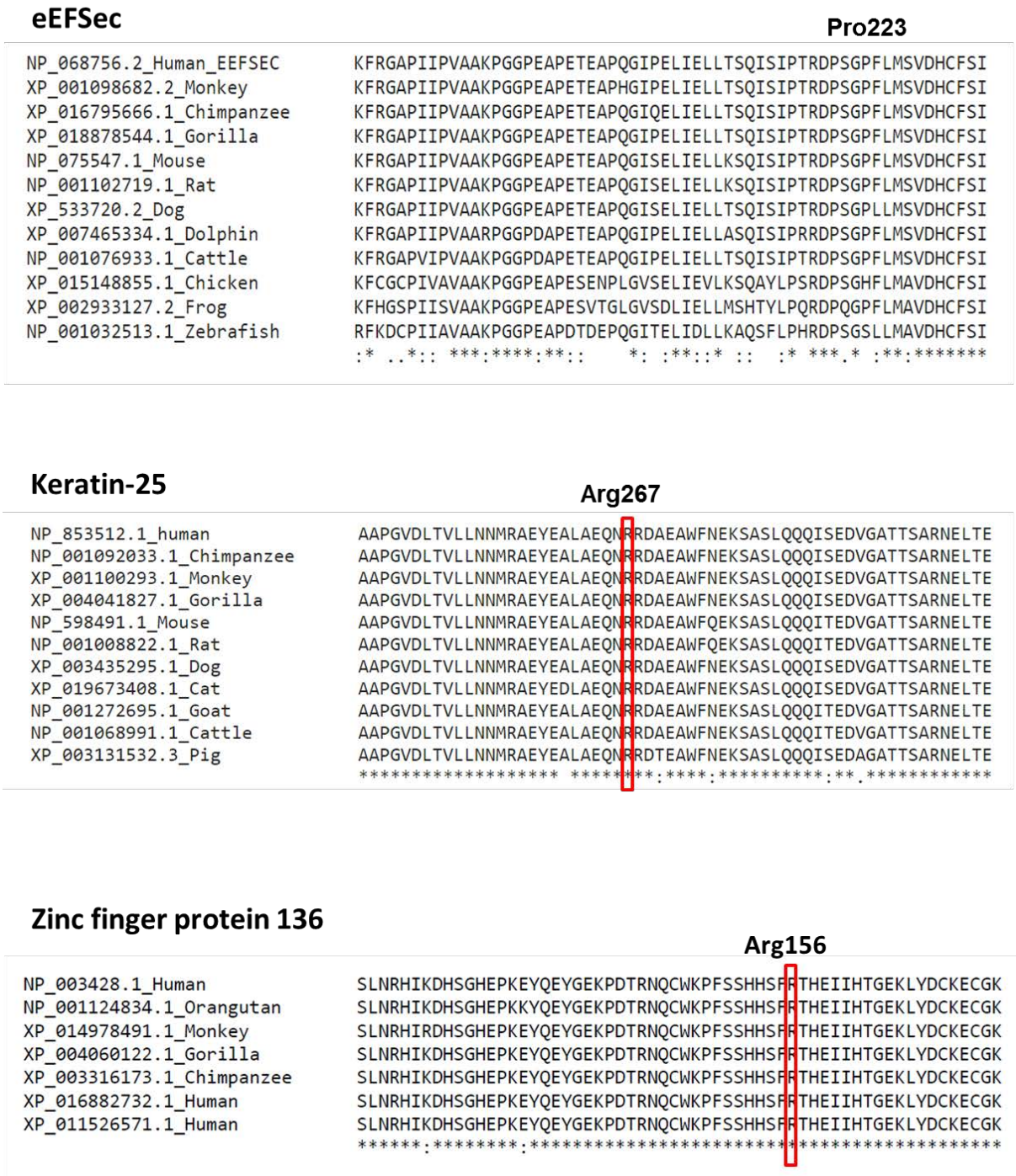
Primers		Sequence	Amplicon (pb)	Annealing temperature	N° Cycles
EEFSEC	F	GAATGCAGTCAGGGAGTTAG	376	61 °C	40
	R	CAGAAGGGAAGACAAGGTAAG			
KRT25	F	GGCTCCGACATCCTCAGA	390	58 °C	40
	R	AGGGGAAAACCAGACGAACT			
ZNF136	F	AAGTCAGCATGGGTCAGTCA	427	62 °C	40
	R	GGTCCATCTCCAGTGTGCTT			
GPRC6A	F	TTGCACACTCTGGCTAATC	654	59 °C	40
	R	GGAATGTGGCATCTCCTAAG			
ELN	F	GCTCAGTCTTCCCATTTGT	283	58 °C	40
	R	TCTCTTCTTCCTCCGATTCT			

Supplementary Table 2: Summary of total of reads, trimmed reads (by quality), and percentage of mapped reads against the human genome.

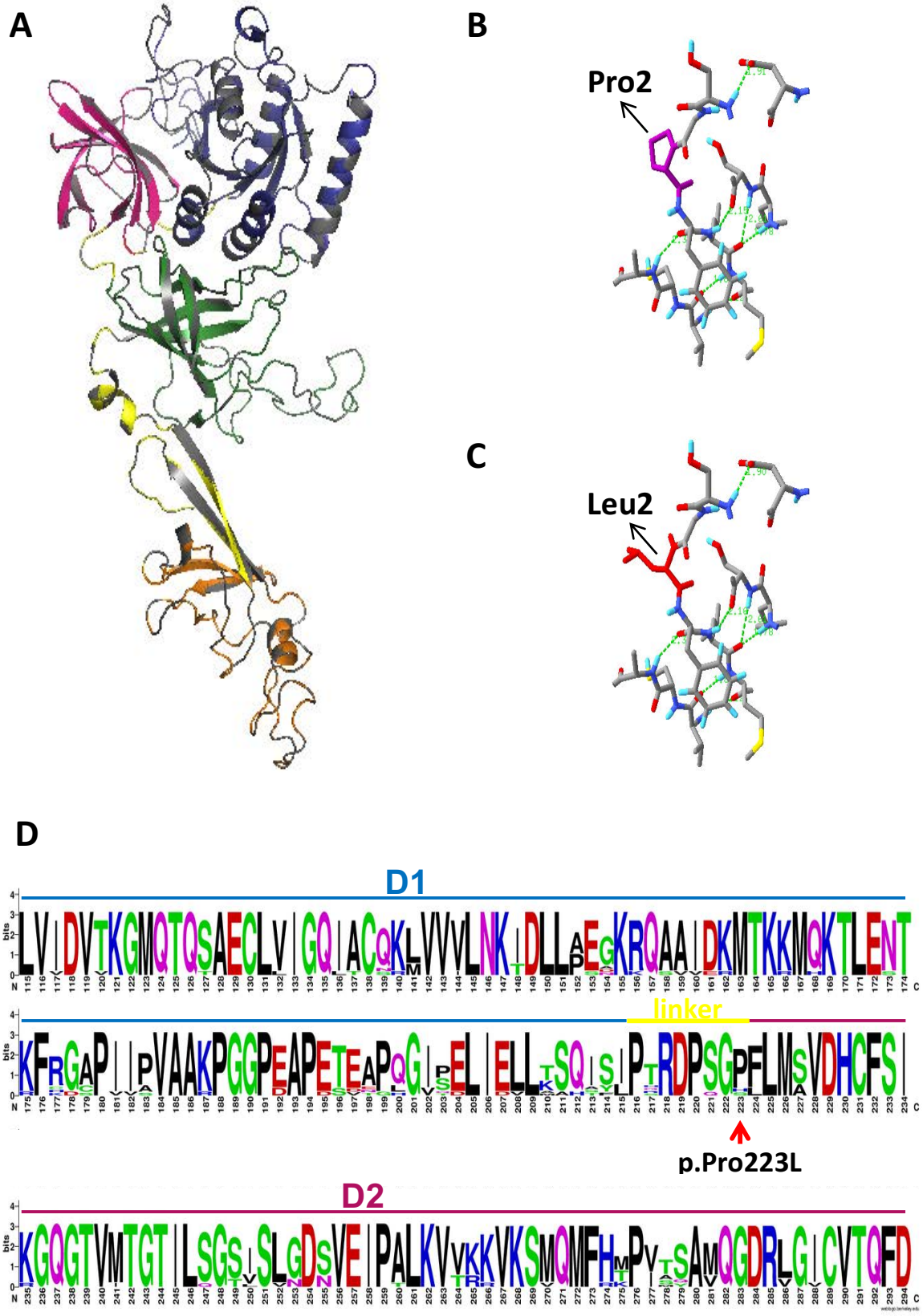
Samples	# total reads	# total trimmed reads	# trimmed reads R1	# trimmed reads R2	#total mapped	%total mapped	# properly paired	% properly paired
F1-1	57494820	57404762	28702381	28702381	56446598	98.18%	55862336	97.31%
F1-2	63766725	63669808	31834904	31834904	62606480	98.18%	61940554	97.28%
F1-3	64904056	64818598	32409299	32409299	63141085	97.28%	62566660	96.53%
F1-4	55804841	55722674	27861337	27861337	55624280	99.68%	55131312	98.94%
F2-1	63466090	63366716	31683358	31683358	62923829	99.15%	62182178	98.13%
F2-2	58649015	58547304	29273652	29273652	57899626	98.72%	57242454	97.77%
F2-3	58408061	58325078	29162539	29162539	57758939	98.89%	57162082	98.01%
F2-4	58329901	58238788	29119394	29119394	57909717	99.28%	57367590	98.50%
Total/Mean	480823509	480093728	240046864	240046864	474310554	98.67%	469455166	97.81%

Supplementary Table 3. Sample Coverage

Library	Total mapped reads	Read Size	Genome Size	Coverage
F1-1	4951805866	100	2937655681	**168.56**
F1-2	5688736807	100	2937655681	**193.65**
F1-3	6104727525	100	2937655681	**207.81**
F1-4	5111165153	100	2937655681	**173.99**
F2-1	5614582941	100	2937655681	**191.12**
F2-2	5227903789	100	2937655681	**177.96**
F2-3	5207314737	100	2937655681	**177.26**
F2-4	5276040785	100	2937655681	**179.60**
			Mean	183.74



Supplementary Figure 1. Multi-species sequence alignment for eEFSec (encoded by the EEFSEC), Keratin-25 (encoded by the KRT25) and Zinc finger protein 236 (encoded by the ZNF236) using generated Clustal Omega (European Bioinformatics Institute, EMBL-EBI; http://www.ebi.ac.uk/Tools/msa/clustalo/). The residues affected by missense variants (p.Pro223Leu, p.Arg267His, and p.Arg156Gly, respectively) are indicated by a red rectangle.



Supplementary figure 2 – Domain organization and 3D models analysis for eEFSec (human Selenocysteine-specific elongation factor). A) Superimposed 3D models from mutant

(p.Pro223Leu) and native eEFSec protein sequences (596 aa). Cartoon representations of the chalice-like structure of the human eEFSec composed of four domains: the N-terminal D1 (residues 1–215, Tr-type domain) labeled and colored in blue, D2 (residues 224–304) labeled and colored in magenta, D3 (residues 310–455) labeled and colored in green, and the C-terminal D4 (residues 477–575) labeled and colored in magenta in orange. Pro223 native and Leu223 mutant residues are highlighted in red (indicate by an arrow). Linker region (residues 469–476 and 576–582) and extreme C-terminus regions are colored in yellow. Localization of sites for phosphoserine (S537), Phosphothreonine (T545) and Omega-N-methylarginine (R556). Nucleotide binding region: P-loop (14GxxxxGKT21), the switch 1 (residues 32–47), switch 2 (92DxxGH96) and the guanine-binding sequence (146NKxD149). Nuclear localization signal (547-553 aa). The Pro residue affected by SNV (p.Pro223Leu), is located in the long loop (216-223 residues) connecting D1 domain with a β-barrel structure of the D2 domain, which is composed of 8 antiparallel b-strands and a flanking, short a-helix (Dobosz-Bartoszek, M. et al.). The models were based on the crystal structure of the Selenocysteine-specific elongation factor (PDB: 5izk.1.A, sequence identity > 99.83%). The models were aligned, visualized, and analyzed using the PyMOL software (PyMOL Molecular Graphics System, Version 1.7.4, Schrödinger, LLC). B and C) Stick models showing internal contacts for codon 223 in the eEFSec native (in B) and mutant (SNV: p.Pro223Leu in C) 3D models assessed by modeling tool Swiss-PdbViewer. No alteration in the internal contacts was predicted when Pro native residue (magenta stick) was replacement by Leu (red stick) at 223 amino acid position. Carbon (gray), Hydrogen (green), nitrogen (blue), oxygen (red), hydrogen bond (green). D) Graphical representation of an amino acid multi-species sequence alignment using a sequence Logo generator (WebLogo) for eEFSec. The graphic represent eEFSec protein sequence conservation, corresponding to 115-294 residues from eEFSec protein, and other 12 different species of mammalians and vertebrate. D1 domain (in blue) and D2 domain (in magenta) and the linker region (in yellow) are indicated in the figure. SNV position (codon 223) is indicated by a red arrow.

3.3 Transcriptome of healthy gingival tissue from edentulous sites in patients with a history of aggressive periodontitis*

Abstract

Background: This study evaluated the transcriptome of healthy gingival tissue from edentulous sites in patients with a history of generalized aggressive periodontitis (GAgP), chronic periodontitis (CP) and in patients with no history of periodontitis (H), using microarray and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis.

Methods: Healthy gingival tissue from edentulous sites was taken from GAgP (n =12), CP (n=12) and H (n=12) patients. Initially, total RNA from 4 tissues samples per group was employed in transcriptomic microarray analysis. Differential gene expression (fold-change), gene ontology (GO; biological process) and pathway analyses were performed. Genes that were differentially expressed and showing a significant role on altered pathways were validated by qRT-PCR analysis on 12 samples per group.

Results: In total, 270 probes sets and 50 GO groups were differentially expressed (up-regulated or down-regulated) between GAgP and H. Natural killer cell receptors and other genes related to the immune system were up-regulated in GAgP, while genes with functions in neural processes and in proliferation/differentiation of keratinocytes were under-expressed. There were 220 probe sets and 75 GO groups that were differentially expressed when comparing CP and GAgP. CP was characterized by the increased expression of genes related to responses to external stimuli and an under-expression of immune system-related genes. qRT-PCR analysis confirmed the microarray results, that KIR2DL4, IL6 and SELE were highly expressed in GAgP than CP or H patients.

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Transcriptome of healthy gingival tissue from edentulous sites in patients with a history of aggressive periodontitis

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The present study demonstrated significant differences in the gene expression profile of gingival tissue from edentulous sites from patients with a history of generalized aggressive periodontitis,

chronic periodontitis, and healthy patients. Aggressive periodontitis presented an overexpression of pro-inflammatory genes.

Abstract

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Conclusion: This study demonstrates differences in the transcriptome of healthy gingival tissue from edentulous sites from GAgP when compared with that of H or CP patients.

Introduction

Aggressive periodontitis (AgP) is characterized by an early age of onset, a high rate of disease progression and the absence of systemic diseases that compromise the host response to infection, which, if left untreated, can lead to early edentulism1. Patients with AgP seem to have a hyper-inflammatory response, with an imbalance in the release of pro- and anti-inflammatory cytokines in the gingival crevicular fluid or serum before and after periodontal therapy, or upon

activation of inflammatory cells by periodontopathogens2-4. Although the hyper-inflammatory response is considered an important feature of this disease, it is not well understood. However, it is recognized that inherent patient susceptibility influences the immune-inflammatory response, playing a large role in the progression of periodontal diseases5,6.

Previous studies have investigated gene expression profiles in periodontitis-affected tissues to clarify their pathogenesis and to identify the genes involved in these diseases7-11. Kebschull et al.8 identified an overexpression of genes related to immune responses, apoptosis and signal transduction, and an under-expression of genes related to epithelial integrity and metabolism in the periodontitis-affected gingiva of AgP patients. Moreover, Kramer et al.9 reported a pronounced induction of the natural- killer (NK) cell-mediated cytotoxicity pathway, especially due to a significant induction of the SLAM7 gene, a prominent activating receptor for NK cells, in periodontal pocket tissues from AgP-affected individuals.

However, although these studies identified some distinctive molecular patterns of AgP, to date it has been impossible to establish a clear biological basis for the distinction between the clinical phenotypes of this disease and chronic periodontitis (CP)9, 12-14. Once gingival gene expression is influenced by several factors, such as the presence of subgingival biofilm and tissue inflammation, the inclusion of diseased-sites does not allow constitutive biological differences between AgP and CP to be determined8, 15.

In fact, a previous study showed that the subgingival microbiota can be a determinant of gene expression in the adjacent gingival tissues of patients with periodontitis, independent of probing pocket depth15. In this study, Papapanou et al.15 demonstrated that even health-associated species (A. naeslundii and V. parvula) modulated the expression of genes related to immune system development, acute inflammatory response, antigen processing and presentation and other biological processes. Thus, gene expression profiles of gingival tissue adjacent to the periodontal pocket or gingival sulcus may reflect the inflammatory process activated by subgingival bacteria rather than biological differences between the different types of periodontitis. Furthermore, previous studies had always used healthy gingival tissues from individuals with periodontitis as controls, which had an impact on different disease comparisons, as gene expression is influenced by inherent or acquired characteristics7, 9.

In this sense, because several characteristics could mask possible constitutive alterations in tissue gene expression between different profiles of periodontal disease, the aim of the present study was to compare the gene expression profiles of patients with a history of

generalized aggressive periodontitis (GAgP) and CP with those of patients who had not experienced any destructive periodontitis, through transcriptomic analysis of healthy gingival tissues from edentulous sites.

Material and Methods

Participants

The study was designed in accordance with the STROBE Statement16, and was approved by the Ethics Committee of the University of Campinas (017/2010). Informed written consent was granted by each patient after explanations were provided. A total of 36 patients were recruited from among patients referred for implant placement to the Graduate Clinic of Piracicaba Dental School, University of Campinas, Piracicaba, Brazil, between March 2011 and January 2012. Patients with a history of GAgP (n=12) or CP (n=12), previously treated by our team of periodontists and who had received regular supportive periodontal therapy (SPT) for at least one year following active periodontal therapy, presenting full-mouth plaque index (FMPI)17, and a full-mouth bleeding score (FMBS)18 less than 30% were selected.

GAgP and CP were treated in previous studies19-22. At the first examination, before periodontal therapy, the diagnosis of GAgP and CP was based on the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions23, 24. Furthermore, these patients needed the presence of at least 20 teeth, with at least 8 teeth presenting probing pocket depth (PPD) ≥5mm with bleeding on probing (BOP) (having at least 2 with PPD ≥7mm), <35 years of age for GAgP, and >35 years for CP. All patients received comprehensive periodontal therapy, which included careful oral hygiene instructions combined with non-surgical periodontal therapy. AgP patients received amoxicillin plus metronidazole for 7 days, or the local application of 10% povidone iodine (PVPI) adjunctive to non-surgical therapy, at least one year before tissue collection. In the SPT, sites presenting PPD ≥5mm and BOP received additional scaling and root planning. Residual pockets (PPD≥5mm) associated with furcation or intrabony defects received periodontal surgery and/or regenerative approaches. None of the teeth adjacent to the biopsy site received regenerative procedures. None of the periodontitis patients presented sites with PPD ≥5mm with BOP at the implant surgery. Patients with no history of periodontitis were also enrolled (H; n=12), following the same criteria as previous studies (i.e. free of interproximal attachment loss and having no PPD of >4mm)25,26. To prevent “not yet CP affected” patients, healthy patients were ≥35 years.

Additional inclusion criteria were as follows: non-smoking status, no use of systemic antibiotics or anti-inflammatory drugs in the previous six months, no systemic diseases that affect the healing process (e.g., diabetes) or any cardiovascular disease that would contraindicate any surgical procedure, and not pregnant or lactating. The following clinical parameters were evaluated at six sites per tooth using a manual probe§ guided by an acrylic stent in all participants before tissue collection: FMPI, FMBS, PPD and relative clinical attachment level (rCAL) at six points around each tooth.

Collection of tissue samples

Healthy gingival tissue samples were obtained from posterior maxillary or mandibular edentulous sites at the moment of implant placement, at least 2mm distant from the adjacent teeth, preventing the influence of the subgingival microbiota27-29. The teeth adjacent to tissue collections (mesial and distal) did not present PPD >3mm, BOP and plaque accumulation. Furthermore, the patients did not use fixed or removable dental prosthesis in the tissue collection area. GAgP and CP patients presented tooth loss due to periodontal disease, while H patients presented tooth loss due to caries, trauma or endodontic reasons. After local anesthesia (Articain 4% with 1:100.000 epinephrine), a crestal incision was made, following by two parallel incisions in the direction of lingual surfaces of the edentulous alveoli, and a tissue sample of approximately 3x3mm was carefully dissected, comprising the oral epithelium and connective tissue. After collection, the tissue samples were rinsed with sterile saline solution, and immediately stored overnight in an RNA stabilization reagent|| at 4oC. Samples were then stored at -80oC for subsequent RNA isolation.

Isolation of total RNA from tissue samples

Tissue specimens were disrupted by a motor pestle and total RNA from the samples was isolated using a solution for RNA extraction¶ following the manufacturer's instructions. The remaining DNA was removed using deoxyribonuclease.# RNA was quantified spectrophotometrically using a spectrophotometer device.** Before microarray and quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments, the quality control of total RNA samples was performed using an automated electrophoresis instrument.†† All samples had RNA integrity values above 8, and were used for further analysis.

Double-stranded cDNA synthesis and microarray hybridization

Initially, total RNA from four randomly chosen tissue samples per group (GAgP = 4; CP = 4; H = 4) was used for microarray analysis. Double-stranded cDNA was synthesized from 5 ẖg of total RNA using a cDNA synthesis kit‡‡, and then the cDNA was purified using a PCR product purification Kit‡‡ according to the manufacturer's instructions. Each cDNA sample was labelled, and then hybridized to a human gene expression array§§ covering 45,033 human transcripts. The array was scanned at 2-μm resolution using a microarray Scanner.||||

Microarray data analysis

A software package‡‡ was used to extract fluorescent intensity signals from the scanned images. The data were analyzed using the Oligo package for the analysis of genomic data in the software R and Bioconductor30. Expression data were normalized and summarized using the Robust Multi-Array (RMA) analysis.

Comparison analyses were then used to differentiate the real changes in gene expression levels between the GAgP and H, and between the CP and H. Differential expression analysis was performed by calculating the fold-change and the t-test of the empirical Bayes method using the Limma package31. Genes showing at least 2-fold changes in expression and a p-value ≤ 0.05 were identified as differentially expressed32. After this, to compare the differential gene expression between GAgP and CP, only those genes differentially expressed in CP-H and GAgP-H comparisons were considered for analysis. This step allowed the identification of genes that were differently expressed between CP and GAgP by excluding genes that were commonly expressed when compared with H individuals. Enrichment analysis of the data was then performed using the Database for Annotation, Visualization and Integrated Discovery 6.7 (DAVID)33,34, using the functional annotation tool for gene ontology (GO) to predict biological processes for identified transcripts (GOTERM BP ALL), and pathway analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The GO biological process database provides controlled terms describing a series of events accomplished by one or more organized assemblies of molecular functions, to identify groups of genes with similar biological functions. Pathway analysis links a set of genes based on the molecular interaction in the cell, such as a pathway or complex, representing a higher-order biological function35. Enriched GO terms and pathways with a significant p-value ($p < 0.05$) generated by a modified Fisher's exact test followed by the Bonferroni test were considered in the results.

qRT-PCR

In order to confirm the results generated by microarray analysis, total RNA from 36 tissue samples (GAgP = 12; CP = 12; H = 12) was used for qRT-PCR. Four genes that were part of important enriched biological process and pathways in GAgP when compared to H and CP were selected. cDNA synthesis and qRT-PCR were performed as previously described by Casarin et al.36. Primers for killer cell immunoglobulin like receptor, two Ig domains and long cytoplasmic tail 4 (KIR2DL4), interleukin-6 (IL6), interleukin-1β (IL1B), selectin E (SELE) and GAPDH are presented in supplementary table 1.

qRT-PCR Statistical Analysis

All qRT-PCR experiments were performed in duplicate. Mean and standard deviation were obtained to establish statistical comparisons. The statistical analysis was performed using statistical software.¶¶ Data were first examined for normality by Shapiro-Wilk test. Data comparison was performed using one-way ANOVA, and a pairwise multiple-comparison test (Tukey test). The level of significance was set at 5%.

Results

Patient characteristics

No differences were observed between groups regarding clinical parameters. The mean age of GAgP patients was 32.25 ± 5.85 years, which was statistically younger than that of CP patients (51.75 ± 3.30 years old). However, there was no difference in age between GAgP and H patients, or between CP and H patients (Table 1). None of the periodontitis patients presented sites with PPD ≥5 mm, and there is no statistical difference in % of sites with PPD = 4 mm and PPD ≤3 mm between the groups. There were no statistical differences between the groups regarding region and arch of the sample collection, as well as in PPD, rCAL and presence of plaque or BOP in the adjacent teeth (Table 1).

Microarray Analysis

The microarray data were processed according to Minimum Information About a Microarray Experiment guidelines (MIAME), and the data set was deposited in Gene Expression Omnibus (GSE79705). The complete list of differentially regulated genes can be viewed in Supplementary

Table 2. Data from the CP-H comparison were used only for adjustment and are not shown in this manuscript.

We first compared the transcriptome of GAgP and H patients. This analysis showed that the levels of 94 transcripts were increased, and those of 176 were decreased in GAgP in relation to H (Supplementary Table 2). Twenty selected up-regulated and down-regulated genes are shown in Table 2. Several constituents of NK cells, such as killer cell immunoglobulin-like receptors (KIR2DL1/CD158A, KIRSDL2/CD158B1, KIR2DL3/CD158B2, KIR2DL4/CD158D, KIR3DL1/CD158E1, KIR3DL2/CD158K/ KIR3DL3/CD158Z, KIR2DS1/CD158H, KIR2DS2/CD158J, KIR2DS3/NKAT7, KIR2DS4/CD158I) and killer cell lectin-like receptors (KLRC1/CD159A, KLRC2/CD159C, KLRC3/NKG2E, KLRC4/NKG2F and KLRD1/CD94), and other genes, such as leukocyte immunoglobulin-like receptor A (LILRA5), Fc fragment of IgG receptor Ia (FCGR1A–CD64), which play an important role in the regulation of the immune response, were identified as up-regulated genes. In contrast, genes related to cellular growth, development and division, and differentiation and cornification of keratinocytes, such as caspase 14, apoptosis-related cysteine peptidase (CASP14), early growth response 3 (EGR3), and calmodulin-like 5 (CALML5), and genes involved in neural processes, such as internexin neuronal intermediate filament (INA), neuroligin 4, Y-linked (NLGN4Y) and 5-hydroxytryptamine receptor 3A (HTR3A), were found to be down-regulated.

GO analysis identified seven significantly up-regulated groups in the GAgP-H comparison, including cellular defense response, immune response, immune system process, defense response, response to stimulus and natural killer (NK) cell activation. Meanwhile, 43 groups, including neurotransmitter transport, nervous system development, development processes and multicellular organismal development, were found to be down-regulated (Figure 1 for the 10 top GO groups, Supplementary Table 2 for the complete list). Notably, three pathways were differentially overexpressed in GAgP: antigen processing and presentation, NK cell-mediated cytotoxicity and graft-versus-host disease. One pathway was down-regulated: endocytosis (table 3).

The comparison between CP and GAgP, after filtering for commonly expressed genes in the CP-H and GAgP-H comparison, showed that 103 transcripts were more highly expressed and 117 were expressed at lower levels in CP than in GAgP (Supplementary Table 2). A list of 20 selected up-regulated and down-regulated genes are shown in Table 4. Genes related to cellular growth, development and division and genes for epithelial components, including psoriasis susceptibility

1 (PSORS1C1), caspase 14 (CASP14), and early growth response 3 (EGR3) were found to be more highly expressed in CP. On the other hand, genes encoding the pro-inflammatory cytokines, interleukin-6 (IL6) and interleukin-1β (IL1B), and selectin E (SELE), an immunoadhesion molecule found in cytokine-stimulated endothelial cells, were detected as genes expressed at lower levels in CP (or inversely, at higher levels in GAgP).

GO analysis identified 19 groups that were more highly expressed in CP than in GAgP, including epidermis and ectoderm development, tissue development, phospholipid catabolic processes and leukotriene metabolic processes. In contrast, 56 groups were expressed at lower levels in CP, including immune response, immune system processes, inflammatory response, biological and cell adhesion (figure 1 for top GOs, supplementary table 2 for complete list). Furthermore, pathway analysis identified one differentially overexpressed pathway in CP: arachidonic acid metabolism, and three under-expressed pathways in CP: hematopoietic cell lineage, cell adhesion molecules and the cytosolic DNA sensing pathway (Table 3).

qRT-PCR

To validate the microarray gene expression pattern, four genes of important enriched GO and pathways in GAgP were selected. KIR2DL4 is part of NK cell mediated cytotoxicity pathway, and of NK cell activation biological process. SELE is part of cell adhesion and cell-cell adhesion biological process, and cell adhesion molecules pathway. Il1B and IL6 are part of immune response. KIR2DL4 and IL6 were more expressed in GAgP when compared with both H and CP (Figure 2). SELE was significantly down-regulated in CP when compared with GAgP and H ($p < 0.05$). These results were similar to those obtained by microarray analysis. However, no statistically significant differences were found in the IL1B mRNA levels between the groups, in contrast with the results of microarrays analysis.

Discussion

In the present study, we hypothesized that patients with AgP would show constitutive differences in gene expression profiles, i.e., under healthy conditions, and without the direct influence of the biofilm, these patients could present “physiologic” alterations when compared with patients with a history of CP and H individuals that could explain the aggressive phenotype. To date, previous studies only investigated the transcriptome of tissue adjacent to periodontal pocket or gingival sulcus to finding biological differences responsible for the periodontitis, and

differences between CP and AgP. However, this analysis could be influenced by several factors, mainly biofilm constitution and tissue inflammation. Thus, the present study is the first to assess the differences of transcriptome of gingival tissues from edentulous sites, to avoid the influence of biofilm. Our findings demonstrate significant differences in gene expression in GAgP versus both CP and H patients, with overexpression of pro-inflammatory genes in GAgP.

The principal finding of this study was the significant increase in gene expression of the NK cell-mediated cytotoxicity pathway in GAgP when compared with the H group. NK cells are lymphocytes that have cytotoxic effects against tumor and virus-infected cells without previous activation. NK cells have been recognized as major producers of cytokines, under many physiological and pathological conditions, and they can regulate other inflammatory cells and also antigen-specific T- and B-cell responses37. In the present study, killer cell immunoglobulin-like receptors (KIRs) and killer cell lectin-like receptors (KLRs) were the major genes up-regulated in GAgP relative to H individuals.

KIRs and KLRs are the two major classes of Human Leukocyte Antigen class I-binding NK receptors, and can be divided into two forms: inhibitory and activating receptors38. Activating receptors detect self-molecules induced under cellular stress or infection, while inhibitory receptors of NK cells bind to the ligands of the target cells, preventing NK-cell activation and cytotoxicity in normal cells. In the present study, we found both activating and inhibitory receptors to be up-regulated in GAgP. The role of NK cells in periodontitis is not completely known39. Although some studies did not show any relationship between periodontitis and the NK cells, other studies have found a correlation between them and periodontal status39. Moreover, in previous studies, some of these activating and inhibitory receptors were found to be more expressed in inflamed gingival tissues of experimental gingivitis26, CP and AgP7,9,35, and ageing healthy gingival tissue of monkeys40, suggesting their role in periodontal disease.

Interestingly, NK cells, which express a high number of inhibitory receptors, were not reactive to normal cells (due to interactions of their inhibitory receptors with MHC-I on normal cells)37,41,42. However, some studies demonstrated that, once activated by an infection or for other reasons, these cells can present a greater responsiveness, such as higher cell degranulation and cytokine production37,41,42. In addition, at sites of infection, there are several cellular changes that can result in the activation of activating receptors, which were also found to be more expressed in GAgP in the present study. Thus, the increase in expression of both kinds of

receptors may indicate an imbalance in NK cell response, a phenomenon that could contribute to the hyper-inflammatory profile observed in aggressive periodontitis2-4,43,44.

This hyper-inflammatory characteristic was also observed when GAgP and CP were compared. Several genes linked to inflammatory response, immune system process and as response to wounding were up-regulated in GAgP (for example, IL-6, leukocyte immunoglobulin-like receptor A, Fc fragment of IgG-Ia and Fc-gamma receptor I B2). Interestingly, Kebschull et al.8 reported the overexpression of similar genes and biological processes in diseased gingival tissues of AgP when compared with CP (for example, FC receptor like-5, IL-6 and other genes related to apoptosis and immune response). It is important to highlight that in the present study, only those genes commonly altered in the CP-H and GAgP-H comparisons were considered in the data analysis, giving more strength to CP and GAgP comparison. Overall, the findings suggest that GAgP patients have a pro-inflammatory profile, even in the healthy gingival tissue from edentulous site. This could represent an at-risk-for-harm response to biofilm accumulation, altering the host response, which could be linked to the well-known severe and fast periodontal breakdown occurring in AgP. Furthermore, this corroborates the idea that CP and AgP may have a distinct pathobiology. Moreover, another important aspect is the fact that, although some previous studies also indicate the different expression of these genes, this natural alteration has never been considered and should be included when conclusions are drawn.

Moreover, GAgP was characterized by the under-expression of genes involved in proliferation, differentiation and cornification of keratinocytes, when compared with both CP and H groups. These biological processes and pathways have already been found to be down-regulated in inflamed gingival tissues of AgP when compared with CP and healthy patients by previous studies8,45. Kebschull et al.8 reported that genes linked to supporting epithelial integrity, epidermis development and ectoderm development (desmocollin 1, laminin γ2, keratin 2) were underexpressed in AgP. Similarly, Guzeldemir-Akcakanat et al.45 also identified a significant decrease in the expression of desmocollin-1 in these patients. Interestingly, a lower expression of genes related to epithelial development and adhesion reported in these previous studies was correlated with phenotype and disease activity7,45,46. These findings in healthy gingival tissues of GAgP patients may represent a disruption of tissue homeostasis, and could have an impact on the clinical development of the disease. Since these results are observed in the absence of tissue inflammation and direct influence of biofilm, it is possible to speculate that these differences in gene expression comes from genetic polymorphisms presented by subjects,

epigenetic alteration, or represent a state of continuous remodeling/late active response after periodontal therapy, or a combination of them.

A previous study reported that some polymorphisms (rs2069827 and rs2069825) influence local and systemic IL-6 responses after periodontal therapy47. There is also evidence that some cytokine genes, like IL-6, are hypomethylated in tissues with periodontitis, which predisposes to its overexpression. On the other hand, the overexpression of IL-6 might exert an epigenetic influence on tissues, promoting changes in the regulatory mechanism of gene expression48. In addition, the gingival tissue of patients with periodontitis could present a continuous process of remodeling or active response after periodontal treatment. In this sense, Beikler et al.49 reported the activation of pathways that regulate tissue damage and repair in sites with residual pockets after a short period of periodontal therapy. However, the relationship between healthy tissue alterations and genetic/epigenetic factors and the "residual effect" of periodontal disease/therapy should be studied in future studies.

Furthermore, GO analyses demonstrated that genes involved in neural process were down-regulated in GAgP when compared with H patients. Offenbacher et al.50 reported that genes of neural activation pathways, which were up-regulated in the induction phase of experimental gingivitis, were linked broadly to vasculature, wound healing and epithelial tissues. Interestingly, in our study, genes linked to neural and epithelial processes were down-regulated in GAgP. Moreover, Davanian et al.10 found that some genes linked to the neuroactive ligand-receptor interaction pathway were uniquely expressed in healthy gingival tissue, but not in periodontitis-affected tissue samples. Thus, these genes may be involved in the homeostatic mechanisms of healthy gingival tissue, and future studies should also include an investigation of these genes to clarify their roles in periodontal disease.

Meanwhile, some points should be considered in the interpretation of the results of the present study. The number of samples analyzed is small, although similar to previous studies11,25,35. To compensate the small sample and to validate microarray gene expression patterns, qRT-PCR evaluated four genes of important GOs and pathways in GAgP in a larger sample. The qRT-PCR results agreed quite well with microarray results. The groups presented differences regarding age, which is expected due GAgP's characteristics, and that may influence the transcriptome. Gonzales et al40 reported differences in gingival transcriptome between aged and younger monkeys. Interestingly, ageing gingival tissue presented up-regulation of NK receptors, which were more expressed in GAgP in the present study. Furthermore, this study

revealed significant difference in the gene expression profile between GAgP, CP and H patients in a specific condition, the healthy gingival tissue from edentulous sites. To clarify the temporal sequence of molecular events involving these genes and pathways in AgP pathogenesis, other clinical conditions, from gingival tissue with subclinical inflammation, gingivitis, gingival tissue associated to periodontal pocket before and after periodontal therapy should be evaluated. Moreover, some genes still demand more studies to identify their role in periodontal destruction and, in the future, shed new light on the etiopathogenesis, treatment and prevention of aggressive periodontitis.

Conclusion

In conclusion, the present study demonstrates inherent differences in the gene expression profiles of healthy gingival tissues from GAgP patients when compared with those of H or CP, remarking on the differences in pathogenesis between them.

Footnotes

§ PCPUNC 15®, HuFriedy, Chicago, IL, USA.

|| RNAlater, Ambion Inc., Austin, TX, USA.

¶ Trizol Reagent, Ambion Inc., Austin, TX, USA.

Turbo DNA-free, Ambion Inc., Austin, TX, USA.

** Nanodrop 2000, Thermo Scientific, Wilmington, DE, USA.

†† 2100 Bioanalyzer Instrument, Agilent Technologies, Palo Alto, CA, USA.

‡‡ Roche Diagnostics, Mannheim, Germany.

§§ NimbleGen Human Gene Expression 12X135K Arrays, Roche Diagnostics, Mannheim, Germany

|||| NimbleGen MS 200 Microarray Scanner

¶¶ BioEstat 5.0 Software, Instituto Sustentável Mamirauá, Belém, PA, Brazil

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Private Reviewer Link – Gene Expression Omnibus

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gzynamkofzwphqr&acc=GSE79705)

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Figure Legends

Figure 1. Gene Ontology analysis. Biological process of probe sets differentially expressed in healthy gingival tissues from GAgP, CP and healthy patients, generated by DAVID software using GOTERM_BP_ALL databases. Gene ontology (GO) terms are represented as different color wedges in the pie charts, with the number of probe sets per group shown in parentheses. *p*: p-value.

Figure 2. mRNA transcript levels (mean ± standard deviation), relative to the *GAPDH*, for *KIR2DL4*, *IL1B*, *SELE* and IL6. Statistical analysis was performed using ANOVA/Tukey tests.

Supplementary files

Supplementary Table 1. Primers used for qRT-PCR reactions

Supplementary table 2. Differentially expressed probes sets, biological process and pathways.

Reviewer access link – Gene Expression Omnibus
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gzynamkofzwphqr&acc=GSE79705

Table 1. Patient characteristics, full-mouth clinical parameters (mean ± standard deviation), and clinical parameters (mean ± standard deviation) of the adjacent teeth surrounding sample collection site and region of sample collection.

Characteristics	GAgP	CP	H	p-value
General and full-mouth clinical parameters				
Age (years)	32.25±5.85 A	51.75 ± 3.30 B	46.25 ± 11.44 AB	0.0153
FMPI (%)	23.10±6.53	17.05 ± 8.42	19.36 ± 5.38	0.2696
FMBS (%)	24.83±9.05	23.53 ± 7.69	19.39 ± 2.31	0.5428
PPD (mm)	2.35±0.05	2.28 ± 0.27	2.13 ± 0.23	0.3504
rCAL (m)	5.44±1.04	5.44 ± 0.90	5.15 ± 0.72	0.8729
PPD ≥ 5 mm [n (%)]	0 (0)	0 (0)	0 (0)	
PPD = 4 mm [n (%)]	7.7 ± 4.1 (5.5 ± 2.8)	7.0 ± 4.9 (5.0 ± 3.7)	3.2 ± 5.2 (2.4 ± 3.7)	0.4045
PPD ≤ 3 mm [n (%)]	131.7 ± 25.0 (94.5 ± 2.8)	128 ± 16.9 (95.0 ± 3.7)	137.7 ± 16.2 (97.6 ± 3.7)	0.7887
Adjacent Teeth				
Plaque (%)	0	0	0	n.s
BOP (%)	0	0	0	n.s
PPD (mm)	2.24 ± 0.27	2.17 ± 0.35	2.06 ± 0.12	0.6668
rCAL (mm)	5.67 ± 0.97	5.75 ± 0.62	4.31 ± 1.04	0.0847
Sample Region				0.8945
Premolar (n)	6	5	6	
Molar (n)	6	7	6	
Dental Arch				0.9693
Maxilla (n)	6	6	5	
Mandible (n)	6	6	7	

Different letters indicate statistical difference by one-way ANOVA / Tukey test

Table 2. Top 20 differentially expressed probes sets in aggressive periodontitis relative to healthy patients

GenBank	Gene Name	Gene Symbol	FC	p
Up-regulated				
NM_182596	hypothetical protein FLJ25037	*FLJ25037*	8.226	0.003
NM_173508	solute carrier family 35, member F3	*SLC35F3*	6.099	0.015
BC068238	hypothetical LOC554223	*LOC554223*	3.572	0.038
NM_024690	mucin 16, cell surface associated	*MUC16*	3.519	0.028
U73394	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4	*KIR2DL4*	3.223	0.016
XM_931396	hypothetical protein LOC643194	*LOC643194*	3.202	0.003
L76661	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	*KIR3DL1*	3.138	0.012
NM_007328	killer cell lectin-like receptor subfamily C, member 1	*KLRC1*	3.083	0.029
NM_014513	killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 5	*KIR2DS5*	3.075	0.015
BC012550	killer cell lectin-like receptor subfamily C, member 1	*KLRC1*	2.925	0.020
BC032422	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	*KIR2DL3*	2.897	0.022
NM_014511	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	*KIR2DL3*	2.855	0.024
NM_013289	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	*KIR3DL1*	2.818	0.007
NM_001956	endothelin 2	*EDN2*	2.800	0.027
NM_020535	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 5A	*KIR2DL5A*	2.760	0.018
BC017784	killer cell lectin-like receptor subfamily C, member 4	*KLRC4*	2.753	0.017
L76667	killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2	*KIR2DS2*	2.700	0.012
NM_015868	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3	*KIR2DL3*	2.689	0.016
NM_002259	killer cell lectin-like receptor subfamily C, member 1	*KLRC1*	2.670	0.021
AF461157	killer cell lectin-like receptor subfamily C, member 3	*KLRC3*	2.670	0.011
Down-regulated				
BC104765	early growth response 3	*EGR3*	-6.364	0.002
NM_003411	zinc finger protein, Y-linked	*ZFY*	-6.333	0.036
BC110817	chromosome Y open reading frame 15A	*CYorf15A*	-5.254	0.043
AK125965	hypothetical protein LOC284116	*LOC284116*	-4.891	0.001
BC112238	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)	*SMPD3*	-4.808	0.003
XM_928193	hypothetical protein LOC284116	*LOC284116*	-4.524	0.001
BC035312	chromosome Y open reading frame 15B	*CYorf15B*	-4.304	0.031
NM_182660	ubiquitously transcribed tetratricopeptide repeat gene, Y-linked	*UTY*	-4.128	0.047
BC032567	neuroligin 4, Y-linked	*NLGN4Y*	-3.984	0.014
NM_021012	potassium inwardly-rectifying channel, subfamily J, member 12	*KCNJ12*	-3.902	0.001
NM_012114	caspase 14, apoptosis-related cysteine peptidase	*CASP14*	-3.870	0.032
NM_024693	enoyl Coenzyme A hydratase domain containing 3	*ECHDC3*	-3.708	0.011
BC009617	enoyl Coenzyme A hydratase domain containing 3	*ECHDC3*	-3.635	0.010
BC100905	ribosomal protein S4, Y-linked 1	*RPS4Y1*	-3.464	0.050
BC096096	histidine ammonia-lyase	*HAL*	-3.418	0.019
BC098106	SLIT and NTRK-like family, member 5	*SLITRK5*	-3.291	0.025
XM_926115	hypothetical protein LOC642657	*LOC642657*	-3.273	0.035
BC096097	histidine ammonia-lyase	*HAL*	-3.210	0.029
AJ003078	5-hydroxytryptamine (serotonin) receptor 3A	*HTR3A*	-3.156	0.026
NM_001676	ATPase, H+/K+ transporting, nongastric, alpha polypeptide	*ATP12A*	-3.103	0.016

FC – Fold Change - describes the ratio of mean expression levels in aggressive periodontitis groups over the mean expression levels in healthy group. Multiple probe sets map to a single gene.

Table 3. Differentially expressed (upregulated and downregulated) pathways, determined by pathway analysis based on Kyoto Encyclopedia of Gene and Genome (KEGG) database.

Pathways	p-value	Counts
Aggressive periodontitis vs Healthy		
Up-regulated		
Antigen processing and presentation	5.2E-20	15
Natural killer cell mediated cytotoxicity	1.3E-13	13
Graft-versus-host disease	2.8E-10	8
Down-regulated		
Endocytosis	3.3E-2	5
Chronic vs Aggressive periodontitis		
Up-regulated		
Arachidonic acid metabolism	1.7E-2	4
Down-regulated		
Hematopoietic cell lineage	1.3E-3	5
Cell adhesion molecules (CAMs)	6.2E-3	5
Cytosolic DNA-sensing pathway	3.9E-2	3

Counts – number of differentially expressed genes (upregulated or downregulated) included in the pathway (KEGG database).

Table 4. Top 20 differentially expressed probes sets in chronic periodontitis relative to aggressive periodontitis patients

GenBank	Gene Name	Gene Symbol	FC	p
Up-regulated				
NM_014068	psoriasis susceptibility 1 candidate 1	PSORS1C1	7.272	0.009
AF484418	psoriasis susceptibility 1 candidate 1	PSORS1C1	6.360	0.006
BC104765	early growth response 3	EGR3	6.290	0.001
BC009617	enoyl Coenzyme A hydratase domain containing 3	ECHDC3	4.505	0.004
NM_012114	caspase 14, apoptosis-related cysteine peptidase	CASP14	4.159	0.025
NM_024693	enoyl Coenzyme A hydratase domain containing 3	ECHDC3	4.148	0.006
NM_021012	potassium inwardly-rectifying channel, subfamily J, member 12	KCNJ12	4.086	0.000
AK125965	hypothetical protein LOC284116	LOC284116	3.874	0.002
NM_006848	coiled-coil domain containing 85B	CCDC85B	3.816	0.027
XM_928193	hypothetical protein LOC284116	LOC284116	3.791	0.002
NM_001676	ATPase, H+/K+ transporting, nongastric, alpha polypeptide	ATP12A	3.639	0.007
NM_001004318	FLJ16165 protein	FLJ16165	3.635	0.024
BC112238	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)	SMPD3	3.538	0.010
BC096096	histidine ammonia-lyase	HAL	3.536	0.016
BC096097	histidine ammonia-lyase	HAL	3.529	0.020
AJ003078	5-hydroxytryptamine (serotonin) receptor 3A	HTR3A	3.464	0.018
NM_000869	5-hydroxytryptamine (serotonin) receptor 3A	HTR3A	3.430	0.021
AY551001	kallikrein 9	KLK9	3.410	0.001
AJ003080	5-hydroxytryptamine (serotonin) receptor 3A	HTR3A	3.399	0.021
NM_004430	early growth response 3	EGR3	3.314	0.001
Down-regulated				
NM_173508	solute carrier family 35, member F3	SLC35F3	-4.259	0.04
BC068238	hypothetical LOC554223	LOC554223	-4.101	0.02
NM_000450	selectin E (endothelial adhesion molecule 1)	SELE	-3.859	0.03
NM_000600	interleukin 6 (interferon, beta 2)	IL6	-3.827	0.03
NM_181985	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5	LILRA5	-3.671	0.01
NM_001005738	formyl peptide receptor-like 1	FPRL1	-3.504	0.01
BC051362	hypothetical LOC554223	LOC554223	-3.456	0.03
BC029125	formyl peptide receptor-like 1	FPRL1	-3.452	0.01
NM_000566	Fc fragment of IgG, high affinity Ia, receptor (CD64)	FCGR1A	-3.441	0.00
NM_001004340	Fc-gamma receptor I B2	LOC440607	-3.348	0.00
NM_007115	tumor necrosis factor, alpha-induced protein 6	TNFAIP6	-3.345	0.00
NM_001017986	Fc-gamma receptor I B2	LOC440607	-3.057	0.00
BC093970	chemokine (C-C motif) ligand 23	CCL23	-3.048	0.03
BC031649	LysM, putative peptidoglycan-binding, domain containing 1	LYSMD1	-3.043	0.00
NM_145898	chemokine (C-C motif) ligand 23	CCL23	-3.032	0.03
NM_005672	prostate stem cell antigen	PSCA	-3.012	0.04
AK024382	Homo sapiens cDNA FLJ14320 fis, clone PLACE3000455.	N/A	-2.992	0.00
NM_005064	chemokine (C-C motif) ligand 23	CCL23	-2.974	0.02
BC098354	killer cell lectin-like receptor subfamily F, member 1	KLRF1	-2.942	0.01
NM_017770	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2	ELOVL2	-2.941	0.04

FC – Fold Change - describes the ratio of mean expression levels in aggressive periodontitis groups over the mean expression levels in healthy group. Multiple probe sets map to a single gene.

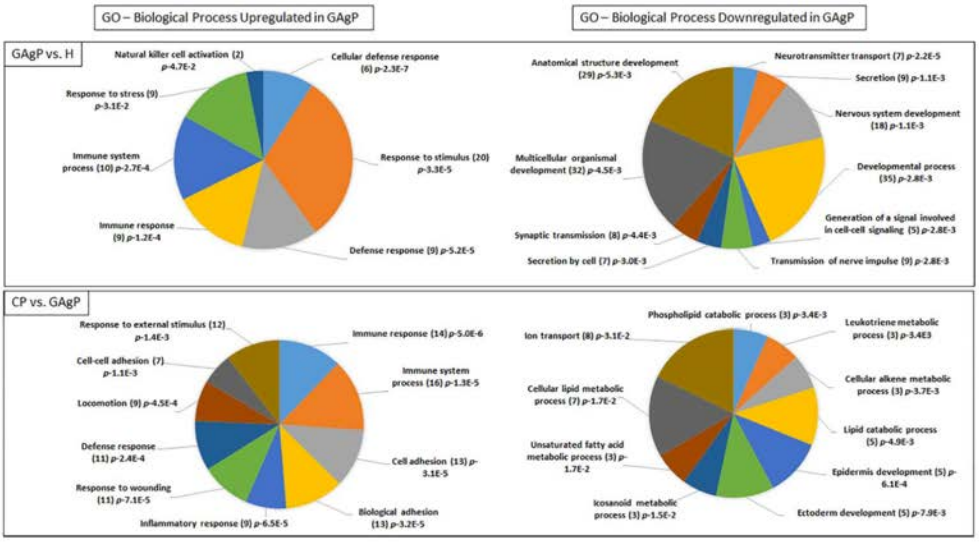

Figure 1. Biological process of probe sets differentially expressed in healthy gingival tissues from GAgP, CP and healthy subjects, generated by DAVID software using GOTERM_BP_ALL databases. Gene ontology (GO) terms are represented as different color wedges in the pie charts, with the number of probe sets per group shown in parentheses. p: p-value.

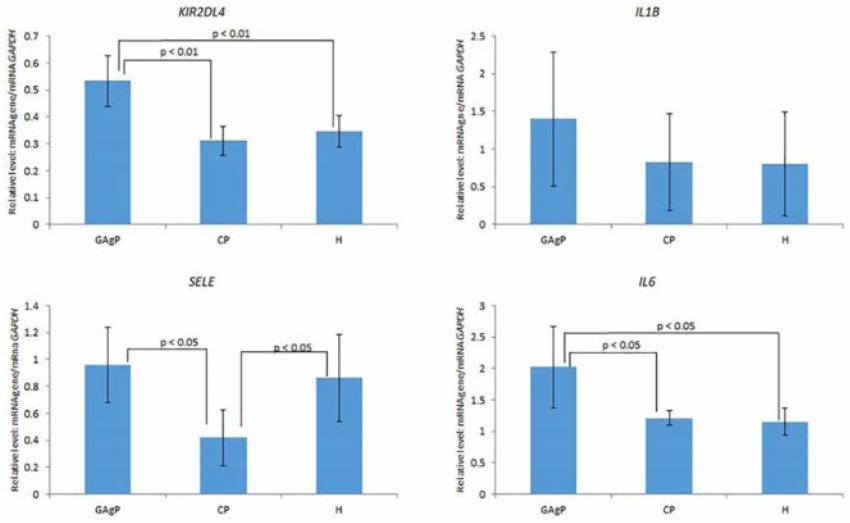

Figure 2. mRNA transcript levels (mean ± standard deviation), relative to the GAPDH, for KIR2DL4, IL1B, SELE and IL6. Statistical analysis was performed using ANOVA/Tukey tests.

3.4 Levels of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, inflammatory cytokines and species-specific immunoglobulin G in generalized aggressive and chronic periodontitis.*

BACKGROUND AND OBJECTIVE: Aggressive periodontitis pathogenesis still is not completely understood in the literature regarding the relationship between microbial and inflammatory aspects. So this study aimed to compare microbial and inflammatory patterns in the gingival crevicular fluid of generalized aggressive and chronic periodontitis patients. MATERIAL AND METHODS: Forty aggressive and 28 chronic periodontitis patients were selected. Biofilm and gingival crevicular fluid were collected from a deep pocket (periodontal probing depth >7 mm) and a moderate pocket (periodontal probing depth = 5 mm) of each patient, and microbiological and immunoenzymatic assays were performed. Real-time PCR was used to determine quantities of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. Enzyme-linked immunosorbent assay (ELISA) was employed to determine gingival crevicular fluid levels of interleukin-1beta, interferon-gamma, prostaglandin E(2) and interleukin-10. In addition, immunoglobulin G (IgG) levels against *A. actinomycetemcomitans* and *P. gingivalis* lipopolysaccharide were also determined by ELISA. Analysis of variance/Tukey test, Mann-Whitney U-test and the Pearson correlation test were used to determine differences and correlations between variables analysed (alpha = 5%).

RESULTS: Patients suffering from generalized aggressive periodontitis had their mouth colonized by higher amounts of *A. actinomycetemcomitans* and *P. gingivalis* than chronic periodontitis patients. Conversely, the gingival crevicular fluid levels of IgG against both pathogens were statistically inferior in aggressive periodontitis patients ($p < 0.05$). With regard to gingival crevicular fluid levels of cytokines, aggressive periodontitis patients presented reduced levels of interleukin-10 ($p < 0.05$). CONCLUSION: In comparison to chronic periodontitis, generalized aggressive periodontitis patients have an imbalance in the host response, with reduced levels of interleukin-10 and IgG, and increased periodontal pathogens.

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Levels of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, inflammatory cytokines and species-specific immunoglobulin G in generalized aggressive and chronic periodontitis

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Casarin RCV, Del Peloso Ribeiro É, 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 Periodont Res 2010; 45: 635–642.*

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Material and Methods: Forty aggressive and 28 chronic periodontitis patients were selected. Biofilm and gingival crevicular fluid were collected from a deep pocket (periodontal probing depth >7 mm) and a moderate pocket (periodontal probing depth $= 5$ mm) of each patient, and microbiological and immunoenzymatic assays were performed. Real-time PCR was used to determine quantities of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. Enzyme-linked immunosorbent assay (ELISA) was employed to determine gingival crevicular fluid levels of interleukin-1β, interferon-γ, prostaglandin E_2 and interleukin-10. In addition, immunoglobulin G (IgG) levels against *A. actinomycetemcomitans* and *P. gingivalis* lipopolysaccharide were also determined by ELISA. Analysis of variance/Tukey test, Mann–Whitney *U*-test and the Pearson correlation test were used to determine differences and correlations between variables analysed ($\alpha = 5\%$).

Results: Patients suffering from generalized aggressive periodontitis had their mouth colonized by higher amounts of *A. actinomycetemcomitans* and *P. gingivalis* than chronic periodontitis patients. Conversely, the gingival crevicular fluid levels of IgG against both pathogens were statistically inferior in aggressive periodontitis patients ($p < 0.05$). With regard to gingival crevicular

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fluid levels of cytokines, aggressive periodontitis patients presented reduced levels of interleukin-10 ($p < 0.05$).

Conclusion: In comparison to chronic periodontitis, generalized aggressive periodontitis patients have an imbalance in the host response, with reduced levels of interleukin-10 and IgG, and increased periodontal pathogens.

Although both chronic and aggressive periodontitis apparently derive from the same aetiological factors, they present clear differences in the development and progression of the disease (1). While chronic disease presents a slow progression, usually compatible with local factors, aggressive periodontitis is defined as an inflammatory disease that has its onset primarily during early-adult years and is characterized by rapid attachment loss and bone destruction (2). In order to explain possible differences between both forms of disease, microbiological and genetic aspects, as well as the role of the host immune response, have been studied; however, mechanisms responsible for these differences remain unclear.

Microorganisms and the host response against their antigens appear to modulate the development of aggressive and chronic periodontitis. Many studies have attributed the presence and high rates of *Aggregatibacter actinomycetemcomitans* (and in some populations, *Porphyromonas gingivalis*) to the different forms of aggressive periodontitis (3–5). Both bacteria present innumerable antigens capable of inducing an inflammatory and robust serum antibody response, consequently leading to periodontal destruction (6). However, at the same time, a deficiency in production of an adequate antibody response against these antigens has also been correlated with generalized aggressive periodontitis and recurrent chronic periodontal disease (3,7).

Despite decades of studies, the role of specific oral bacterial antibodies in the development and progression of aggressive and chronic periodontitis is still unclear. While some findings have suggested a protective character of antibodies in disease progression (6,8–10), others have shown that their simple presence is not related to periodontal protection against microbial virulence (11,12). One possible explanation could be the role of pro- or anti-inflammatory cytokines released by host defense cells during the production of specific antibodies (13).

Levels of cytokines in aggressive periodontitis vary depending on the type and severity of disease. Macrophages derived from blood cells of localized aggressive periodontitis patients have shown a hyper-responsiveness, releasing high levels of pro-inflammatory cytokines, such as prostaglandin E_2 (PGE_2), interleukin-1β (IL-1β) and interleukin-6 (IL-6; 13). At the same time, the cytokine profile of generalized aggressive periodontitis patients seems contradictory in the literature. While some studies have suggested that these patients present lower responsiveness of protective cells (3,14), other studies show no difference (15) or increased proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α; 16). Moreover, the presence of *A. actinomycetemcomitans*, usually associated with aggressive periodontitis, is correlated with lower levels of serum interleukin-10 (IL-10), a cytokine that controls pro-inflammatory cytokine release by the host (17), modulating periodontal destruction.

Recent studies have tried to create a model in order to connect the cytokines and production of specific antibodies (13). However, only a few studies have evaluated these patterns simultaneously, especially in the gingival crevicular fluid, which could result in more conclusive results. Therefore, the present study was undertaken to evaluate the presence of *P. gingivalis* and *A. actinomycetemcomitans* and levels of specific antibodies against these periodontal pathogens in generalized aggressive and chronic periodontitis patients. The cytokine profile in these subjects was also determined in order to understand possible interactions between these factors in the pathogenesis of periodontal destruction.

Material and methods

Population screening

Subjects were selected after a screening examination that included a full medical and dental history, an intra-oral examination, a full-mouth periodontal probing and radiographic evaluation. Subjects were divided by the type of periodontal disease, using the following criteria.

Group A – Generalized chronic periodontitis—Patients presented as follows: (i) diagnosis of severe chronic periodontitis characterized by the presence of periodontal pockets with a clinical attachment loss of ≥5 mm, bleeding on probing and radiographic bone loss; (ii) age over 35 years; (iii) at least eight teeth with a probing pocket depth of ≥5 mm and bleeding after pocket probing and at least two of the eight qualifying teeth must have probing pocket depth ≥7 mm; and (iv) minimum of 20 teeth in both jaws (wisdom teeth excluded).

Group B – Generalized aggressive periodontitis—Patients presented as follows: (i) diagnosis of generalized aggressive periodontitis, following the American Academy of Periodontology (AAP) classification (2), defined as the presence of periodontal pockets with a clinical attachment loss and radiographic bone loss in at least three teeth different from first molars and incisors; (ii) age less than 35 years; (iii) at least eight teeth with a moderate probing pocket depth of ≥5 mm and bleeding

after pocket probing and at least two of the eight qualifying teeth must have probing pocket depth ≥ 7 mm (deep probing pocket depth); (iv) minimum of 20 teeth in both jaws (wisdom teeth excluded); and (v) systemic general health (according to patient; absence of diabetes mellitus, cardiovascular, liver, cerebral or pulmonary disease).

Patients were not included in the study if presented with any of the following: (i) periapical alterations on qualifying teeth; (ii) medical disorders that required prophylactic antibiotic coverage or that could influence the response to treatment; (iii) scaling and root planning in the preceding 6 months; (iv) consumption of drugs known to affect periodontal status (antibiotic, anti-inflammatory, anticonvulsant, immunosuppressant and calcium channel blocker) within the past 6 months; (v) orthodontic therapy; (vi) pregnancy; and (vii) smoking habit (or former smokers).

Considering the above-mentioned criteria, a total of 28 patients presenting generalized chronic periodontitis and 40 subjects presenting generalized aggressive periodontitis were selected for analysis. During 4.5 years, about 1750 subjects were examined and among them, 40 generalized aggressive periodontitis patients were recruited to the present study (approximately 2.5% of the total number of subjects). As shown in Table 1, both groups presented a female majority (82 and 72.5% for chronic and aggressive, respectively). No differences between groups could be seen concerning the full-mouth plaque or bleeding on probing indexes nor in clinical attachment level (6.10 ± 0.20 and 6.39 ± 0.72, chronic and aggressive periodontitis, respectively). The mean age of aggressive periodontitis patients was 27.62 ± 0.88 years, which was statistically younger than that of chronic periodontitis patients (48.11 ± 1.52 years old, $p < 0.0001$).

Table 1. Patient characteristics (means ± SEM) in each group

	Chronic	Aggressive	*p* value
Age	48.11 ± 1.52	27.62 ± 0.88	< 0.0001*
Sex (% female)	82.0%	72.5%	0.52
Full-mouth plaque index	36.96 ± 2.65	38.10 ± 2.07	0.72
Full-mouth bleeding on probing index	29.65 ± 2.64	26.35 ± 1.69	0.38
Clinical attachment level	6.10 ± 0.20	6.39 ± 0.72	0.69

*Mann–Whitney *U*-test (α = 5%).

Sample collection

After a full-mouth examination, all sites presenting periodontal probing depth ≥5 mm were analysed regarding pulpar alterations and presence of furcation defects (if present, the teeth were excluded). Among them, one site presenting probing pocket depth of 5 mm and another pocket with a probing pocket depth of 7 mm were randomly selected (by a paper draw) for subgingival biofilm collection. Following the careful removal of the supragingival biofilm, the areas were washed with a water spray, isolated with cotton rolls and gently dried. A sterile paper point (no. 35) (Dentsply, Ribeirão Preto, Brazil) was inserted into the bottom of the periodontal pocket for 30 s. The paper points were placed into sterile tubes containing 300 μL of reduced transport fluid (18).

Gingival crevicular fluid was collected from the same microbiological sampling sites. Sixty to 90 s after subgingival biofilm collection, the teeth were washed again, and the area was isolated and gently dried. Gingival crevicular fluid was collected by placing filter paper strips (Periopaper; Oraflow, Plainview, NY, USA) into the pocket until a slight resistance was perceived, and then left there for 15 s. Immediately, the volume of the sample was measured with the aid of a calibrated electronic gingival fluid measuring device (Periotron 8000; Oraflow). After volume measurements, the strips were placed into sterile tubes containing 400 μL of phosphate-buffered saline (PBS) with 0.05% Tween-20 (19). Strips contaminated by visible blood were discarded and a new collection was made after 30 s.

All samples (subgingival biofilm and gingival crevicular fluid) were immediately stored at 20°C. One examiner made all clinical measurements and collected all microbial and gingival crevicular fluid samples.

Microbiological evaluation

For detection and quantification of periodontopathic bacteria, the real-time PCR technique was used. The presence and concentration of *P. gingivalis* and *A. actinomycetemcomitans* were evaluated in each site using specific primers reported in the literature (20). The primers used for *P. gingivalis* were 5′ CATAGATATCACGAGGA ACTCCGATT, 3′ AAACTGTTAGC AACTACCGATG TGG; and for *A. actinomycetemcomitans* 5′ GAACC TTACCTACTCTTGACATCCGAA, 3′ TGCAGCACCTGTCTCAAAGC.

Each paper point was placed separately in plastic tubes containing 0.01 M Tris–EDTA solution, pH 8 (TE). The DNA was then extracted from the subgingival biofilm, as described previously (21). Reaction efficiency was optimized, and primer final concentrations of 0.5 mM for *P. gingivalis* and *A. actinomycetemcomitans* were chosen. Real-time PCR was performed in the LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany) with the Fast-Start DNA Master SYBR Green I kit (Roche Diagnostics GmbH). For each run, water was used as the negative control. Briefly, amplification was performed in a 10 μL final volume containing 2.5 μL of template DNA. The concentration of the DNA used in each run was always 10 μg/mL (22). The amplification profiles were as follows: 95/10, 55/5 and 72/4 [temperature (°C)/time (s)] and 40 cycles for *P. gingivalis*; and 95/10, 55/5, 72/3 and 40 cycles for *A. actinomycetemcomitans*. Melting peaks were used to determine the specificity of the PCR (20). Absolute quantification of target bacteria in clinical samples was performed using *P. gingivalis* (ATCC 33277) and *A. actinomycetemcomitans* (JP2) as controls. Standard curves were

made with these controls (20). The standard curves were used to convert cycle threshold scores into the number of bacterial cells using controls with known amounts of bacteria-specific DNA. The level of detection was set to 10^3 bacteria per plaque sample for all target bacteria. The determination of DNA content in controls was based on the genome size of each bacteria and the mean weight of one nucleotide pair (23).

Cytokine levels in gingival crevicular fluid

Aliquots of each gingival crevicular fluid sample were assayed by an ELISA using commercially available kits (R&D Systems Inc., Minneapolis, MN, USA) for IL-1β, PGE_2, interferon-γ (IFN-γ) and IL-10, according to the manufacturer's instructions. Previously, samples were diluted with the diluent of the kit. The dilution was used to calculate the concentration of each gingival crevicular fluid substance. This concentration was calculated with a standard curve, which was prepared using the standard proteins in the kit. The standard curve range used for IL-1β measurement was 8–0.125 pg/mL; for PGE_2 125– 19.26 pg/mL; for IFN-γ 1000–15.60 pg/mL and for IL-10 50–0.78 pg/mL. The ELISAs were run in duplicate, and mean values were used to calculate concentrations of each cytokine.

Levels of IgG antibodies in gingival crevicular fluid

An ELISA was used to determine the levels of total IgG antibodies against *A. actinomycetemcomitans* and *P. gingivalis* in gingival crevicular fluid, according to the following protocol. A 96-well plate was covered (37°C for 75 min) with *A. actinomycetemcomitans* (JP2) and *P. gingivalis* (ATCC 33277) lipopolysaccharide (kindly donated by Dr Fatiha Chandad). After blocking free sites, samples and standard were incubated for 60 min at 37°C. Then, an alkaline phosphatase-conjugated antibody was added and the plates incubated for 60 min at 37°C. After that, a substrate solution (4-nitrophenyl phosphate disodium salt hexahydrate; Sigma) was added and incubated (60 min at 37°C) and the reaction was stopped (2 N H_2SO_4). The plate was read at a wavelength of 450 nm within 30 min and the values expressed as ELISA units. All microbiological and immunological laboratory procedures were performed blind, without knowledge of the clinical status of the study subjects or of the periodontal sites sampled.

Statistical analysis

The homogeneity of data was tested through the Shapiro–Wilk test. Since a heterogeneity was achieved for all parameters, non-parametric Mann–Whitney *U*-tests and Wilcoxon tests were used for inter- and intragroup analysis, respectively. To determine the equality of sex between groups, chi-square test was used. In order to correlate the subgingival levels of *A. actinomycetemcomitans* and *P. gingivalis* with species-specific IgG, the Pearson rank correlation test was used. A *p*-value of ≤ 0.05 was required for statistical significance.

Results

Cytokine levels

Table 2 shows that gingival crevicular fluid levels of IL-1β and PGE_2 of aggressive periodontitis patients were similar to those of chronic periodontitis patients in moderate and deep pockets ($p > 0.05$). With regard to IL-10 levels, aggressive periodontitis patients presented statistically lower levels compared with chronic periodontitis patients in moderate (0.45 ± 0.07 and 2.08 ± 0.63 pg/mL, respectively) and in deep pockets (0.33 ± 0.06 and 2.11 ± 0.74 pg/mL, respectively). Moreover, a comparison between strata of pockets shows that in aggressive periodontitis lower levels of IL-10 were found in deep pockets ($p = 0.04$), in contrast to observations in chronic periodontitis subjects ($p = 0.82$).

Gingival crevicular fluid IFN-γ levels were similar between the two types of periodontitis (Table 2). However, only aggressive periodontitis presented a statistical difference between moderate (0.67 ± 0.07 pg/mL) and deep pockets (0.46 ± 0.05 pg/mL).

Microbiological and immunological assays

Real-time PCR analysis revealed that aggressive periodontitis pockets presented higher amounts of *P. gingivalis* and *A. actinomycetemcomitans* when compared with chronic periodontitis pockets (Table 3). The *A. actinomycetemcomitans* log concentration in moderate pockets was 3.06 ± 0.46 and in deep pockets was 2.75 ± 0.46; these values were statistically higher than those found in chronic periodontitis (0.99 ± 0.34 and 1.40 ± 0.45 for moderate and deep pockets, respectively). No difference between pocket strata was found between aggressive and chronic periodontitis.

Conversely, quantities of IgG against *A. actinomycetemcomitans* were statis-

Table 2. Mean (pg/mL ± SEM) of interleukin-1β, prostaglandin E_2, interferon-γ and interleukin-10 levels in moderate (periodontal probing depth 5 mm) and deep pockets (probing pocket depth ≥7 mm) of chronic and aggressive periodontitis

	Chronic		Aggressive	
	Moderate	Deep	Moderate	Deep
Interleukin-1β	117.31 ± 19.18	99.64 ± 12.11	124.22 ± 30.22	131.22 ± 30.28
Prostaglandin E_2	462.16 + 93.78	540.85 + 165.08	328.92 + 88.03	324.78 + 88.41
Interferon-γ	1.20 ± 0.25	0.96 ± 0.24	0.67 ± 0.07	0.46 ± 0.05[a]
Interleukin-10	2.08 ± 0.63	2.11 ± 0.74	0.45 ± 0.07[b]	0.33 ± 0.06[ab]

[a]Statistical intragroup difference between moderate and deep pockets (Wilcoxon test, $p < 0.05$).
[b]Statistical intergroup difference between chronic and aggressive periodontitis (Mann–Whitney *U*-test, $p < 0.05$).

Table 3. Amounts (log_{10} ± SEM) of *A. actinomycetemcomitans* and *P. gingivalis* and IgG against them (ELISA units ± SEM) in moderate and deep pockets of chronic and aggressive periodontitis

	Chronic	Aggressive
A. actinomycetemcomitans		
Moderate		
log_{10}	0.99 ± 0.34	3.06 ± 0.46[a]
IgG	216.79 ± 47.34	114.52 ± 20.58[a]
Deep		
log_{10}	1.40 ± 0.45	2.75 ± 0.46[a]
IgG	276.94 ± 107.90	94.29 ± 14.09[a]
P. gingivalis		
Moderate		
log_{10}	3.47 ± 0.54	5.15 ± 0.53[a]
IgG	76.46 ± 16.65	75.84 ± 13.26
Deep		
log_{10}	3.59 ± 0.51	5.65 ± 0.55[a]
IgG	95.72 ± 25.89	59.34 ± 8.26

[a]Statistical difference between chronic and aggressive periodontitis (Mann–Whitney *U*-test, $p < 0.05$).

tically lower in aggressive compared with chronic periodontitis, both in moderate (114.52 ± 20.58 and 216.79 ± 47.34 ELISA units, respectively) and in deep pockets (94.29 ± 14.09 and 276.94 ± 107.90 ELISA units, respectively). No statistical difference was observed between the two types of periodontitis or pocket strata with regard to the total IgG amounts against *P. gingivalis* (Table 3).

Immunoglobulin G levels and microbiological/cytokine quantities in gingival crevicular fluid

Table 4 displays the results of the correlation between species-specific IgG levels and the amounts of *A. actinomycetemcomitans* and *P. gingivalis* in chronic and aggressive periodontitis. As it can be seen from Table 4, the Pearson correlation test did not find significant correlations between the periodontopathic bacteria and the amount of bacteria-specific IgG ($p > 0.05$).

Immunoglobulin G levels against *A. actinomycetemcomitans* were also correlated with the gingival crevicular fluid levels of IL-1β, IFN-γ, PGE_2 and IL-10 (Table 5). A stronger and statistically positive relationship between *A. actinomycetemcomitans*-specific IgG and IFN-γ, PGE_2 and IL-10 could be seen in aggressive periodontitis, both in moderate and in deep pockets. The amounts of IL-10, PGE_2 and IFN-γ were greater in the gingival crevicular fluid when the quantity of *A. actinomycetemcomitans*-specific IgG was also higher. In chronic periodontitis, a significant positive correlation was observed between IFN-γ and PGE_2 levels and in IgG against *A. actinomycetemcomitans* in deep pockets (Table 5).

With regard to *P. gingivalis*-specific IgG, a significant correlation with cytokine levels was only observed in aggressive periodontitis, in both strata of pockets. Higher levels of IL-1β, PGE_2, IFN-γ and IL-10 correlated with higher quantities of *P. gingivalis*-specific IgG (Table 6). No correlation was observed in chronic periodontitis.

Table 4. Correlation between gingival crevicular fluid levels of bacterial species-specific IgG and amounts (log_{10}) of *A. actinomicetemcomitans* and *P. gingivalis* in moderate and deep periodontal pockets

	Chronic log_{10} × IgG against		Aggressive log_{10} × IgG against	
	Moderate	Deep	Moderate	Deep
A. actinomicetemcomitans	−0.20	−0.21	−0.10	−0.18
P. gingivalis	0.21	−0.03	0.08	−0.11

Pearson rank correlation test ($p > 0.05$).

Discussion

Several studies have attempted to explain the differences in development and progression of aggressive and chronic periodontitis. However, discrepant results found in the literature regarding microbial and cytokine profile and production of antibodies complicates the comprehension of the differences between the bacteria–host relationship of each form of the disease. Therefore, this study aimed to evaluate the local presence, i.e. in the gingival crevicular fluid, of *A. actinomycetemcomitans* and *P. gingivalis* and their IgG species-specific antibodies, as well as inflammatory cytokine levels, in patients suffering from generalized aggressive and chronic periodontitis.

In the present study, higher concentrations of both microorganisms were found in periodontal pockets of aggressive than of chronic periodontitis. Although some studies did not find any difference in microbial profiles between the diseases (24), others corroborate the present results, showing higher levels of *A. actinomycetemcomitans* in aggressive periodontitis (5,25). A previous study evaluated the bacterial profile of aggressive periodontitis in a Brazilian population, finding 72% of aggressive periodontitis individuals infected with *A. actinomycetemcomitans*; a percentage that was statistically greater than that found in chronic periodontitis (41.6%; 4). Our results also corroborate other microbiota screening studies carried out in Chilean (26) and Japanese periodontitis patients (27).

The relationship between *P. gingivalis* and aggressive periodontitis is uncertain in the literature. The 1999 AAP Periodontal Disease Classification states that *P. gingivalis* may be associated with aggressive periodonti-

Table 5. Correlation (r) between IgG against *A. actinomycetemcomitans* and gingival crevicular fluid level of interleukin-1β, prostaglandin E_2, interferon-γ and interleukin-10 (pg/mL) in chronic and aggressive periodontitis (moderate and deep pockets)

	Chronic IgG against *A. actinomicetemcomitans*		Aggressive IgG against *A. actinomicetemcomitans*	
	Moderate	Deep	Moderate	Deep
Interleukin-1β	0	−0.14	0.1	0.26
Prostaglandin E_2	0.34	0.54*	0.44*	0.62**
Interferon-γ	0.32	0.56*	0.57**	0.68**
Interleukin-10	0.01	−0.15	0.56**	0.37*

Pearson rank correlation test, $*p < 0.02$ and $**p < 0.001$.

Table 6. Correlation (r) between *P. gingivalis*-specific IgG and gingival crevicular fluid level of interleukin-1β, prostaglandin E_2, interferon-γ and interleukin-10 (pg/mL) in chronic and aggressive periodontitis (moderate and deep pockets)

	Chronic IgG against *P. gingivalis*		Aggressive IgG against *P. gingivalis*	
	Moderate	Deep	Moderate	Deep
Interleukin-1β	0.08	−0.05	0.39*	0.44*
Prostaglandin E_2	0.38	0.02	0.86**	0.84**
Interferon-γ	−0.17	0.19	0.79**	0.80**
Interleukin-10	0.31	0.01	0.65**	0.79**

Pearson rank correlation test, $*p < 0.02$, $**p < 0.001$.

tis in some populations (1), as confirmed in other studies (28). However, only a few studies have been able to find differences in the prevalence of *P. gingivalis* between aggressive and chronic disease (29,30). In our study, using quantitative analyses (real-time PCR), significantly higher quantities of this pathogen were observed in aggressive periodontitis.

It is well established that both *A. actinomycetemcomitans* and *P. gingivalis* present antigens that lead to a robust host immunological response. Several studies have shown that whole cells, lipopolysaccharide, fimbriae and carbohydrates from both bacteria are capable of increasing the production of immunoglobulin, especially subclass G (13,31,32). Localized aggressive periodontitis usually shows greater quantities of IgG, especially against *A. actinomycetemcomitans*, than generalized aggressive and chronic disease (12,33–35), although some studies did not find significant differences between the diseases (12,31–42).

Studies have found a positive relationship between the presence of periodontal pathogens and local levels of IgG (12,43). However, the present study demonstrated discrepant correlations between levels of anti-*P. gingivalis* and anti-*A. actinomycetemcomitans* IgG and the amounts of both bacteria in the periodontal pockets. With regard to *P. gingivalis*, even though aggressive periodontitis presented higher amounts of these bacteria, the IgG level was inferior but not statistically significant. In the case of *A. actinomycetemcomitans*, although statistically higher quantities of the bacteria were found in the subgingival area, the IgG level detected was significantly lower, suggesting a poorer IgG production of aggressive periodontitis patients.

Recently, the influence of the levels of cytokines on IgG production has been investigated, and a modulator potential of several cytokines has been discovered. Among these, IL-1β, IFN-γ, PGE_2 and IL-10 have all been shown to influence the production of immunoglobulins in different manners (13,32). Considering that aggressive periodontitis presented lower levels of IL-10 production, its role on infectious diseases, such as periodontitis, should be considered.

Interleukin-10 is essential to B-cell differentiation and proliferation, reducing apoptosis of immune cells and stimulating Cd8+ and natural killer cells, representing an important role in the adaptative response (44–46). At the same time, its overexpression is implicated in infectious persistence and augmentation of host susceptibility to some bacteria (46–50). Considering this, the altered IL-10 production in aggressive periodontitis should be further clarified in order to identify its relationship to disease pathogenesis.

Our results show that aggressive periodontitis subjects presented higher amounts of *A. actinomycetemcomitans* and *P. gingivalis* than chronic periodontitis patients. Conversely, the gingival crevicular fluid levels of IgG against both pathogens and the IL-10 cytokine levels were lower in aggressive periodontitis patients. These results indicate that aggressive and chronic periodontitis pathologies present several host response differences, possibly explaining their difference in progression and development. However, we must be careful when considering the results. First of all, some other factors should be considered in future studies to confirm and explain the results, for example, comparing both diseases to healthy individuals and validating the results by repetitive sampling analyses would be necessary. In addition, the influence of race also could contribute to identify populations with an imbalanced host response, as addressed by other studies (1,12).

Conclusion

In comparison to chronic periodontitis, generalized aggressive periodontitis patients have an imbalance in the host response, with reduced levels of anti-inflammatory cytokines and IgG, and increased periodontal pathogens. However, this imbalanced host response should be further clarified with additional studies which could contribute to a better under-

standing of periodontitis pathogenesis phenomena.

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3.5 Full-mouth ultrasonic debridement associated with povidone iodine rinsing in GAgP treatment: a randomised clinical trial*

Abstract

OBJECTIVE: This study evaluated the clinical, immunological and microbiological results of full-mouth ultrasonic debridement (FMUD) with 10 % povidone iodine (PVPI) as the cooling liquid in the treatment of generalised aggressive periodontitis (GAgP).

MATERIAL AND METHODS: Twenty-eight patients presenting GAgP were randomly assigned to one of the following groups for evaluation: FMUD+SS (n=14)--single session of FMUD with 0.9 % saline solution as cooling agent and FMUD+PVPI (n=14)--single session of FMUD with PVPI solution as cooling agent. Probing depth (PD), relative clinical attachment level (RCAL), relative position of gingival margin, plaque index (FMPI) and bleeding score (FMBS), immunological (interleukin-10 and interleukin-1β concentrations in gingival crevicular fluid) and microbiological (Aa and Pg amounts) parameters were evaluated at baseline, first, third and sixth months after treatment.

RESULTS: The two groups presented reduction of FMPI and FMBS and had statistically significant PD reductions, RCAL gains and gingival recession ($p<0.05$). Both therapies reduced Pg levels in deep and in moderate pockets ($p<0.05$). FMUD+PVPI reduced Aa levels in deep pockets. However, no inter-group differences in clinical, immunological and microbiological parameters were observed ($p>0.05$).

CONCLUSIONS: It could be concluded that 10 % PVPI used as an irrigant solution in FMUD decreased Aa levels in deep pockets but had no additional benefits when compared with saline solution irrigation in terms of clinical, microbiological and immunological results.

CLINICAL RELEVANCE:

The FMUD is a valid option for the treatment of GAgP, but the use of 10 % PVPI did not improve the results of the periodontal therapy.

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ORIGINAL ARTICLE

Full-mouth ultrasonic debridement associated with povidone iodine rinsing in GAgP treatment: a randomised clinical trial

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Abstract
Objective This study evaluated the clinical, immunological and microbiological results of full-mouth ultrasonic debridement (FMUD) with 10 % povidone iodine (PVPI) as the cooling liquid in the treatment of generalised aggressive periodontitis (GAgP).
Material and methods Twenty-eight patients presenting GAgP were randomly assigned to one of the following groups for evaluation: FMUD+SS (n=14)—single session of FMUD with 0.9 % saline solution as cooling agent and FMUD+PVPI (n=14)—single session of FMUD with PVPI solution as cooling agent. Probing depth (PD), relative clinical attachment level (RCAL), relative position of gingival margin, plaque index (FMPI) and bleeding score (FMBS), immunological (interleukin-10 and interleukin-1β concentrations in gingival crevicular fluid) and microbiological (*Aa* and *Pg* amounts) parameters were evaluated at baseline, first, third and sixth months after treatment.
Results The two groups presented reduction of FMPI and FMBS and had statistically significant PD reductions, RCAL gains and gingival recession (p<0.05). Both therapies reduced *Pg* levels in deep and in moderate pockets (p<0.05). FMUD+PVPI reduced *Aa* levels in deep pockets. However, no inter-group differences in clinical, immunological and microbiological parameters were observed (p>0.05).
Conclusions It could be concluded that 10 % PVPI used as an irrigant solution in FMUD decreased *Aa* levels in deep pockets but had no additional benefits when compared with saline solution irrigation in terms of clinical, microbiological and immunological results.
Clinical relevance The FMUD is a valid option for the treatment of GAgP, but the use of 10 % PVPI did not improve the results of the periodontal therapy.

Keywords Aggressive periodontitis · Povidone iodine · Non-surgical periodontal therapy · Full-mouth ultrasonic debridement

Introduction

Generalised aggressive periodontitis (GAgP) is a rare disease occurring in 2.5 to 5 % of the population [1]. However, in spite of its low frequency, its treatment presents a challenge for clinicians, as it presents severe and rapid destruction and a low response to periodontal therapy [2, 3]. Randomised clinical trials focused on achieving more predictable results for GAgP treatment have shown that an antimicrobial approach combined with different non-surgical periodontal treatments (i.e. conventional quadrant scaling and root planing, one-stage full-mouth disinfection and full-mouth ultrasonic debridement) could promote additional benefits. Systemic intake of amoxicillin and metronidazole, adjunctively with non-surgical scaling, has been demonstrated to be the best antimicrobial choice [4–12].

However, in spite of the good results of this combined approach, the literature describes non-adherence by patients to systemic antimicrobial therapy as well as risks of systemic side effects such as nausea, diarrhoea and stomach ache [5].

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Moreover, Guerrero et al. [13] reported that non-adherence to or cessation of antimicrobial therapy negatively influences the results of clinical treatment. In attempts to minimise patient influence on the results of therapy and reduce the systemic side effects of antimicrobial therapy, local antimicrobial therapy seems to be a viable alternative for the treatment of generalised aggressive periodontitis. Systematic review shown additional reduction in probing depth when locally administered adjunctive antimicrobials were used in the periodontal therapy in chronic periodontitis [14]. In this vein, povidone iodine (PVPI) is an interesting alternative, as it has a wide antibacterial spectrum [15–18] and, in vitro, acts rapidly against periodontal pathogens [19]. PVPI also has low financial cost, low systemic toxicity and does not lead to bacterial resistance [20–22].

Periodontal treatment with PVPI as an adjunct to non-surgical periodontal treatment has already been tested in chronic periodontal disease [23]. Systematic and meta-analytical reviews reported minor additional benefit in pocket depth reduction at the third month after treatment with PVPI as an adjunct to conventional scaling and root planing in the treatment of chronic periodontitis [24]. However, this alternative therapy has not yet been tested in GAgP treatment. So, this study tested the hypothesis that there were clinical, microbiological and immunological additional benefits in the use of povidone iodine in the treatment of GAgP patients.

Aim of this study

The aim of this study was to perform a randomised clinical trial to evaluate the clinical, immunological and microbiological results of full-mouth ultrasonic debridement (FMUD) with 10 % povidone iodine solution as a cooling liquid in the treatment of generalised aggressive periodontitis.

Materials and methods

Study design

This study was designed as a parallel, controlled, randomised clinical trial to assess clinical, microbiological and immunological outcomes of FMUD with PVPI as a cooling liquid for the treatment of GAgP. The study was approved by the Ethics Committee of the University of Campinas (024/2006). The first treatment occurred in October 2008, and examinations ended in December 2010.

Study population

The sample evaluated in this study was initially comprised of 34 patients seeking treatment in the Graduate Clinic of Piracicaba Dental School, University of Campinas, Piracicaba, Brazil. The study inclusion criteria were (1) diagnosis of GAgP [2], (2) presence of ≥20 teeth, (3) presence of ≥8 teeth with probing depth (PD) ≥5 mm with bleeding on probing (BOP) and at least 2 teeth with PD ≥7 mm, (4) good general health and (5) <35 years of age. Patients were excluded from the study if they (1) were pregnant or lactating, (2) were suffering from any other systemic diseases (e.g. cardiovascular, diabetes) or conditions affecting periodontal treatment with PVPI, (3) had received antimicrobials in the previous 3 months, (4) were taking long-term anti-inflammatory drugs, (5) had received a course of periodontal treatment within the last 6 months or (6) smoked. Written informed consent was obtained from included participants.

Prior to experimental study, patients underwent 1-month supragingival therapy, which included one session of prophylaxis, supragingival calculus removal and oral hygiene instructions.

Calibration, randomisation and power calculation

Initially, we selected three non-study patients presenting with GAgP. The designated examiner (HFV) measured relative clinical attachment level (RCAL) and PD in all three patients twice within 24 h, with an interval of ≥1 h between examinations. The intraclass correlation coefficient was calculated for each parameter, resulting in 90 % reproducibility for RCALand 91 % for PD.

The randomisation was done by means of an opaque envelope with 34 cards with "PVPI" written on 17 and "saline solution" written on the other 17. Before the treatment, the envelope was opened, and the cooling liquid was revealed. After randomisation, the envelope and lot were discarded. The blinding was broken at the end of the follow-up period.

Sample size calculation was done before the study with statistical software (SAS v.9.1, SAS Institute, Cary, NC, USA). This analysis indicated that with 14 patients in each group, the study would have 80 % power to detect a 1-mm difference in the RCAL of patients in the two groups, considering a SD of 0.8 mm [25].

Periodontal treatment

The patients were randomly assigned to one of two treatment protocols:

1. FMUD+SS (n=17)—one 45-min session of full-mouth ultrasonic subgingival debridement by means of an ultrasonic scaler (Cavitron Select—Dentsply International Inc., Long Island City, NY, USA) and subgingival tips (25 K FSI®-SLI®-10S—Dentsply International Inc.), with 0.9 % saline solution (SS) as a cooling liquid for the ultrasonic device; or

2. FMUD+PVPI (n=17)—the same treatment as in the FMUD+SS group, but the cooling liquid was 10 % povidone iodine (Riodeine—Indústria Farmacêutica Rioquímica Ltda, São José do Rio Preto, SP, Brazil).

Subgingival treatment was performed by the same operator (RCVC), with the patients under local anaesthesia [2 % lidocaine with epinephrine, 1:100,000 (DFL Indústria e Comércio S.A., Rio de Janeiro, RJ, Brazil)]. After the treatment, patients were instructed to take analgesic pills (sodium dipyrone 500 mg, Medley Indústria Farmacêutica Ltda, Campinas, SP, Brazil) for any discomfort inherent to treatment during the first 72 h. Supportive therapy was scheduled, with monthly recalls until the sixth month after treatment. At the first, third and sixth months, clinical, microbiological and immunological evaluations were performed, ending with a session for prophylaxis, supragingival calculus removal and oral hygiene instructions when needed. After the sixth month, teeth that still presented PD ≥5 mm were retreated by conventional scaling and root planing with curettes and local anaesthesia.

Clinical evaluations

Clinical evaluations were assessed before periodontal therapy (baseline) and 1, 3 and 6 months afterward by means of a manual probe (PCPUNC 15®—Hu Friedy, Chicago, IL, USA). The exams were done by the calibrated examiner (HFV), who was blinded until the end of the study.

The following clinical parameters were evaluated: (1) full-mouth plaque index (FMPI), according to Ainamo and Bay [26], and full-mouth bleeding score (FMBS), according to Mühlemann and Son [27]; (2) PD, distance from the bottom of the pocket to the gingival margin; (3) relative gingival margin position (RGMP), distance from the gingival margin to the stent margin and (4) RCAL, distance from the bottom of the pocket to the stent margin.

The measurements were standardised by means of an individually manufactured acrylic stent in which a groove was made to determine the specific site of probing (recorded to the nearest 0.5 mm). All teeth presenting at least one site with PD ≥5 mm were preselected for clinical evaluation, and all presenting pulpal disease or furcation lesions were excluded. From the selected teeth—those presenting more than one site with PD ≥5 mm—only the deepest site was selected. These sites were designated as qualifying sites.

Biofilm and gingival crevicular fluid collection

Subgingival biofilm samples were collected from two sites in each patient, from a pocket presenting 5 mm≤PD<7 mm (moderate pocket) and from another pocket presenting PD ≥7 mm (deep pocket). Following supragingival biofilm removal and relative isolation with cotton rolls, a sterile paper point (#35) (Dentsply Indústria e Comércio, Petrópolis, RJ, Brazil) was inserted into the bottom of the periodontal pocket for 30 s. The paper points were placed in sterile tubes containing 300 μL of 0.5 mM Tris-EDTA.

Gingival crevicular fluid (GCF) was collected from two sites initially presenting 5 mm≤PD<7 mm (moderate pocket) and another two sites presenting PD ≥7 mm (deep pocket). Sixty seconds after subgingival biofilm collection, the area was isolated and gently dried. We collected GCF by placing filter paper strips (Periopaper, OraflowInc, New York, USA) into the pocket until the clinician (HFV) perceived a slight resistance and then leaving them in place for 15 s. This time of 60 s was considered to minimise the chance of contamination of paper strips with blood, but when it occurred, the paper strip was discarded and another sample was collected. The fluid volume was measured with a calibrated electronic GCF measuring device (Periotron 8000, OraflowInc, New York, USA). After volume measurements, the strips were placed in sterile tubes containing 400 mL phosphate-buffered saline (PBS) with 0.05 % polysorbate 20 (Tween 20, Sigma-Aldrich, St. Louis, MO, USA). Both subgingival biofilm and GCF samples were immediately stored at −20 °C.

Microbiological evaluations

The presence and concentrations of *Porphyromonas gingivalis* (*Pg*) and *Aggregatibacter actinomycetemcomitans* (*Aa*) were evaluated with specific primers as reported previously [12, 25], by the real-time polymerase chain reaction (PCR) technique.

Initially, DNA was extracted from the subgingival biofilm. Real-time PCR was performed with the 'hot start' reaction mix for PCR (FastStart DNA Master SYBR Green I, Roche Diagnostics, Mannheim, Germany). The concentration of the DNA used in each run was 10 mg/mL. The amplification profiles were as follows: 95 °C/10 min, 55 °C/5 min and 72 °C/4 min, 40 cycles for *Pg* and 95 °C/10 min, 55 °C/5 min and 72 °C/3 min, 40 cycles for *Aa*. Absolute quantification of target bacteria in clinical samples was performed with *Pg* (ATCC 33277) and *Aa* (JP2) as controls.

The determination of DNA genome copies in controls was based on the genome size of each bacterium and the mean weight of one nucleotide pair. The microbiological analyses were performed separately (moderate and deep pockets).

GCF cytokine levels

Aliquots of each GCF sample were examined by an enzyme-linked immunosorbent assay (ELISA), commercially available in a kit (R&D Systems Inc., Minneapolis, MN, USA) for interleukins IL-1β and IL-10, according to the manufacturer's instructions. Previously, samples were diluted with the

diluent found in the kit. The dilution was considered to calculate the concentration of each GCF substance.

This concentration was calculated with a standard curve, prepared as recommended by the manufacturer. The ELISA assays were run in duplicate, and mean values were used to calculate the concentration of each cytokine. The immunological analyses were performed separately in moderate and deep pockets.

Statistical analysis

Statistical analysis was performed by a professional (GMBA) not involved with the treatment and only for patients who had completed the follow-up (per the protocol). The null hypothesis tested was that FMUD with povidone iodine added no clinical, microbiological or immunological benefits to the treatment of GAgP patients compared with the use of FMUD with saline solution.

The homogeneity of groups at baseline (age, FMPI, FMBS, PD, RCAL and RGMP) was tested by Student's *t* test. For clinical, microbiological and immunological variables presenting normality by the Shapiro-Wilk test ($p>0.05$), repeated-measures ANOVA was used to detect intra- and inter-group differences, with the patient as the statistical unit. When a statistically significant difference was found, an analysis of that difference was determined by the Tukey test.

Variables that did not presented normality were analysed by the Friedman test to detect intra-group differences and by the Mann-Whitney test to detect inter-group differences. The experimental level of significance for all tests was determined to be 5 %. The software used included BioEstat 5.0 (Instituto Sustentável Mamirauá, Belém, PA, Brazil) and SAS 9.1 (The SAS Institute, Cary, NC, USA).

Results

In total, 1280 patients were examined in the Periodontal Clinic of Piracicaba Dental School during the study, but only 34 patients met the inclusion criteria for this study. After the first examination, 34 patients were selected to initiate the protocols, and all were randomly allocated to one of two groups (Fig. 1). In each group, 3 patients were excluded from the analysis, and the follow-up period ended with 28 patients: 14 in the FMUD+SS solution group and 14 in the FMUD+PVPI group.

Table 1 shows the initial demographical and clinical characteristics of subjects included in this study, confirming the homogeneity between groups. Statistically significant differences were not seen when parameters like age ($p=0.98$) and gender ($p=0.38$) and initial values of FMPI ($p=0.78$), FMBS ($p=0.71$), RCAL ($p=0.44$), PD ($p=0.29$) and RGMP ($p=0.37$) were compared between groups.

During the follow-up period, FMPI and FMBS were statistically reduced in the first month and were maintained until the sixth month (Fig. 2). Differences between groups were not seen during the evaluation.

Changes in post-therapy clinical parameters are given in Table 2. PD reduction ($p<0.0001$), RCAL gain ($p<0.0001$) and increase in RGMP ($p<0.0001$) were noted only in the first month after therapy in both groups and were maintained until the sixth month, with no difference between groups concerning RCAL, PD and RGMP at any period of evaluation ($p>0.99$).

When the analyses were made with moderate pockets and deep pockets considered separately, it was seen that both groups promoted RCAL gains, PD reduction and increases in RGMP (Table 3). In both pocket strata, no between-group differences were seen ($p>0.05$). In moderate pockets, PD reduction and RCAL gain after 6 months were 1.35 and 0.76 mm in FMUD+SS ($p<0.0001$) and 1.30 and 0.54 mm in FMUD+PVPI ($p<0.0001$). In deep pockets, PD reduction and RCAL gain after 6 months were 2.74 and 1.75 mm in FMUD+SS ($p<0.0001$) and 2.26 and 1.13 mm in FMUD+PVPI ($p<0.0001$).

In terms of the immunological parameters, in moderate pockets, the two groups did not present IL-1β reductions (Table 4) compared with baseline levels ($p>0.05$), but in deep pockets, the FMUD+SS group presented statisctically reduction of IL-1β in the first month ($p=0.0095$), and these lower levels were maintained until the sixth month ($p=0.0092$). No statistically difference was observed between groups regarding IL-1β levels. The IL-10 levels showed a tendency to increase after therapy (Table 4). For moderate pockets of FMUD+SS group, after 3 months, statistically significantly higher levels were noted than those observed at baseline ($p=0.005$), but at sixth month, these values were not different from baseline ($p=0.8751$). For FMUD+PVPI, no differences in IL-10 levels were observed nor in moderate nor in deep pockets. In spite of that, there were no between-group differences at any period of evaluation ($p>0.05$) (Table 4).

Microbiological analysis (Table 5) showed that *Aa* levels were reduced in FMUD+SS group 3 months after therapy in moderate pockets ($p=0.05$), and no statistically significant difference was seen between groups at any time ($p>0.05$). In deep pockets, *Aa* levels did not present significant reductions in the FMUD+SS group during the follow-up period ($p=0.06$), but in the FMUD+PVPI group, statistical reduction was seen after 3 and 6 months of follow-up ($p<0.05$). No significant differences were observed between groups ($p>0.05$).

There were significant reductions in *Pg* levels at the third and sixth months of follow-up ($p<0.05$), in moderate as well as in deep pockets. However, no significant between-group differences in *Pg* levels were seen ($p>0.05$).

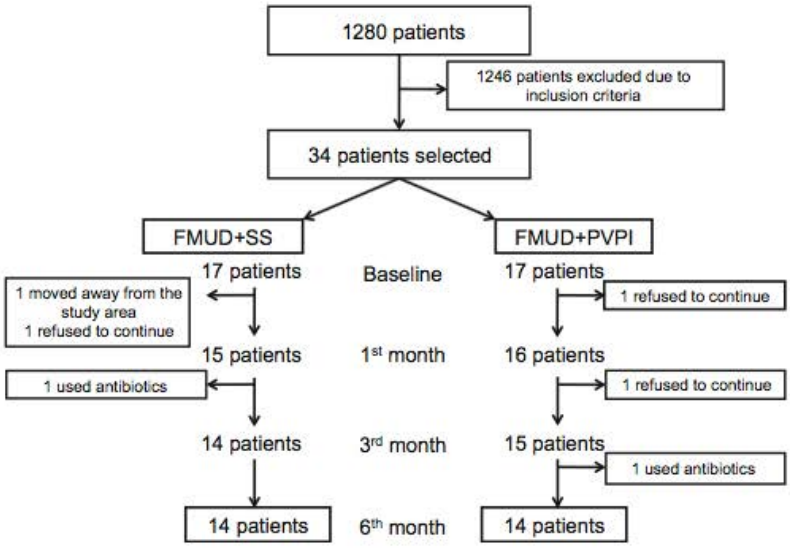

Fig. 1 Flowchart of patients included in the study protocol

Discussion

Aggressive periodontitis has an intricate etiopathology, and many factors are not completely understood [28]. However, in addition to the inconclusive understanding of its pathogenesis, GAgP also presents a reduced response to periodontal treatment [3], making this disease challenging to treat. Different treatment protocols for this disease have been tested [4–12, 29–33], and the most predictable outcomes have been obtained when antimicrobials were associated with mechanical treatment to treat patients with GAgP. Therefore, this study aimed to evaluate the effect of povidone iodine used as a cooling liquid for an ultrasonic scaler in full-mouth ultrasonic debridement.

Few clinical trials have been reported to use povidone iodine in periodontal therapy [23, 34–39]. Those studies evaluated patients with chronic periodontitis treated by different PVPI concentrations and showed discrete benefits when PVPI was associated with traditional periodontal therapy. In comparisons of scaling and root planing (SRP) plus povidone iodine with SRP alone, an additional benefit in PD reduction was seen when povidone iodine was used (0.28 mm, IC 95 %=0.08–0.48 mm, p=0.007) after 6 months of follow-up [24]. Sahrmann et al. [24] observed better clinical results with povidone iodine rinsing and two scaling and root planing interventions, performed at

Table 1 Initial demographical and clinical characteristics of subjects included in each group

Parameters	Treatment		p value
	FMUD+SS (n=14)	FMUD+PVPI (n=14)	
Age (years)	28.57 (±4.59)	28.54 (±4.14)	0.98
Gender F/M	9/5	12/2	0.38
FMPI (%)	29.12 (±11.19)	28.06 (±8.89)	0.78
FMBS (%)	40.93 (±23.06)	43.98 (±18.97)	0.71
RGMP (mm)	2.47 (±1.01)	2.15 (±0.78)	0.37
PD (mm)	6.24 (±0.43)	6.12 (±0.33)	0.29
RCAL (mm)	8.71 (±0.91)	8.36 (±0.79)	0.44

The gender parameter was evaluated by Fisher's exact test. The other parameters were analysed by Student's t test

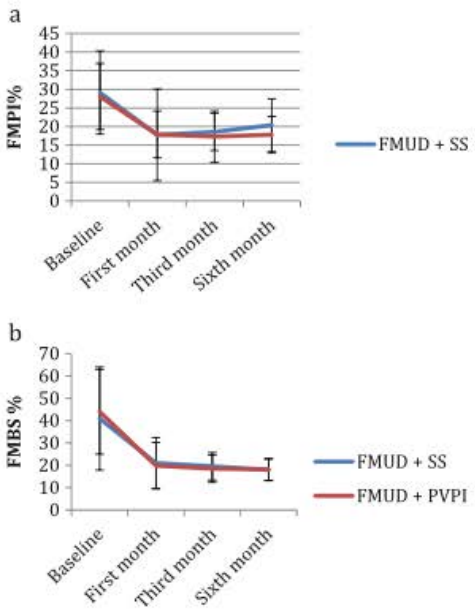

Fig. 2 Mean values (±SD) of full-mouth plaque index (FMPI) (**a**) and full-mouth bleeding score (FMBS) (**b**) of the FMUD+SS (n=14) and FMUD+PVPI (n=14) groups during the 6-month follow-up period. In FMPI as in FMBS, statistically significant differences were evaluated by ANOVA followed by Tukey's test (α=0.05). No inter-group differences were observed (p>0.05). In both groups, the parameters were statistically reduced after the first month compared with baseline (p<0.05)

Table 2 Comparison between groups based on clinical parameters (mean±SD) evaluated: relative gingival margin position (RGMP), relative clinical attachment level (RCAL) and probing depth (PD) during follow-up

Parameters/group	Follow-up period			
	Baseline	First month	Third month	Sixth month
RGMP (mm)				
FMUD+SS (n=14)	2.47 (±1.01) Ab	3.17 (±0.84) Aa	3.34 (±0.98) Aa	3.38 (±0.82) Aa
FMUD+PVPI (n=14)	2.15 (±0.78) Ab	3.13 (±0.69) Aa	3.13 (±0.68) Aa	3.12 (±0.70) Aa
RCAL (mm)				
FMUD+SS (n=14)	8.71 (±0.91) Aa	7.56 (±1.00) Ab	7.38 (±1.00) Ab	7.53 (±0.79) Ab
FMUD+PVPI (n=14)	8.36 (±0.79) Aa	7.91 (±0.72) Ab	7.69 (±0.96) Ab	7.63 (±0.95) Ab
PD (mm)				
FMUD+SS (n=14)	6.24 (±0.43) Aa	4.39 (±0.54) Ab	4.04 (±0.50) Ab	4.19 (±0.65) Ab
FMUD+PVPI (n=14)	6.12 (±0.33) Aa	4.78 (±0.47) Ab	4.56 (±0.56) Ab	4.51 (±0.49) Ab

Data from all qualifying sites are given

Different lowercase letters in rows and *different uppercase letters* in columns indicate statistically significant differences by repeated-measures ANOVA followed by Tukey's test (p<0.05)

least 1 month apart. Recently, Sahrmann et al. [39] presented that additional PD reduction was obtained 3 months after scaling and root planing with PVPI gel as adjunct substance. The authors suggested that the gel formula has a longer substantivity than the PVPI solution; therefore, it would remain for a longer period of time inside the periodontal pocket, consequently improving the clinical results. Differently from both studies previously mentioned, in the present study, the PVPI solution was only used during the initial debridment with the reinstrumentation of residual pockets performed after 6 months. The use of PVPI solution did not promote additional clinical benefits.

Table 3 Comparison between groups based on clinical parameters (median (interquartile ranges)) evaluated, including relative gingival margin position (RGMP), relative clinical attachment level (RCAL) and probing depth (PD) of moderate (5 mm≤PD<7 mm) and deep (PD≥7 mm) pockets

Initial pocket strata	Parameter/group	Follow-up period		
		Baseline	Third month	Sixth month
Moderate pocket	RGMP (mm)			
	FMUD+SS	2.92 (2.33–3.25) Ab	3.42 (2.67–3.92) Aa	3.25 (3.17–3.75) Aa
	FMUD+PVPI	2.13 (1.63–2.89) Ab	2.98 (2.44–3.54) Aa	2.90 (2.53–3.51) Aa
	RCAL (mm)			
	FMUD+SS	8.00 (7.83–8.57) Aa	7.25 (6.67–7.58) Ab	7.17 (6.50–8.00) Ab
	FMUD+PVPI	7.54 (7.29–8.30) Aa	7.13 (6.73–7.84) Ab	7.13 (6.58–7.78) Ab
	PD (mm)			
	FMUD+SS	5.36 (5.00–5.50) Aa	3.50 (3.28–4.33) Ab	3.57 (3.25–4.83) Ab
	FMUD+PVPI	5.50 (5.25–5.62) Aa	4.00 (3.83–4.52) Ab	4.00 (3.79–4.68) Ab
Deep pocket	RGMP (mm)			
	FMUD+SS	2.33 (1.28–3.00) Ab	2.83 (2.20–4.25) Aa	3.30 (2.20–4.00) Aa
	FMUD+PVPI	2.00 (1.75–2.50) Ab	3.00 (2.75–3.50) Aa	3.06 (2.75–3.50) Aa
	RCAL (mm)			
	FMUD+SS	9.38 (8.57–10.00) Aa	7.25 (6.71–8.33) Ab	7.25 (6.58–8.50) Ab
	FMUD+PVPI	9.00 (8.90–10.18) Aa	8.06 (7.58–9.28) Ab	8.31 (7.68–9.00) Ab
	PD (mm)			
	FMUD+SS	7.29 (7.20–7.50) Aa	4.50 (4.14–5.00) Ab	4.67 (4.07–5.00) Ab
	FMUD+PVPI	7.25 (7.03–7.45) Aa	5.31 (4.56–5.87) Ab	5.06 (4.62–5.50) Ab

For the parameters moderate RCAL and deep RGMP, *different lowercase letters* in rows indicate a statistically significant difference by the Friedman test (p<0.05), and *different uppercase letters* in columns indicate a statistically significant difference by the Mann-Whitney test (p<0.05). For the other parameters, different *lowercase letters* in rows and *different uppercase letters* in columns indicate statistically significant differences by repeated-measures ANOVA followed by Tukey's test (p<0.05)

Table 4 Comparison between groups based on immunological variables (mean±SD) evaluated: interleukin-1 beta and interleukin 10 of moderate (5 mm≤PD<7 mm) and deep (PD≥7 mm) pockets

Initial pocket strata	Parameter/group	Follow-up period			
		Baseline	First month	Third month	Sixth month
Moderate pockets	IL-1β (pg/mL)				
	FMUD+SS	33.55±25.64 Aa	22.00±14.38 Aa	20.23±15.19 Aa	20.51±20.97 Aa
	FMUD+PVPI	25.00±18.71 Aa	10.05±6.23 Aa	11.06±8.09 Aa	12.76±8.1) Aa
	IL-10 (pg/mL)				
	FMUD+SS	0.22±0.36 Ab	0.47±0.75 Aab	1.59±2.67 Aa	0.52±0.87 Aab
	FMUD+PVPI	0.32±0.66 Aa	0.71±0.75 Aa	0.80±0.91 Aa	0.85±1.04 Aa
Deep pockets	IL-1β (pg/mL)				
	FMUD+SS	53.21±39.10 Aa	20.41±12.02 Ab	25.79±25.84 Ab	19.68±20.43 Ab
	FMUD+PVPI	30.44±23.33 Aa	12.39±9.83 Aa	12.40±9.26 Aa	18.67±8.14 Aa
	IL-10 (pg/mL)				
	FMUD+SS	0.08±0.14 Aa	0.29±0.50 Aa	0.44±0.59 Aa	0.27±0.45 Aa
	FMUD+PVPI	0.05±0.07 Aa	0.31±0.37 Aa	0.39±0.40 Aa	0.31±0.41 Aa

Different lowercase letters in rows and *different uppercase letters* in columns indicate statistically significant differences by repeated-measures ANOVA followed by Tukey's test ($p<0.05$)

Using the proposed protocol of the present study, povidone iodine presented in moderate pockets a RCAL gain of 0.54± 0.52 mm and PD reduction of 1.30±0.47 mm (while in control group, these values were, respectively, 0.76±1.15 and 1.35± 1.13 mm). In deep pockets, the RCAL gains were 1.75±0.98 and 1.13±0.94 mm, and PD reductions were 2.74±0.75 and 2.26±0.98 mm in the FMUD+SS and FMUD+PVPI groups, respectively. This absence of additional clinical benefits of subgingival irrigation has been described by Krück et al. [38], who treated patients with chronic periodontitis by scaling and root planing, followed immediately by pocket irrigation with 0.9 % sodium chloride solution, 0.12 % chlorhexidine digluconate or 7.5 % povidone iodine. This study showed no statistical difference between the irrigation substances regarding PD reduction, clinical attachment level gain and bleeding on probing reduction. Koshy et al. [40] and Zanatta et al. [23], using the same non-surgical therapy protocol with an ultrasonic device and povidone iodine or water irrigation to

Table 5 Between-group comparisons based on microbiological variables: quantitative presence (median (interquartile ranges)) of *Aggregactibacter actinomycetemcomitans* (*Aa*) and *Porphyromonas gingivalis* (*Pg*) in moderate (5 mm≤PD<7 mm) and deep (PD≥7 mm) pockets

Initial pocket strata	Parameter/group	Follow-up period		
		Baseline	Third month	Sixth month
Moderate pocket	*Aa**			
	FMUD+SS	5.46 (3.96–5.99) Aa	2.04 (0–4.60) Ab	4.12 (0.75–4.95) Aab
	FMUD+PVPI	5.04 (4.69–5.78) Aa	3.76 (2.44–4.23) Aa	4.13 (1.43–5.26) Aa
	*Pg**			
	FMUD+SS	5.82 (5.49–7.25) Aa	1.19 (0–4.82) Ab	0 (0–4.20) Ab
	FMUD+PVPI	5.42 (2.42–5.85) Aa	0.49 (0–2.20) Ab	0 (0–1.65) Ab
Deep pocket	*Aa***			
	FMUD+SS	5.49 (0–5.72) Aa	3.30 (0–5.15) Aa	3.38 (0–4.34) Aa
	FMUD+PVPI	5.33 (3.97–5.47) Aa	3.77 (1.92–4.18) Ab	3.45 (1.47–3.93) Ab
	*Pg**			
	FMUD+SS	5.83 (5.15–6.74) Aa	0 (0–2.90) Ab	2.21 (0–4.02) Ab
	FMUD+PVPI	4.87 (4.33–5.62) Aa	2.25 (0–3.61) Ab	1.10 (0–3.12) Ab

**Different lowercase letters* in rows and *different uppercase letters* in columns indicate statistically significant differences by repeated-measures ANOVA followed by Tukey's test ($p<0.05$)

***Different lowercase letters* in rows indicate statistically significant differences by the Friedman test ($\alpha=0.05$), and *different uppercase letters* in columns indicate statistically significant differences by the Mann-Whitney test ($\alpha=0.05$)

treat generalised chronic periodontitis, also failed to found additional clinical benefits with PVPI irrigation.

The FMUD+PVPI group presented PD reduction and RCAL gain of 1.30 and 0.54 mm in moderate pockets after 6 months. In deep pockets, the values of PD reduction and RCAL gain after 6 months were 2.26 and 1.13 mm in that group. Studies investigating the association of scaling and root planing with systemic antibiotics (amoxicillin+metronidazole) found PD reductions and clinical attachment gains of 1.4 and 1.6 [7] and 2 and 2 mm [11] in moderate pockets. In deep pockets, the same studies found PD reductions and clinical attachment gains of 3.7 and 4.2 mm [7] and 4.0 and 3 mm. Although the present study did not investigate the comparison between topical and systemic antibiotics, it seems plausible that the concentrations of antibiotics in gingival crevicular fluid after periodontal therapy could explain the lower values found in the present study. During systemic antibiotic therapy, the concentrations of drugs in gingival crevicular fluid are greater than in a single topical use of PVPI, which, in deep pockets, could probably reduce the bacterial challenge, thereby aiding the host response.

The values found in this study were similar to those found in controlled groups of some studies that tested GAgP treatments. Guerrero et al. [5] presented, in the control group (scaling and root planing+placebo), PD reductions of 1.0 and 1.8 mm, and clinical attachment gains of 0.8 and 1.3 mm in moderate and deep pockets, respectively. Hughes et al. [41] showed, in deep pockets, a PD reduction of 2.11±2.01 mm and a clinical attachment gain of 1.77±2.15 mm after scaling and root planing. Similar values were also found by Haas et al. [9] in moderate (PD reduction of 1.25±0.17 mm and clinical attachment gain of 0.74±0.28 mm) and deep pockets (PD reduction of 2.76±0.51 mm and clinical attachment gain of 1.35±0.34 mm) in patients treated by scaling and root planing plus placebo. Mestnik et al. [11] showed PD reductions and clinical attachment gains of approximately 1.0 and 1.0 mm in moderate pockets and 2.50 and 2.00 mm in deep pockets 3 months post-scaling and root planing alone. The results found in this study are in agreement with those presented in the literature, supporting the idea that the protocol used in this study could be indicated for the treatment of GAgP.

After treatment, there was an increase on the relative position of the gingival margin values on all groups; however, no difference was observed between the groups. In spite of this increase, one cannot infer that gingival recession occurred, since this parameter was represented by the distance from the gingival margin until the stent used during the clinical examinations and not the cement-enamel junction. Instead, these results indicate that both therapies were able to reduce the gingival margin edema, which is aimed during periodontal therapy.

Concerning the immunological parameters, IL-1β is a pro-inflammatory cytokine, and its level could be used as a bone loss predictor in patients with periodontitis [42]. Thus, the reduction in IL-1β is desirable after periodontal therapy. For the anti-inflammatory cytokine IL-10, one of its functions is the suppression of tissue destruction [43], which makes its increase after treatment an expected goal of therapy [44]. In this study, both groups promoted this tendency to reduce IL-1β and no difference was observed between groups. Difference was found only in deep pockets for FMUD+SS group. Tendency to increase IL-10 levels were also seen in both groups, but no differences in intra- or inter-group evaluations were observed. Other authors who evaluated these inflammatory markers also presented high inter-individual variability of GCF markers and have not shown statistically significant differences between groups with chronic [25] and GAgP disease [12, 45, 46].

On the microbiological evaluation, povidone iodine therapy did not promote statistical reduction of *Aa* levels in moderate pockets, and no statistically significant difference was seen between groups during the evaluation period. Interestingly, in deep pockets, the FMUD+PVPI group showed a statistically significant reduction in *Aa* levels on the 3-month evaluation. These results are in agreement with Krück et al. [38] who showed that PVPI could exert some effect on *Aa* reduction 3 months after the therapy. Nonetheless, in the present study, both groups remained with similar outcomes and no statistical difference. Although no difference could be seen between groups, *Aa* is a key pathogen in GAgP disease and should be a focus of disease control, as its presence has been associated with further clinical attachment loss. It has been shown to persist in periodontal sites when these were treated only by mechanical therapy, with significant reductions when antimicrobials were adjunctively used [12].

Regarding the *Pg* level, no significant additional reduction could be noticed with the use of PVPI. Studies that have presented additional benefits of PVPI use in the reduction of *Pg* levels [38, 39] performed different treatment protocols. Krück et al. [38] found significant *Pg* reduction using one session full-mouth scaling and root planing with curretes and ultrasonic device instrumentation 5 min per tooth associated with 10 mL povidone iodine irrigation per quadrant, and the patients were instructed to rinse their mouth twice daily with 10 mL of 0.12 % chlorhexidine digluconate for 10 days. Sahrmann et al. [39] found lower levels of *Pg* in the PVPI group compared to the baseline values, the treatment protocol included scaling and root planing associated with PVPI gel application. Sahrmann et al. [47] also presented that povidone iodine gel seems to be a good way to use this farmacol being detectable in the pocket 15 min after the application, allowing a prolonged remnant effect in the pocket.

In the present study, the FMUD protocol could also negatively influence PVPI action in the subgingival region, as, in this protocol, all teeth were instrumented within 45 min,

which represents less than 2 min per tooth. It has been reported in the literature that PVPI acts against periodontal bacteria in a very low time exposure [19], but Sahrmann et al. [24] described that factors like GCF and bleeding can dilute and wash the PVPI from the periodontal pocket, negating its effective action. Sahrmann et al. [47] described that povidone iodine solution applied immediately after scaling and root planing presents significant concentration decrease within 1 min, which is probably influenced by the bleeding and GCF. The use of specific subgingival ultrasonic inserts seems to be insufficient for spraying the irrigation solution all the way to the bottom of the periodontal pocket [48]. Differently from the use of a syringe, in the ultrasonic insert, the liquid was released at the middle of the insert. This characteristic could influence the extent of PVPI irrigation inside the pocket, which might have affected its antimicrobial action.

Since this is the first study enrolling generalised aggressive periodontitis subjects treated by povidine iodine adjuctive to mechanical therapy, there are still answer to be searched for. Thus, future studies including new treatment approaches should consider including this hard-to-treat population and assess the benefits of PVPI in this kind of patients. Also, different methods of PVPI application should be tested to evaluate its effect on microbiological and, consequently, clinical/immunological results.

Conclusion

We concluded that 10 % PVPI used as an irrigant solution in FMUD decreased *Aa* levels in deep pockets but had no additional benefits when compared with saline solution irrigation in terms of clinical, microbiological and immunological results.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures in this study were performed in accordance with the ethical standards of the Ethics Committee of Piracicaba Dental School (protocol 024/2006), and written informed consent was obtained from all individual participants included in the study.

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3.6 The combination of amoxicillin and metronidazole improves clinical and microbiologic results of one-stage, full-mouth, ultrasonic debridement in aggressive periodontitis treatment.*

Abstract

BACKGROUND: The aim of the present study is to assess clinical, microbiologic, and immunologic benefits of amoxicillin/metronidazole (AM) when performing full-mouth ultrasonic debridement (FMUD) in generalized aggressive periodontitis (GAgP) treatment.

METHODS: Twenty-four GAgP patients were divided into two groups: the FMUD group (n = 12), which received FMUD plus placebo, and the FMUD+AM group (n = 12), which received FMUD and 375 mg amoxicillin plus 250 mg metronidazole for 7 days. The following clinical outcomes were tested: plaque and bleeding on probing indices, pocket probing depth (PD), relative gingival margin position (GMP), and relative clinical attachment level (CAL). Total amount of *Porphyromonas gingivalis* (Pg), *Aggregatibacter actinomycetemcomitans* (Aa), *Tannerella forsythia* (Tf), and gingival crevicular fluid (GCF) concentration of interleukin (IL)-10 and IL-1β were also determined. All clinical, microbiologic, and immunologic parameters were assessed at baseline and at 3 and 6 months post-therapy. The ANOVA/Tukey test was used for statistical analysis (α = 5%).

RESULTS: Amoxicillin/metronidazole used as an adjunct to the FMUD protocol added clinical and microbiologic benefits to GAgP treatment (P <0.05). FMUD+AM groups presented an additional PD reduction in initially deep PDs at the 3-month follow-up (3.99 ± 1.16 mm and 3.09 ± 0.78 mm for FMUD+AM and FMUD, respectively; P <0.05), a lower number of residual pockets at the 3- and 6-month follow-ups, and a statistical reduction in amounts of Aa (P <0.05). Analysis of Tf and Pg amounts, as well as IL-10 and IL-1β GCF concentrations failed to demonstrate a difference between the groups (P >0.05).

CONCLUSION: It may be concluded that amoxicillin/metronidazole improves clinical and microbiologic results of FMUD in GAgP treatment.

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The Combination of Amoxicillin and Metronidazole Improves Clinical and Microbiologic Results of One-Stage, Full-Mouth, Ultrasonic Debridement in Aggressive Periodontitis Treatment

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Background: The aim of the present study is to assess clinical, microbiologic, and immunologic benefits of amoxicillin/metronidazole (AM) when performing full-mouth ultrasonic debridement (FMUD) in generalized aggressive periodontitis (GAgP) treatment.

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Conclusion: It may be concluded that amoxicillin/metronidazole improves clinical and microbiologic results of FMUD in GAgP treatment. *J Periodontol 2012;83:988-998.*

KEY WORDS

Aggressive periodontitis; anti-infective agents; debridement; randomized clinical trial; ultrasonic.

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A review of the literature discussing antimicrobial therapy as an adjunct to mechanical therapy in the treatment of aggressive periodontitis indicates that its use could provide additional benefits.[1,2] In these patients, amoxicillin associated with metronidazole was shown to be the most predictable antimicrobial protocol with regard to clinical and microbiologic benefits. Xajigeorgiou et al.[3] evaluated different antimicrobial protocols as adjuncts to traditional quadrant scaling and root planing. Comparing doxycycline, metronidazole, and the combination of amoxicillin and metronidazole (AM), the authors concluded that there was a greater reduction in probing depth (PD) when amoxicillin and metronidazole were prescribed in combination. Other studies[4-7] also demonstrated better results when using this combination of antimicrobials in aggressive periodontitis treatment.

Organized subgingival biofilm is resistant to the action of antimicrobials,[8] and researchers have presented data that demonstrate better results from the immediate intake of an antimicrobial after the conclusion of mechanical therapy, and a possible benefit resulting from completing this mechanical therapy in a shorter time, preferably <1 week).[9] In this context, the association of antimicrobial therapy with one-stage, full-mouth, ultrasonic debridement (FMUD) could be an important alternative. The FMUD protocol is based on the bacterial translocation concept (i.e., the potential of microbials presented in non-treated sites and other oral niche colonize previously-treated sites) and the knowledge that extensive scaling and root planing is not necessary to reestablish periodontal health.[10,11] The purpose of this protocol is to debride all sites presenting signs of periodontitis within a short period of time using an ultrasonic scaler. Studies have shown that this protocol has the potential to provide results similar to those obtained with traditional quadrant scaling and root planing in the treatment of chronic periodontitis.[12-16] In addition, this protocol is less time consuming[12,13,16] and demonstrates a better cost-benefit ratio. Recently, Aimetti et al.[17] assessed the clinical and microbiologic changes in generalized aggressive periodontitis (GAgP) patients treated with one-stage, full-mouth disinfection (OSFMD) protocol. The 6-month follow-up indicated that OSFMD could be a viable approach to deal with severe GAgP because it promoted a reduction in PD and a gain in clinical attachment level (CAL). However, to the best of our knowledge, no study has evaluated the additional benefits of AM in this protocol for treating aggressive periodontitis.

Thus, the aim of the present study was to determine the clinical, microbiologic, and immunologic effects of systemic antimicrobials in a one-stage FMUD protocol in treating GAgP.

MATERIALS AND METHODS

Study Design

This study is designed as a parallel, masked, randomized, placebo-controlled clinical trial to assess the clinical, microbiologic, and immunologic outcomes of FMUD with AM for the treatment of GAgP. The study was approved by the Ethics Committee of the University of Campinas (06/0024). The first treatment occurred in February 2006, and examinations ended in December 2008.

Population Screening

Potential study participants were selected from patients referred to the Graduate Clinic of Piracicaba Dental School, University of Campinas, Piracicaba, Brazil. The study inclusion criteria were: 1) diagnosis of GAgP;[18] 2) presence of ≥20 teeth; 3) presence of ≥8 teeth presenting PD ≥ 5 mm with bleeding on probing (BOP) and ≥2 teeth with PD ≥ 7 mm (including incisors and first molars, in addition to two other non-contiguous teeth between them); 4) good general health; and 5) <35 years of age. Patients who: 1) were pregnant or lactating; 2) were suffering from any other systemic diseases (e.g., cardiovascular, diabetes); 3) received antimicrobials in the previous 3 months; 4) were taking long-term anti-inflammatory drugs; 5) received a course of periodontal treatment within the last 6 months; or 6) smoked were excluded from the study. Written informed consent was obtained from included participants.

Prior to experimental study, patients underwent a 1-month supragingival therapy, which included one session of prophylaxis, supragingival-calculus removal, and oral hygiene instructions.

Clinical Parameters

All clinical parameters were assessed by a single examiner (RCVC) before periodontal therapy (baseline) and at 3 and 6 months afterward using a manual probe.‖

The following clinical parameters were evaluated: 1) full-mouth plaque index (FMPI), according to Ainamo and Bay[19] and full-mouth bleeding score (FMBS), according to Mühlemann and Son[20]; 2) PD, distance from the bottom of pocket to gingival margin; 3) relative gingival margin position (GMP), distance from the gingival margin to stent margin; and 4) relative CAL, distance from the bottom of the pocket to the stent margin.

The measurements were standardized using an individually manufactured acrylic stent in which a groove was made to determine the specific site of probing (recorded to the nearest 0.5 mm). All teeth presenting ≥1 site with PD ≥5 mm were preselected for clinical evaluation, and all presenting pulpar disease or furcation lesions were excluded. From

‖ PCP-UNC 15, Hu-Friedy, Chicago, IL.

selected teeth, acrylic guides were prepared, and in teeth presenting >1 site with PD ≥ 5 mm, the deepest one was selected. These sites were designated as qualifying sites.

Calibration, Randomization, and Power Calculation

Initially, a total of three non-study patients presenting with GAgP were selected. The designated examiner (RCVC) measured relative CAL and PD in all patients twice within 24 hours with an interval of ≥1 hour between examinations. The intraclass correlation was calculated for each parameter, resulting in 91% reproducibility for relative CAL and 94% for PD. This calibration was repeated annually.

Patients were allocated to groups according to a computer-generated list (under responsibility of MZC). This code was not broken until the follow-up was concluded. The use of a placebo ensured both the patients' and examiner's masking to the antimicrobial therapy. A person other than those responsible for treatment and medication delivery (EDPR) and the clinical examination (RCVC) was responsible for patient allocation.

Power calculation was done before the study with a statistical software program.[¶] This analysis indicated that with 12 patients in each group, the study would have 80% power to detect a 1-mm difference in the CAL of patients in the two groups. After the completion of the study, the authors considered the standard deviation of each group in the present clinical trial and confirmed that the power value was 80% to detect a 1-mm difference in the CAL of patients in the two groups.[16]

Non-Surgical Treatment

After random allocation, patients were subjected to one of the two treatment protocols: 1) FMUD group (n = 12): One session of FMUD with a time limit of 45 minutes, using an ultrasonic scaler[#] with subgingival tips[**] (previously described in Zanatta et al.,[14] Ribeiro Edel et al.,[21] and Del Peloso Ribeiro et al.[16]) plus two distinct placebo pills prescribed on the day of treatment, and taken every 8 hours for 7 days. 2) FMUD+AM group (n = 12): Same treatment as FMUD group but members took 375 mg amoxicillin and 250 mg metronidazole prescribed on the day of treatment, every 8 hours for 7 days.[22]

Both antimicrobial and placebo pills were produced by the same company.[††] To maintain patient masking, the placebo pills used looked identical to those containing antimicrobials (amoxicillin pill was blue and metronidazole was red). Compliance with pill intake was also determined via an interview with patients after the period of intake. Moreover, the patients were instructed to take one 500-mg sodium dipyrone every 6 hours if they felt pain or discomfort.

Adverse Effects Evaluation

At the end of periodontal treatment, patients were asked to fill out a questionnaire that measured pain by means of a visual analog scale. This scale consisted of a horizontal line marked to the left as "no pain" and at the right as "unbearable pain." In this scale, the patients were instructed to make a mark correspondent to their experience of pain during and after treatment. In addition, patients were asked to record the number of analgesics taken, their body temperature, any oral ulcerations, and gastrointestinal discomfort.

Biofilm and Gingival Crevicular Fluid Collection

Subgingival biofilm samples were collected from two sites in each patient, from a pocket initially presenting PD = 5 mm (moderate pocket) and from another pocket presenting PD ≥7 mm (deep pocket). Following the supragingival biofilm removal and relative isolation with cotton rolls, a sterile paper point (#35) was inserted into the bottom of the periodontal pocket for 30 seconds. The paper points were placed into sterile tubes containing 300 μL 0.5-mM Tris-EDTA.

Gingival crevicular fluid (GCF) was collected from two sites presenting initially PD = 5 mm (moderate pocket) and another two sites presenting PD ≥ 7 mm (deep pocket). Sixty seconds after the subgingival biofilm collection, the area was isolated and gently dried. GCF was collected by placing filter paper strips[‡‡] into the pocket until the clinician (RCVC) perceived a slight resistance and then leaving them in place for 15 seconds. The fluid volume was measured with a calibrated electronic GCF measuring device.[§§] After volume measurements were taken, the strips were placed into sterile tubes containing 400 μL phosphate-buffered saline (PBS) with 0.05% polysorbate 20.[‖‖] Both subgingival biofilm and GCF samples were immediately stored at –20°C.

Microbiologic Evaluation

The presence and concentration of *Porphyromonas gingivalis* (*Pg*), *Aggregatibacter actinomycetemcomitans* (*Aa*), and *Tannerella forsythia* (*Tf*) were evaluated using specific primers reported by a previous researcher in the literature[23] using a real-time polymerase chain reaction (PCR) technique.

Initially, DNA was extracted from the subgingival biofilm. A real-time PCR was performed using the hot start reaction mix for PCR.[¶¶] The concentration of the DNA used in each run was 10 μg/mL. The

¶ SAS v. 9.1, SAS Institute, Cary, NC.
# Cavitron, DENTSPLY, York, PA.
** FSI-SLI, DENTSPLY.
†† PharmaTerra, Bellevue, WA.
‡‡ PerioPaper, Oraflow, Plainview, NY.
§§ Periotron 8000, Oraflow.
‖‖ Tween 20, Sigma-Aldrich, St. Louis, MO.
¶¶ FastStart DNA Master SYBR Green I, Roche Diagnostics, Mannheim, Germany.

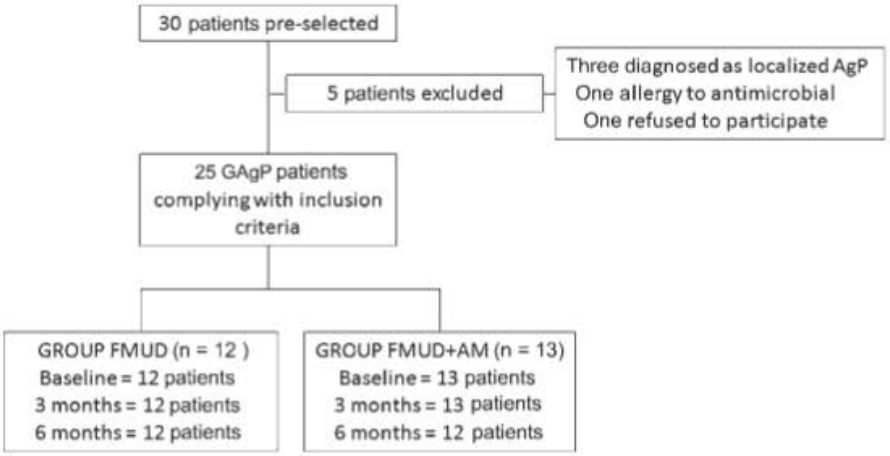

Figure 1.
Flowchart of the study. AgP = aggressive periodontitis.

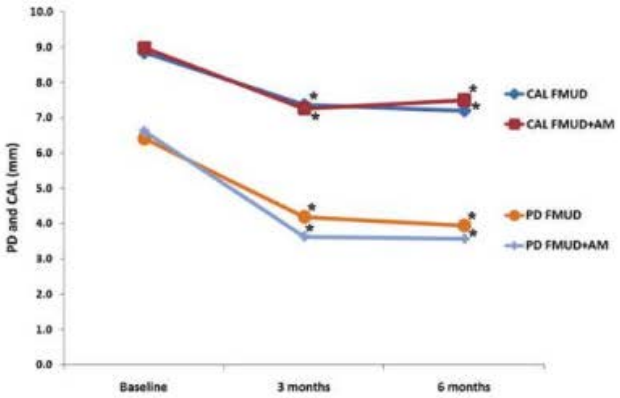

Figure 2.
Full mouth PD and CAL (mm) variation in FMUD and FMUD+AM groups during experimental period.
**Statistically significant difference from baseline (ANOVA/Tukey test, P ≤0.05).*

amplification profiles were as follows: 95°/10′, 55°/5′, 72°/4′, 40 cycles for *Pg*; 95°/10′, 55°/5′, 72°/3′, 40 cycles for *Aa*; and 95°/10′, 55°/5′, 72°/3′, 45 cycles for *Tf*. Absolute quantification of target bacteria in clinical samples was performed using *Pg* (ATCC 33277), *Aa* (JP2), and *Tf* (ATCC 43037) as controls. The determination of DNA genome copies in controls was based on the genome size of each bacteria and the mean weight of one nucleotide pair. The microbiologic analyses were separately performed (moderate and deep pockets).

GCF Cytokine Levels

Aliquots of each GCF sample were examined using an enzyme-linked immunosorbent assay (ELISA) commercially available in a kit## for interleukin (IL)-1β and IL-10, following the manufacturer's instructions. Previously, samples were diluted with the diluent found in the kit. The dilution was considered to calculate the concentration of each GCF substance.

This concentration was calculated with a standard curve, prepared as recommended by the fabricant. The standard curve range used for IL-1β was 1,000 to 15.60 pg/mL, and for IL-10 the range was 50 to 0.20 pg/mL. The ELISA assays were run in duplicate, and mean values were used to calculate concentrations of each cytokine. The immunologic analyses were performed separately in moderate and deep pockets.

Data Management and Statistical Analyses

The statistical analysis considered the per-protocol population (i.e., patients who completed the follow-up). The null hypothesis tested was that FMUD associated with AM added no clinical, microbiologic, or immunologic benefits to the treatment of GAgP patients compared with FMUD alone. To test this hypothesis, a statistical software program was used.

The homogeneity of groups at baseline (PD and relative CAL, primary variable) was tested using the one-way analysis of variance (ANOVA)/Tukey test. For clinical parameters, a repeated-measures ANOVA was used to detect intragroup differences in clinical parameters (relative GMP, PD, relative CAL), considering the patient as a statistical unit. The results of relative GMP, PD, and relative CAL refer strictly to the qualifying sites. When a statistical difference was found, an analysis of the difference was determined using the Tukey method. The Student *t* test was used to determine the differences between groups regarding changes in clinical parameters and the percentage of residual pockets. The Friedman test was used to detect intragroup differences, and the Kruskal-Wallis test was used for intergroup analysis of full-mouth plaque and bleeding indices in all periods. The experimental level of significance was determined to be 5%.

R&D Systems, Minneapolis, MN.

Table 1.

Patients' Characteristics at Baseline

Characteristics	FMUD	FMUD+AM
Age (years) (mean ± SD)	28.3 ± 5.9	28.8 ± 6.2
Females (%)	58.3	75
FMPI (mean ± SD)	25.8 ± 7.1	23.8 ± 4.6
FMBS (mean ± SD)	34.3 ± 6.1	39.3 ± 6.5
Number of teeth at baseline	22.9 ± 2.4	23.3 ± 2.4
Full-mouth percentage of sites presenting PD ≥5 mm	42.1 ± 4.7	39.7 ± 5.6
Number of sites evaluated (per patient)	8.4 ± 0.8	8.3 ± 0.9
Number of moderate pockets (PD 5 to 6 mm)	4.5 ± 1.2	4.3 ± 1.5
Number of deep pockets (PD ≥7 mm)	3.9 ± 1.7	4.0 ± 1.8
Relative GMP (mm) (mean ± SD)	2.43 ± 1.42	2.37 ± 1.42
PD (mm) (mean ± SD)	6.42 ± 0.54	6.61 ± 0.80
Relative CAL (mm) (mean ± SD)	8.85 ± 0.93	8.99 ± 1.56

At baseline, no significant differences were observed in any characteristic analyzed (ANOVA/Tukey test for age, sex, and clinical parameter comparison; Kruskal-Wallis test for FMPI and FMBS analysis).

Table 2.

FMPI and FMBS Score at Baseline and at 3 and 6 Months (mean ± SD)

Index	Group	Baseline	3 Months	6 Months
FMPI	FMUD	25.8 ± 7.1	20.5 ± 3.3	19.4 ± 3.0
	FMUD+AM	23.8 ± 4.6	18.8 ± 3.9	15.6 ± 3.1
FMBS	FMUD	34.3 ± 6.1	11.7 ± 2.9*	11.0 ± 3.0*
	FMUD+AM	39.3 ± 6.5	11.1 ± 3.3*	10.3 ± 2.0*

* Statistically significant difference from baseline (Friedman test; $P<0.05$).

RESULTS

Patient Accountability

Figure 1 illustrates the study flowchart. Patients were examined, and 30 patients who met the inclusion criteria were initially recruited. Five of them were excluded (three were diagnosed with localized aggressive periodontitis, one had an allergy to antimicrobials, and one refused to participate), and 25 were randomly assigned to one of two groups. All participants received the allocated procedure; however, during follow-up, one patient became pregnant and was dropped from the FMUD+AM group.

Patient Characteristics at Baseline

No differences were observed between groups regarding age, sex, or oral hygiene status (full-mouth plaque and bleeding indices). With regard to the clinical parameters, the groups presented similar values of CAL and PD (Table 1). All patients in the groups presented a similar percentage of pockets presenting a PD of ≥5 mm ($P>0.05$). The number of qualifying sites included in the follow-up in each pocket strata were also similar, with no significant differences ($P>0.05$).

Treatment Assessments

Plaque and bleeding indices. The authors found no significant differences in mean FMPI and FMBS between groups during any period of the evaluation ($P>0.05$) (Table 2). The plaque index showed no significant change during the study, but there was a statistically significant reduction in the bleeding index in the third month in all patients, which was maintained throughout the study (Table 2).

Clinical parameters. When the FMUD protocol was associated with AM therapy, the authors noted some benefits with regard to a reduction in PD, especially in deep pockets ($P<0.05$) (Table 3). In deep pockets, AM promoted a superior reduction in PD statistically different from that observed in the placebo group (3.99 ± 1.16 and 3.09 ± 0.78 mm, respectively

Table 3.
Comparison Between Clinical Outcomes (mean ± SD) of FMUD and FMUD+AM Groups at Different Periods of Evaluation in Moderate and Deep Pockets

Pocket Strata	Clinical Parameter (mm)	Groups	Baseline	3 Months	Difference Between 0 and 3 Months	6 Months	Difference between 0 and 6 Months
Moderate pockets (PD = 5 to 6 mm)	Relative GMP	FMUD	2.64 ± 1.11	3.05 ± 0.83*	−0.41 ± 0.66	3.09 ± 0.92*	−0.45 ± 0.58
		FMUD+AM	2.40 ± 1.86	3.16 ± 1.85*	−0.56 ± 0.63	3.31 ± 1.73*	−0.74 ± 0.73
	PD	FMUD	5.19 ± 0.32	3.53 ± 0.74*	1.65 ± 0.81	3.67 ± 1.10*	1.52 ± 1.19
		FMUD+AM	5.06 ± 0.16	3.23 ± 0.58*	1.84 ± 0.62	3.47 ± 0.59*	1.59 ± 0.60
	Relative CAL	FMUD	7.83 ± 1.06	6.58 ± 0.95*	1.24 ± 0.79	6.76 ± 1.41*	1.07 ± 1.26
		FMUD+AM	7.46 ± 1.86	6.39 ± 2.10*	1.28 ± 0.23	6.79 ± 1.91*	0.81 ± 0.68
Deep pockets (PD ≥7 mm)	Relative GMP	FMUD	2.45 ± 1.01	3.38 ± 1.08*	−0.93 ± 0.52	3.42 ± 0.91*	−0.97 ± 0.57
		FMUD+AM	2.20 ± 1.26	3.63 ± 1.39*	−1.37 ± 0.67	4.19 ± 1.60*	−1.67 ± 0.97
	PD	FMUD	7.54 ± 0.57	4.46 ± 0.83*	3.09 ± 0.78†	4.08 ± 0.90*	3.28 ± 0.60
		FMUD+AM	7.73 ± 0.90	3.77 ± 1.10*	3.99 ± 1.16	3.60 ± 1.38*	4.13 ± 1.44
	Relative CAL	FMUD	10.00 ± 1.29	7.84 ± 1.24*	2.16 ± 0.75	7.50 ± 1.17*	2.49 ± 1.02
		FMUD+AM	9.93 ± 1.60	7.40 ± 2.10*	2.61 ± 1.02	7.70 ± 2.60*	2.34 ± 1.57

* Statistically significant difference from baseline (ANOVA/Tukey test; α = 5%).
† Statistically significant difference between groups (Student *t* test; α = 5%).

[*P* <0.05]). Regarding the other parameters (relative GMP and relative CAL), no additional benefits were noted in patients who received antimicrobial therapy (Fig. 2).

Residual Pockets (PD ≥5 mm and BOP)

After 3 and 6 months, patients in the FMUD+AM group presented the lowest percentage of residual pockets, that is, PD ≥ 5 mm and BOP, differing statistically from the FMUD group (Table 4). At 3 months, the FMUD group presented with 28.8% of periodontal pockets still showing signs of disease whereas the FMUD+AM group presented with only 12.3% (*P*<0.05). At the 6 months, a similar result could be seen. The FMUD+AM group presented with a statistically lower percentage of residual pockets than the FMUD group, that is, 10.7% versus 26.0%, respectively (*P*<0.05).

Microbiologic Results

Aa. In moderate and deep pockets, *Aa* was statistically reduced only when antimicrobials were prescribed as an adjunct to FMUD. In moderate pockets, a significant reduction in *Aa* levels was observed during the 6-month follow-up exam in the FMUD+AM group (*P* <0.05), but there were no differences between the groups (*P* >0.05) (Fig. 3A).

Regarding initially deep pockets, the levels of harbored *Aa* were reduced at 3 and 6 months in the patients that received AM as an adjunct to mechanical therapy (*P* <0.05). However, no differences were noticed between groups in any one period of evaluation (*P* >0.05).

Pg. Patients in both groups of FMUD showed statistically reduced *Pg* amounts at the 3- and 6-month follow-ups (*P*<0.05) in initially moderate and deep pockets (Fig. 3B).

Tf. Concerning *Tf* amounts in moderate and deep pockets, a phenomenon similar to that seen with *Pg* was found (Fig. 3C). Again, patients in both groups showed reduced *Tf* amounts at 3 and 6 months (*P* <0.05), but there was no difference between them (*P* >0.05).

Immunologic Results

IL-1β. GCF IL-1β levels are displayed in Figure 4A. It was seen that patients in the FMUD and FMUD+AM groups had a reduction of the IL-1β level at the 3- and 6-month follow-ups in moderate and deep pockets. However, no differences between the groups were observed (*P* >0.05).

IL-10. IL-10 was significantly increased in moderate pockets at only the 3-month follow-up in the FMUD and FMUD+AM groups (*P* <0.05) and returned to the baseline level at the 6-month follow-up (*P* <0.05). No differences between the groups were observed (Fig. 4B).

Adverse Effects

The degree of pain after treatment did not differ between groups (Table 5). Moreover, with regard to analgesic intake, fever, oral ulceration, or gastrointestinal discomfort, both protocols were similar ($P>0.05$).

DISCUSSION

The present study assesses the FMUD protocol associated with adjunctive antimicrobial therapy in GAgP treatment. The results show that patients treated with the protocol that included AM reaped additional benefits with regard to a reduction in PD, frequency of residual pockets, and reduction in *Aa* compared with patients who received the FMUD alone.

Previous researchers[3,24,25] have reported that different antimicrobial therapies used in aggressive periodontitis treatment have shown good results. Azithromycin, doxycycline, metronidazole, and amoxicillin are antimicrobials used in regimens that showed potential clinical benefits in GAgP treatment.[3,24,25] However, based on the action in *Aa* infections,[26-28] and on the intimate relationship of this bacterium with GAgP,[23,29] the association of AM appears to be the regimen of choice. Pavicić et al.[30] demonstrated a synergism of these agents when they are used to reduce *Aa*. Xajigeorgiou et al.,[3] evaluating different antimicrobial regimens, showed significant benefits of the AM association with regard to the numbers of sites presenting PD >6 mm, when compared with a placebo group. In another clinical trial, Guerrero et al.[5] also demonstrated a superior clinical result when AM was adjunctively prescribed. In deep pockets, the full-mouth disinfection of patients treated with AM revealed higher values of PD reduction and gain of CAL than those in the placebo group. This preferable action of antimicrobial therapy in deep pockets, as observed in the present study, was a commonly achieved goal in previous studies,[31] although some other studies found additional benefits (i.e., CAL gain and PD reduction) in initially moderate pockets.[32]

Regarding microbiologic analysis, the association of FMUD+AM was able to reduce *Aa* statistically. Interestingly, mechanical therapy alone did not reduce it statistically. *Aa* presents with the capacity to invade periodontal tissues, as well as to adhere to the root surface,[33,34] which makes its suppression difficult. Xajigeorgiou et al.[3] showed that the association of these antimicrobials was the only protocol that reduced the levels of *Aa* significantly. Recently, Mestnik et al.,[35] reporting the short-term (3-month follow-up) microbiologic changes after treatment with SRP+AM or SRP+placebo, showed that mean counts of *Aa* were significantly lower in the test compared with the placebo group in sites with initially deep PD. These results corroborate those of the present study, although some others did not show additional benefits regarding *Aa* reduction to AM.[36]

Regarding *Tf* and *Pg*, it could be seen that both pathogens were statistically reduced after FMUD with and without antimicrobials association. It suggests that adequate scaling and root planing and biofilm control is enough to promote their reduction and a re-establishment of periodontal health, also in GAgP patients. This result is similar to that shown by previous studies using different techniques for detection.[35,36] In relation to immunologic results, both groups presented similar changes during the experimental

Table 4.

Percentage (mean ± SD) of Residual Pockets (PD ≥5 mm and BOP) After 3 and 6 Months of Treatment at Different Groups

	Residual Pockets (%)		
Period	FMUD	FMUD+AM	*P* Value*
3 months	28.8 ± 9.8	12.3 ± 10.9	0.03
6 months	26.1 ± 11.6	10.7 ± 11.3	0.04

* Student *t* test (α = 5%).

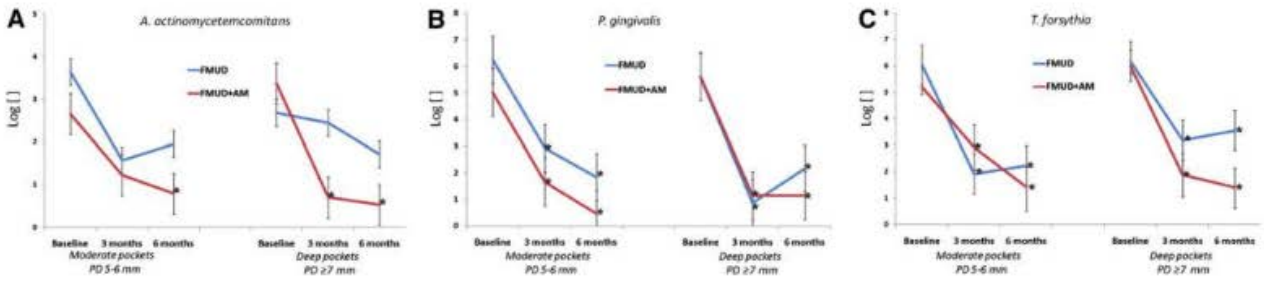

Figure 3.

Total amounts (log [ ] ± SD) of ***A)*** *Aa,* ***B)*** *Pg, and* ***C)*** *Tf at baseline and at 3 and 6 months post-treatment with FMUD and FMUD+AM. Vertical bars represent standard deviation (SD).* *******Statistically significant difference from baseline (ANOVA/Tukey test; P ≤0.05).*

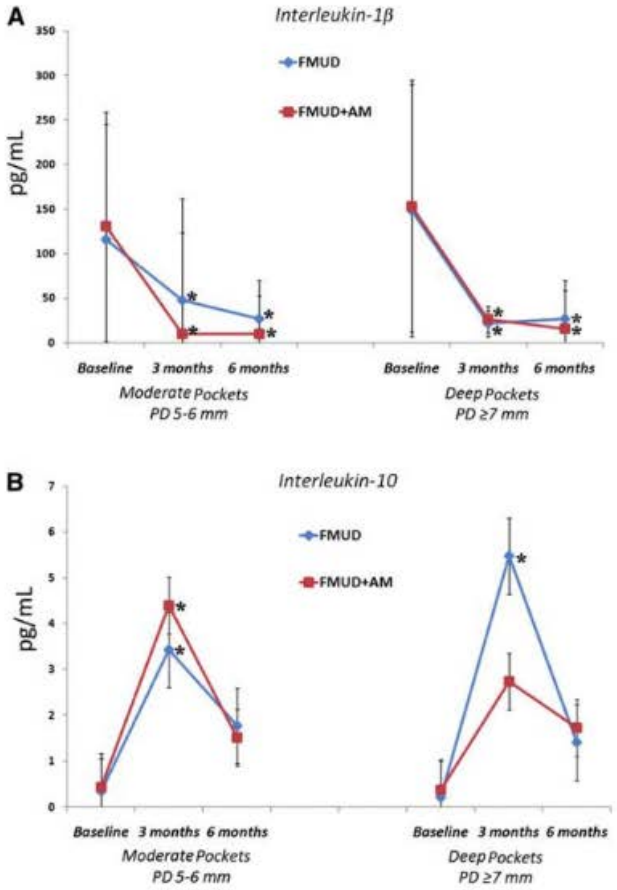

Figure 4.
*GCF concentration (pg/mL ± SD) of **A)** IL-1β and **B)** IL-10 at baseline and at 3 and 6 months post-treatment with FMUD and FMUD+AM. Vertical bars represent standard deviation (SD). *Statistically significant difference from baseline (ANOVA/Tukey test; P ≤0.05).*

period. Immunologic evaluation is important because the balance between local levels of cytokines, stimulated in response to periodontopathogenic bacteria and their products, determines the outcome of the immune response. This means that cytokines play an important role in the initiation and progression of periodontitis. IL-1β, a proinflammatory marker, was reduced at 3 months and 6 months, whereas IL-10, an anti-inflammatory cytokine, was higher at 3 months than at baseline. Other studies achieving the same markers also showed a reduction in IL-1β and an increase of IL-10 over time; however, the authors did not identify any differences between groups.[16,21] Del Peloso Ribeiro et al.[16] discussed the high interindividual variability of GCF markers and did not show any statistically significant difference.

Therefore, it should be pointed out that the antimicrobial therapy promoted additional benefits when associated with one-stage FMUD. This result could be attributed to the disruption of biofilm in all periodontal sites immediately before the antimicrobial intake period. It is well established that, as an organized structure, biofilm is protected against antimicrobial action.[8] This idea gains importance when one considers the treatment sequence of the quadrant debridement, when one quadrant per week is treated, associated with antimicrobials intake. In this protocol, some of the periodontal pockets had not yet been scaled or had been scaled some weeks before antimicrobial intake, depending on whether antimicrobials were prescribed at the first or last session of scaling. This situation could lead to a reduced action of antimicrobials in non-scaled or reorganized biofilm areas and facilitate the recolonization of sites due to bacteria originating from other periodontal pockets. Thus, additional benefits may be achieved when full-mouth scaling is performed. Based on this idea, review of some of the literature indicated that these types of mechanical protocols (based on disinfection of all periodontal sites in only one or some sessions) could be preferable when antimicrobials are prescribed.[9]

The full-mouth disinfection (FMD) protocol was initially described by Quirynen et al.[37] and involves the administration of scaling and root planing of all sites within 24 hours along with an adjunct prescription of antiseptics to avoid bacterial translocation. Mongardini et al.[38] first evaluated the original protocol in "early-onset" periodontitis, showing additional reductions in PDs and CAL gains. This protocol, with some modifications, was recently evaluated as a GAgP treatment in association with antimicrobial therapy. Guerrero et al.[5] showed reductions in PD of 1.5 mm (moderate pockets) and 3.1 mm (deep pockets) in FMD+AM and 1.0 mm (moderate pockets) and 1.8 mm (deep pockets) in FMD+placebo. In a Brazilian population, two clinical trials with patients with aggressive periodontitis showed clinical and microbiologic benefits when AM was prescribed as an adjunct to FMD.[35,39] However, the full-mouth disinfection protocol recommends extensive scaling and root planing, which could possibly cause significant removal of root substance and dentin hypersensitivity.

Another recent study with a Brazilian population[36] assessed AM adjunctive to mechanical therapy. The results showed a similar periodontal improvement in GAgP patients treated by a combination of AM or placebo, mechanical therapy, and chemical agents (pocket irrigation with 0.2% chlorhexidine [CHX] gel and rinse, gargle, and tongue brushing with a 0.12% CHX solution). However, in the present study, we opted for a more conservative root instrumentation, that is, full-mouth ultrasonic debridement, performed with an ultrasonic scaler in a short period of time. When compared with the results of previous FMD-protocol studies, FMUD promotes a similar reduction

Table 5.

Adverse Effects Occurrence

Adverse Effects Parameters	FMUD	FMUD+AM
Level of pain, day 0* (VAS score)	2.20 (0 to 5)	2.00 (0.4 to 6.2)
Level of pain, day after* (VAS score)	1.29 (1 to 3)	1.33 (0 to 2.4)
No. of analgesics, day 0*	0.33 (0 to 2)	0.42 (0 to 2)
No. of analgesics, day after*	0 (0 to 0)	0 (0 to 0)
Occurrences of fever, day 0	2	1
Occurrences of fever, day after	0	0
Occurrences of oral ulceration	0	0
Occurrences of gastrointestinal discomfort	0	2

Day 0 = same day of treatment; day after = next day after treatment; VAS = visual analog scale.
No significant differences were observed between groups at any parameter evaluated (Kruskal-Wallis test for pain and analgesic intake evaluation and χ^2 test for fever, oral ulceration, and gastrointestinal discomfort analysis).
* Mean (minimum – maximum).

in PD (1.59 ± 0.6 mm and 4.13 ± 1.44 mm in moderate and deep pockets, respectively). Additionally, in the present study, a lower number of sites presenting PD ≥5 mm after therapy is found when antimicrobial therapy was prescribed.

Periodontal pockets presenting a PD ≥5 mm and BOP have been described as a risk indicator for future attachment loss[40,41] and have been used as a threshold to determine the need for re-treatment in some studies.[16,21] In this study, FMUD and the use of AM promoted a lower percentage of residual pockets, that is, sites with PD ≥5 mm and BOP, than FMUD without antimicrobial therapy. Guerrero et al.,[5] Mestnik et al.,[35] and Heller et al.[36] also indicate a lower percentage of sites presenting PD >5 mm when AM was prescribed adjunctively to mechanical therapy. This result could be associated with a lower re-treatment requirement throughout follow-up, an important factor when analyzing the clinical significance of treatment.

Moreover, the antimicrobial therapy protocol should be studied further. There is no consensus in the literature regarding dose, type, and period of intake of antimicrobial therapy. In the present study, AM (375 mg amoxicillin and 250 mg metronidazole for a period of 7 days) is used following the steps of the previous study by Winkel et al.[22] It is important to remember that bactericidal agents could present a synergic effect when associated (as shown with the AM combination[27,30]), and high dosages could lead to higher adverse effects. With this in mind, the results of the present study show that this dosage could promote some additional benefits with no significant adverse effects. Recent studies[5,35,36] have shown that different dosages could be used to obtain clinical and microbiologic benefits. Guerrero et al.[5] prescribed 500 mg amoxicillin and 500 mg metronidazole and discussed that this dose was adequate for achieving a local concentration that provided an adequate antimicrobial effect, which was confirmed in further studies.[35,42] Another point related to number of patients included in the present study. Although GAgP is a rare condition, a larger population could confirm without any bias. Future studies, with larger and different populations, presenting results of a long-term follow-up and also testing different dosages of antimicrobials should be conducted to clarify and confirm the results.

CONCLUSION

Within the limits of this study, it could be concluded that AM associated with FMUD promotes some clinical and microbiologic benefits to improve the outcome of treatment for generalized aggressive periodontitis.

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3.7 Amoxicillin/metronidazole associated with nonsurgical therapy did not promote additional benefits in immunologic parameters in generalized aggressive periodontitis: A randomized controlled clinical trial.*

This randomized, blinded, placebo-controlled clinical trial compared the levels of interferon γ (IFN-γ), prostaglandin E2 (PGE2), and interleukin 6 (IL-6) in the gingival crevicular fluid (GCF) from generalized aggressive periodontitis (GAgP) patients treated with nonsurgical therapy associated or not with amoxicillin/metronidazole adjunctive.

METHOD AND MATERIALS:

Thirty-nine GAgP patients were followed during 6 months. The patients were randomly allocated to one of the groups: experimental (scaling and root planing plus 375 mg amoxicillin and 250 mg metronidazole for 7 days) and control (scaling and root planing + placebo). Probing pocket depth (PPD), relative clinical attachment level (rCAL), gingival margin position (GMP), and IL-6, IFN-γ, and PGE2 levels in GCF were evaluated at baseline, and at 3 and 6 months after treatment.

RESULTS:

Both therapies promoted PPD reductions, rCAL gains, and recession in GMP at the end of the study, with the experimental group presenting an additional PPD reduction in fullmouth analysis and deep pockets at the 3- and 6-month follow-ups ($P < .05$). During the period of the study, only the experimental group promoted a reduction in PGE2 levels in deep pockets at 3 and 6 months, while IFN-γ and IL-6 levels remained unchanged. However, the differences in the immunologic parameters were not statistically significant among the groups.

CONCLUSION:

It can be concluded that amoxicillin/ metronidazole associated with nonsurgical therapy promotes an additional PPD reduction in the treatment of GAgP; however, this therapy did not promote additional benefits in the evaluated immunologic parameters.

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Amoxicillin/metronidazole associated with nonsurgical therapy did not promote additional benefits in immunologic parameters in generalized aggressive periodontitis: A randomized controlled clinical trial

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Objective: This randomized, blinded, placebo-controlled clinical trial compared the levels of interferon γ (IFN-γ), prostaglandin E_2 (PGE_2), and interleukin 6 (IL-6) in the gingival crevicular fluid (GCF) from generalized aggressive periodontitis (GAgP) patients treated with nonsurgical therapy associated or not with amoxicillin/metronidazole adjunctive. **Method and Materials:** Thirty-nine GAgP patients were followed during 6 months. The patients were randomly allocated to one of the groups: experimental (scaling and root planing plus 375 mg amoxicillin and 250 mg metronidazole for 7 days) and control (scaling and root planing + placebo). Probing pocket depth (PPD), relative clinical attachment level (rCAL), gingival margin position (GMP), and IL-6, IFN-γ, and PGE_2 levels in GCF were evaluated at baseline, and at 3 and 6 months after treatment. **Results:** Both therapies promoted PPD reductions, rCAL gains, and recession in GMP at the end of the study, with the experimental group presenting an additional PPD reduction in full-mouth analysis and deep pockets at the 3- and 6-month follow-ups ($P < .05$). During the period of the study, only the experimental group promoted a reduction in PGE_2 levels in deep pockets at 3 and 6 months, while IFN-γ and IL-6 levels remained unchanged. However, the differences in the immunologic parameters were not statistically significant among the groups. **Conclusion:** It can be concluded that amoxicillin/metronidazole associated with nonsurgical therapy promotes an additional PPD reduction in the treatment of GAgP; however, this therapy did not promote additional benefits in the evaluated immunologic parameters. *(doi: 10.3290/j.qi.a34723)*

Key words: aggressive periodontitis, antimicrobials, interleukin-6, prostaglandin E_2, interferon-γ, periodontal therapy

Generalized aggressive periodontitis (GAgP) is a rapidly progressive periodontal disease that is characterized by severe and rapid destruction of the supporting of the teeth, which, if left untreated, can lead to early edentulism.[1-3] The attachment loss and bone destruction result from interactions between specific subgingival micro-

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bial species and the susceptible host, leading to the release of inflammatory mediators that mediate tissue destruction.[4,5] The immune responses to subgingival biofilm are orchestrated by cytokines including pro-inflammatory cytokines, T-helper type 1 (Th1) cytokines, and anti-inflammatory cytokines.[6]

Initially, a Th1 immune response occurs with the release of pro-inflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) into the gingiva.[7-9] When released in high concentrations, these cytokines can stimulate the production and release of other inflammatory mediators such as IL-6, matrix metalloproteinases (MMPs), and prostaglandin E_2 (PGE_2).[10] The Th1 response is maintained by interferon γ (IFN-γ), which inhibits the T-helper (Th2) response.[8,11] This inflammatory process can gradually lead to the destruction of connective tissue and alveolar bone.[12,13]

IL-6, one of the most studied inflammatory markers of periodontal disease,[14] is involved in the inflammation-induced tissue destruction of the periodontium and was found in elevated levels in inflamed gingival tissues and gingival crevicular fluid (GCF) of patients with gingivitis and chronic and in refractory periodontitis.[14-17] PGE_2 has been implicated as a key inflammatory mediator in periodontal diseases.[18] Levels of PGE_2 in GCF could be used as a predictor for periodontal attachment loss;[19] thus, one of the results of periodontal therapy should be a reduction in the levels of this mediator.[20,21] IFN-γ, the main cytokine secreted by Th1 subsets and natural killer cells, induces the macrophage activation and the production of a series of pro-inflammatory mediators, such as TNF-α. Increased numbers of IFN-γ in the GCF have been associated with progression of chronic periodontitis.[22,23]

Focusing on GAgP, studies have shown that this disease has an intimate relationship with an imbalance between pro- and anti-inflammatory cytokines in response to periodontopathogenic bacteria and their products.[6,24] Moreover, there is some evidence that imbalance between some pro-inflammatory cytokines and anti-inflammatory cytokines can be partially reversed by periodontal treatment.[15,24] However, GAgP presents a reduced response to nonsurgical periodontal treatment, adding to etiopathology specificities the need to develop more predictable therapies.

In this regard, adjunctive use of antibiotics during treatment of patients with aggressive periodontitis provides some additional benefits compared with mechanical therapy alone.[25,26] In these patients, the combination of amoxicillin with metronidazole has shown to be the most predictable antimicrobial protocol with regard to clinical and microbiologic benefits.[27,28] Recently, Casarin et al[25] reported that GAgP patients treated with ultrasonic debridement plus amoxicillin/metronidazole promoted clinical and microbiologic benefits with regard to a reduction in pocket probing depth (PPD), frequency of residual pockets, and reduction in *Aggregatibacter actinomycetemcomitans* levels compared to patients who received mechanical therapy alone. However, immunologic response to periodontal treatment in aggressive periodontitis is much less understood.

Therefore, in view of the personal immunologic characteristics of GAgP and the clinical and microbiologic benefits of adjunctive use of amoxicillin/metronidazole, this study compared the levels of IFN-γ, PGE_2, and IL-6 in the GCF of GAgP patients treated with nonsurgical therapy associated with and without antimicrobial therapy.

METHOD AND MATERIALS

Study design

The present study was designed as a parallel, randomized, blinded, and placebo-controlled clinical trial of 6 months duration to assess the immunologic outcomes of nonsurgical periodontal therapy with amoxicillin/metronidazole for the treatment of GAgP. The study design was, before commencement, approved by the Ethics Committee of the University of Campinas (06/0024), and was conducted in accordance with the Declaration of Helsinki. All patients received a detailed description of the proposed treatment and provided informed written consent to participate in the study. The first treatment occurred in February 2006, and all 6-month follow-up visits were completed in December 2008.

Population screening

All subjects were selected from individuals referred to the Graduate Clinic of Piracicaba Dental School, University of Campinas, after examinations that include a full medical and dental history, an intraoral examination, full-mouth periodontal probing, and radiographic evaluation.

Subjects who were invited to participate met the following inclusion criteria:

- diagnosis of GAgP[1]
- presence of at least 20 teeth
- at least eight teeth presenting PPD ≥ 5 mm with bleeding on probing (having at least two with PPD ≥ 7 mm)
- good general health < 35 years of age.

Exclusion criteria were as follows:

- pregnancy or lactation
- systemic diseases (cardiovascular, diabetes, others)
- consumption of antimicrobials or anti-inflammatory drugs within the past 3 months
- scaling and root planing in the preceding 6 months
- smoking
- orthodontic therapy
- sensitivity/allergy to amoxicillin or metronidazole.

Sample size, randomization, and allocation concealment

A sample-size calculation was assessed with a statistical software (G*Power 3.1.7, Universität Kiel), included an α error of 5%, 80% power, and a standard deviation (SD) of 1.0 mm, and a difference of 1 mm in the relative clinical attachment level (rCAL) of patients in the two groups was considered clinically significant. It was indicated that a sample of 17 patients per group would be needed. Considering that some patients could be lost during follow-up, 24 patients per group were included.

Patients were randomized into two groups according to a computer-generated list (under responsibility of MZC). This code was not broken until all data had been collected. Thus, the treatment group was not revealed to the clinical examiner or the statistician. The use of a placebo ensured both patients' and the examiner's blindness to the antimicrobial therapy. A person other than those responsible for treatment and medication delivery (EDPR) and the clinical examination (RCVC) was responsible for subject allocation.

Periodontal therapy

Patients initially received detailed information on the etiology of periodontal disease and instructions for self-performed plaque control measures, with a non-traumatic sulcular brushing technique, and interdental cleaning with dental floss and interdental toothbrushes. Oral hygiene instructions were given in order to lower plaque accumulation to approximately 20% to 30% of dental surfaces. In the initial sessions, patients also had plaque retentive factors (caries, excess of restorations, and supragingival calculus) removed. The baseline measurements were done 30 days after this initial phase.

After random allocation, patients were subjected to one of the two treatment protocols:

- Control group (n = 18): scaling and root planing alone using periodontal curettes (Hu-Friedy) and/or an ultrasonic scaler (Cavitron, Dentsply) with subgingival tips (FSI-SLI, Dentsply) plus two distinct placebo pills, every 8 hours for 7 days, prescribed on the same day of treatment.
- Experimental group (n = 21): same treatment as control group plus 375 mg of amoxicillin and 250 mg of metronidazole, every 8 hours for 7 days,[29] prescribed on the same day of treatment.

Both antimicrobial and placebo pills were produced by the same company (Pharmaterra). In order to maintain patient blindness, the placebo pills used were identical to those containing antimicrobials (the amoxicillin pill was blue and metronidazole was red). Compliance with pill intake was also determined via an interview with patients after the period of intake. Moreover, the patients were instructed to take 500 mg sodium dipyrone if they felt pain or discomfort, respecting the limit of one pill every 6 hours.

Supportive therapy was scheduled, with weekly recalls during the first month, and monthly until the

sixth month after treatment. During these appointments, supragingival calculus removal, prophylaxis, and oral hygiene instructions were performed when needed. After the sixth month, teeth that still presented PPD ≥ 5 mm were retreated by scaling and root planing with curettes and local anesthesia.

Clinical examination

Clinical measurements were evaluated at baseline and at 3 and 6 months post-therapy with a manual probe (PCP15, Hu-Friedy). The following clinical parameters were measured:

- full-mouth plaque index (FMPI), according to Ainamo and Bay,[30] and full-mouth bleeding score (FMBS), according to Muhlemann and Son[31]
- PPD: distance from the bottom of pocket to the gingival margin
- GMP: distance from the gingival margin to the stent margin
- rCAL: distance from the bottom of the pocket to the stent margin.

All teeth presenting at least one site with PPD ≥ 5 mm were selected for clinical evaluation. The sites to be analyzed were standardized using an individually manufactured acrylic stent in which a groove was made to determine the site, recorded to the nearest 0.5 mm. Teeth that showed no involvement of furcation or pulp disease were selected.

Examiner calibration

The same examiner (RCVC), who was masked to the therapies, carried out all measurements of clinical evaluation. To perform the intra-examiner calibration, three non-study subjects presenting GAgP were selected. The designated examiner (RCVC) measured rCAL and PPD in all patients twice within 24 hours, with an interval of at least 1 hour between the examinations. The intra-class correlation was calculated for each parameter, resulting in 91% reproducibility for rCAL and 94% for PPD. This calibration was repeated annually.

Evaluation of adverse effects

At the end of periodontal treatment, patients were asked to fill out a questionnaire that measured pain by means of a visual analog scale. This scale consisted of a horizontal line marked to the left as "no pain" and at the right as "unbearable pain". In this scale, the patients were instructed to make a mark correspondent to their experience of pain during and after treatment. Patients also were asked to record their body temperature, any oral ulcerations, gastrointestinal disorder, or other adverse events.

GCF collection

GCF samples were collected from four sites in each patient, two from pockets presenting initially PPD = 5 to 6 mm (moderate pocket) and two from pockets presenting PPD ≥ 7 mm (deep pocket). After removal of the supragingival biofilm, teeth were washed and the area was isolated and gently dried.[32] GCF was collected by placing filter paper strips (Periopaper, Oraflow) into the pocket until a slight resistance was perceived and then left in place for 15 seconds; fluid volume was measured with a calibrated electronic gingival fluid measuring device (Periotron 8000, Oraflow). After volume measurements, the strips were placed into sterile tubes containing 400 µL of phosphate-buffered saline (PBS) with 0.05% Tween-20. Strips contaminated by visible blood were discarded and another sample was collected after 30 seconds. All samples were immediately stored at −20°C.

GCF cytokine levels

Aliquots of each GCF sample were assayed by a colorimetric competitive binding enzyme-linked immunosorbent assay (ELISA) for PGE_2, and sandwich ELISA for IFN-γ and IL-6, using commercially available kits (R&D Systems) according to the manufacturer's instructions. Previously, samples were vortexed for 30 seconds and centrifuged for 5 minutes at 1,500 *g*, and then samples were diluted with the diluent of the kit. The dilution was considered to calculate the concentration of each GCF substance. The absorbance of each well was read in a microplate spectrophotometer (VersaMax, Molecular Devices) at 450 nm, and the concentration was cal-

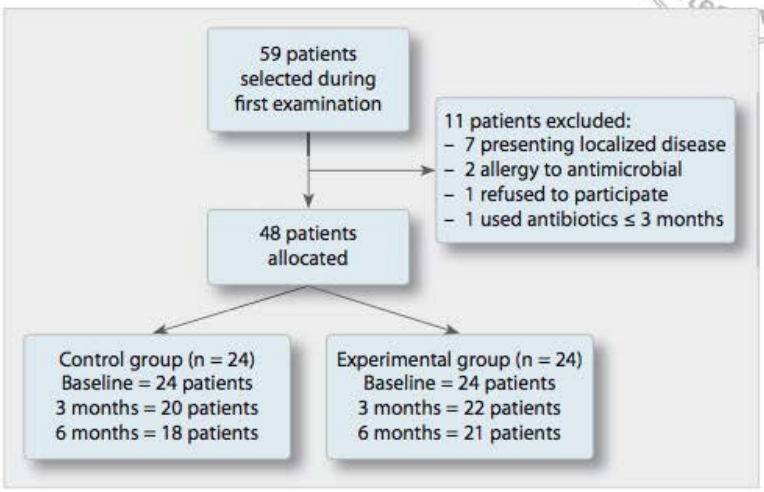


Fig 1 Flowchart of the study.

culated with a standard curve, which was prepared using the standard proteins in the kit. The prepared measurement range of the standard curve for PGE_2 was 125 to 19.26 pg/mL; for IFN-γ the range was 1,000 to 15.60 pg/mL, and for IL-6 was 4,050 to 0.99 pg/mL. All samples, standards, and control (PBS) were assayed in duplicate and mean values were used to calculate concentrations of each cytokine. Calculations of the protein concentrations in each site (pg/mL) were established by dividing the total amount of the each mediator by the GCF volume. Samples with cytokine levels below the detection limit of the assay were scored as 0 pg/mL. The immunologic analyses were performed separately in moderate and deep pockets.

Statistical analysis

The statistical analysis considered the per-protocol population (ie, patients who completed the follow-up). The primary outcome measurement of the study was rCAL. Secondary outcomes included:

- GCF markers
- PPD
- GMP
- FMPI
- FMBS
- adverse effects.

The homogeneity of data was tested through the Shapiro-Wilk test. Since the data achieved normality, parametric methods were used for the comparisons. Clinical parameters were compared by analysis of variance (ANOVA), and a pair-wise comparison was performed by the Tukey test, considering the patient as a statistical unit. The Student *t* test was used to determine the differences between groups regarding changes in clinical parameters (PPD, GMP, rCAL). Friedman and Dunn's multiple comparison tests were used to perform intragroup comparisons, whereas the Mann-Whitney test was used for intergroup analyses regarding cytokine levels. The Kruskal-Wallis test was used for intergroup analysis for pain analysis; and chi-square test for fever, oral ulceration, and gastrointestinal discomfort analysis. An experimental level of significance was determined at 5%. The software used included BioEstat 5.0 (Instituto Sustentável Mamirauá) and SAS 9.1 (SAS Institute).

RESULTS

Patient accountability

Figure 1 illustrates the study flowchart. Patients were examined, and 59 patients who met the inclusion criteria were initially recruited. Eleven of them were excluded (seven were diagnosed with localized aggressive peri-

Table 1 Patients' characteristics at baseline (no significant differences were observed in any characteristic analyzed [Tukey test])

Characteristic	Control group	Experimental group
Age (mean ± SD; years)	27.5 ± 5.5	28.5 ± 5.1
Gender (% female)	67	72
FMPI (mean ± SD)	38.2 ± 6.8	28.5 ± 11.1
FMBS (mean ± SD)	32.6 ± 7.6	35.6 ± 6.7
PPD (mean ± SD; mm)	6.4 ± 0.4	6.5 ± 0.5
rCAL (mean ± SD; mm)	8.6 ± 1.0	8.6 ± 1.4

Table 2 Mean (± SD) of full mouth plaque index (FMPI) and bleeding score (FMBS) at baseline, and 3 and 6 months

Index	Group	Baseline	3 months	6 months
FMPI	Control	38.2 ± 6.8	27.8 ± 6.1	24.4 ± 3.9
	Experimental	28.5 ± 11.1	24.2 ± 6.6	22.5 ± 5.1
FMBS	Control	32.6 ± 7.6	12.8 ± 3.0*	12.5 ± 5.5*
	Experimental	35.6 ± 6.7	12.2 ± 4.5*	11.0 ± 3.8*

*Indicates statistically significant difference to baseline (Tukey test, $P < .05$).

Table 3 Comparison between clinical outcomes (mean ± SD) of control and experimental groups at different periods of evaluation

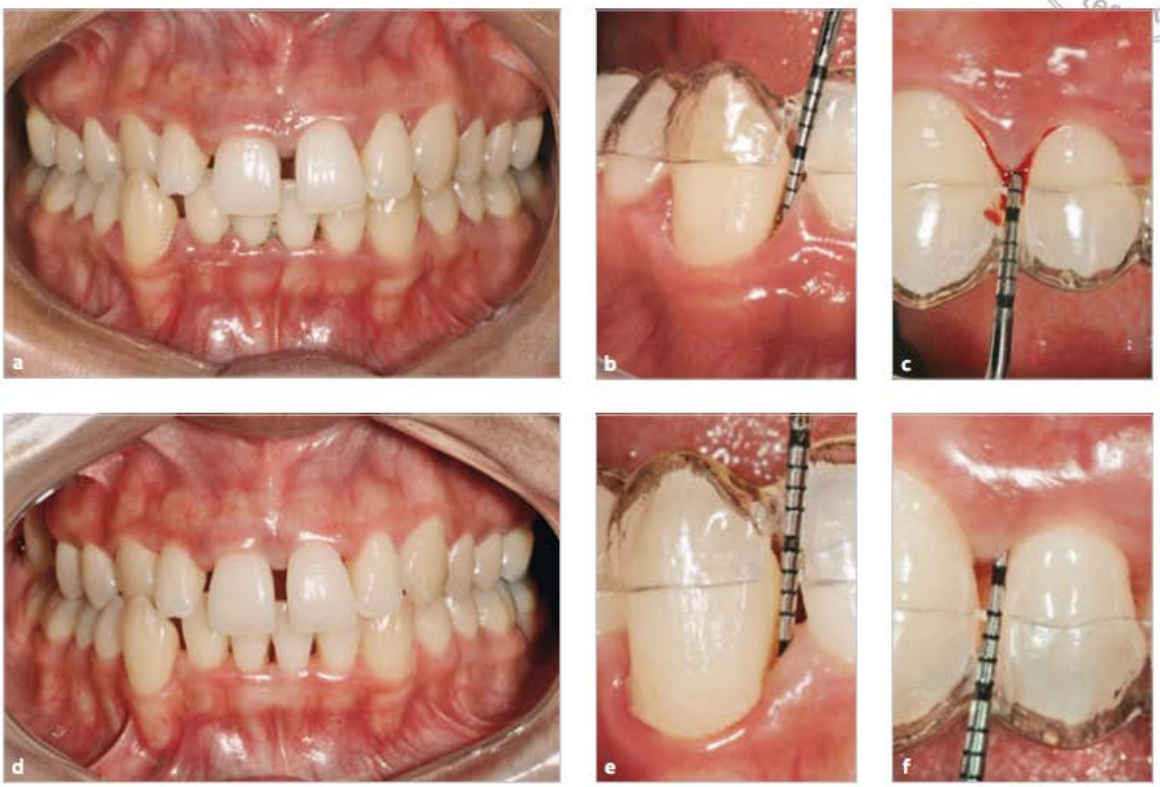
Evaluation	Parameter	Group	Baseline	0–3 months	0–6 months
Full-mouth	GMP	Control	2.2 ± 0.9^A	−0.7 ± 0.5^A	−0.7 ± 0.5^A
		Experimental	2.1 ± 1.3^A	−1.0 ± 0.4^A	−1.1 ± 0.6^A
	rCAL	Control	8.6 ± 1.0^A	1.4 ± 0.8^A	1.5 ± 0.9^A
		Experimental	8.6 ± 1.4^A	1.9 ± 0.6^A	1.7 ± 0.9^A
	PPD	Control	6.4 ± 0.4^A	2.2 ± 0.6^B	2.1 ± 0.7^B
		Experimental	6.5 ± 0.5^A	2.9 ± 0.7^A	2.7 ± 0.8^A
Moderate pockets, 5–6 mm	GMP	Control	2.3 ± 1.0^A	0.6 ± 0.6^A	0.6 ± 0.5^A
		Experimental	2.1 ± 1.5^A	0.6 ± 0.5^A	0.6 ± 0.7^A
	rCAL	Control	7.5 ± 1.1^A	1.0 ± 0.9^A	0.9 ± 1.0^A
		Experimental	7.2 ± 1.5^A	1.1 ± 0.6^A	1.0 ± 0.8^A
	PPD	Control	5.3 ± 0.3^A	1.6 ± 0.8^A	1.5 ± 1.0^A
		Experimental	5.0 ± 0.2^A	1.7 ± 0.7^A	1.7 ± 0.6^A
Deep pockets, ≥ 7 mm	GMP	Control	2.2 ± 0.9^A	0.8 ± 0.8^A	0.9 ± 0.8^A
		Experimental	2.1 ± 1.1^A	1.3 ± 0.6^A	1.3 ± 0.9^A
	rCAL	Control	9.6 ± 1.1^A	2.0 ± 1.0^A	2.2 ± 1.0^A
		Experimental	9.9 ± 1.5^A	2.4 ± 1.0^A	2.3 ± 1.2^A
	PPD	Control	7.5 ± 0.6^A	2.9 ± 0.9^A	3.0 ± 0.9^A
		Experimental	7.8 ± 0.9^A	3.7 ± 1.2^B	3.8 ± 1.3^B

Different letters (capital for intergroup analysis) indicate statistical difference by Student *t* test ($P < .05$).

odontitis, two had an allergy to antimicrobials, one refused to participate, and one used antibiotics ≤ 3 months), and 48 were randomly assigned to participate in the study and received the allocated procedure. Six patients in the control group were lost during follow-up (three due to address changes, two due to the administration of antibiotic for medical reasons, and one patient became pregnant); and three patients in the experimental group were lost during follow-up (one patient became pregnant, and two due to address changes). Thus, 18 subjects in the control group and 21 subjects in the experimental group were included in the statistical analyses.

Patient characteristics at baseline

No differences were observed between groups regarding age, gender, or oral hygiene status (full-mouth



Figs 2a to 2f Clinical aspects of full-mouth *(a)* and PPD measurements *(b and c)* at baseline, and at 6 months of follow-up *(d to f)* in the experimental group.

plaque and bleeding indices). With regard to the clinical parameters, the groups presented similar values of clinical attachment loss and PPD (Table 1).

Clinical results

No significant differences in mean FMPI and FMBS were found between groups during any period of the evaluation ($P > .05$) (Table 2). Both groups showed, at 3 and 6 months, an increase in GMP, a reduction in PPD, and a gain in CAL in all pockets strata ($P < .05$). When the scaling and root planing was associated with amoxicillin/metronidazole therapy, there was a superior reduction in PPD in full-mouth analysis, and especially in deep pockets at 3 and 6 months, statistically different from that observed in the control group (Table 3). There was no difference between groups in the reduction of moderate pockets. At 6 months, amoxicillin/metronidazole promoted a reduction in PPD in deep pockets of 3.8 ± 1.3 mm, while the control group promoted 3.0 ± 0.9 mm of PPD reduction. Regarding the other parameters (GMP and rCAL), no additional benefits were noted in patients who received antimicrobial therapy for full mouth analysis, moderate or deep pockets.

Figure 2 illustrates the clinical aspects and PPD measurements at baseline, and at 6 months of follow-up, for a patient of the experimental group.

Cytokine levels

Table 4 shows GCF levels of each cytokine at baseline and 3 and 6 months post-therapy. An intergroup analysis demonstrated no significant difference between the control and experimental groups in the concentration of PGE_2, IL-6, and IFN-γ in GCF, at all periods of

Table 4 GCF concentration (pg/mL) of PGE_2, IL-6, and IFN-γ at baseline, and 3 and 6 months after treatment in the control and experimental groups (mean ± SD)

Evaluation	Parameter	Group	Baseline	3 months	6 months
Moderate pockets	PGE_2	Control	$464.5.8 \pm 321.6^{Aa}$	452.3 ± 297.5^{Aa}	295.2 ± 270.2^{Aa}
		Experimental	365.9 ± 265.9^{Aa}	341.9 ± 158.7^{Aa}	304.3 ± 208.5^{Aa}
	IL-6	Control	1.13 ± 1.01^{Aa}	1.0 ± 0.9^{Aa}	1.1 ± 1.8^{Aa}
		Experimental	1.5 ± 0.8^{Aa}	1.1 ± 1.9^{Aa}	1.2 ± 1.5^{Aa}
	INF-γ	Control	0.81 ± 0.9^{Aa}	1.2 ± 1.6^{Aa}	1.6 ± 2.3^{Aa}
		Experimental	0.7 ± 0.4^{Aa}	1.8 ± 3.0^{Aa}	1.2 ± 1.6^{Aa}
Deep pockets	PGE_2	Control	276.7 ± 102.2^{Aa}	315.7 ± 156.5^{Aa}	178.0 ± 127.1^{Aa}
		Experimental	337.3 ± 197.5^{Aa}	225.0 ± 137.5^{Ab}	114.9 ± 105.7^{Ab}
	IL-6	Control	0.6 ± 0.8^{Aa}	0.3 ± 0.3^{Aa}	0.8 ± 0.6^{Aa}
		Experimental	0.8 ± 0.9^{Aa}	0.4 ± 0.6^{Aa}	0.6 ± 0.3^{Aa}
	INF-γ	Control	0.4 ± 0.4^{Aa}	0.7 ± 0.9^{Aa}	0.8 ± 1.1^{Aa}
		Experimental	0.4 ± 0.3^{Aa}	0.9 ± 0.9^{Aa}	1.3 ± 1.2^{Aa}

Intragroup comparison was performed by Friedman and Dunn's multiple comparison tests (different non-capital letters indicate statistical difference). Intergroup analysis was performed by Mann-Whitney test (different capital letters indicate significant differences between groups).

Table 5 Adverse effects occurrence

Adverse effects parameters	Control group	Experimental group
Pain (treatment day; mean ± SD)	1.66 ± 1.58	1.94 ± 1.64
Pain (day after; mean ± SD)	0.67 ± 0.96	0.89 ± 0.92
Fever (treatment day; n)	2	2
Fever (day after; n)	0	0
Oral ulceration (n)	0	0
Gastrointestinal discomfort (n)	1	4

No significant differences were observed between groups at any parameter evaluated (Kruskal-Wallis test for pain and χ^2 test for fever, oral ulceration, and gastrointestinal discomfort analysis).

evaluation for moderate and deep pockets ($P > .05$). Only the experimental group in deep pockets had a statistically significant decrease in the levels of PGE_2 at 3 and 6 months ($P < .05$). Additionally, intragroup analysis demonstrated that IL-6 and IFN-γ levels in deep pockets, and all cytokine levels in moderate pockets did not change during the follow-up ($P > .05$).

Adverse effects

The degree of pain after treatment did not differ between groups (Table 5). Moreover, with regard to fever, oral ulceration, or gastrointestinal discomfort, both protocols were similar ($P > .05$).

DISCUSSION

The present study assessed the effects of nonsurgical therapy associated with adjunctive amoxicillin/metronidazole in the levels of IFN-γ, PGE_2, and IL-6 in the GCF of GAgP patients. The results demonstrated that there were a reduction in PGE_2 levels and a superior reduction in PPD at 3 and 6 months, in deep pockets, when amoxicillin and metronidazole were prescribed.

The clinical outcome of the present study of a superior reduction in PPD at 3 and 6 months only in full-mouth analysis (0.7 mm and 0.6 mm, respectively) and deep pockets (0.7 mm and 0.8 mm, respectively)

provided additional evidence of a benefit of an adjunctive combination of amoxicillin and metronidazole associated with nonsurgical periodontal therapy. Although modest, these values were similar to those reported in a recent meta-analysis, which found a superior PPD reduction in full-mouth measurements (0.39 ± 0.16 mm at 3 months, and 0.51 ± 0.09 mm at 6 months), and initially deep pockets (0.88 ± 0.27 mm at 3 months, and 1.09 ± 0.39 mm at 6 months), in GAgP patients treated with nonsurgical periodontal therapy plus amoxicillin/metronidazole.[33] Indeed, previous reports showed that an association of amoxicillin and metronidazole showed significant additional clinical benefits with regard to deep pocket reduction, especially in deep pockets, and numbers of sites presenting residual pocket that bled on probing in GAgP.[3,25,34]

Using the proposed protocol of the present study no statistical significant differences were found in the CAL between the groups. This result was different from that found in the literature.[33,35] Although nonsignificant, the values of additional CAL gain observed in this study for full-mouth analysis (0.5 mm at 3 months, and 0.2 mm at 6 months) were similar to those reported in a recent meta-analysis (0.5 ± 0.4 mm at 3 months, and 0.36 ± 0.1 mm at 6 months) favoring the use of amoxicillin/metronidazole.[33] However, when we separated the rCAL gain for moderate and deep pockets, we found lower mean values than indicated by Keestra el al.[33]

Additionally, no significant differences in FMPI were found within and between the groups during any period of the evaluation. It should be highlighted that all patients received a full-mouth prophylaxis and oral hygiene instructions 30 days before baseline in order to ensure adequacy and to equilibrate oral conditions of patients before the beginning of the study. Moreover, all patients received supportive periodontal therapy during the course of the study. However, although there were no changes in FMPI, there was a statistically significant reduction in the bleeding index in the third month in all patients, which was maintained throughout the study, without differences between the groups. This is a common finding in antimicrobial studies,[25,35,36] although recently Keestra et al,[33] in a meta-analysis, indicated an additional reduction (10.07 ± 5.85% and 8.85 ± 5.51% at 3 and 6 months, respectively) in bleeding on probing in favor of amoxicillin/metronidazole.

This therapeutic approach could alter the composition of subgingival biofilm. In recent studies, only the association of mechanical debridement with amoxicillin/metronidazole was able to reduce levels of *A actinomycetemcomitans*.[25,34] Based on these clinical and microbiologic results,[25,34] it was expected that amoxicillin and metronidazole could also reduce the levels of pro-inflammatory cytokines, since periodontopathogenic bacteria and their products are the key stimulators in the local release of cytokines and inflammatory mediators in GCF.[20] In the present study only the combination of amoxicillin/metronidazole promoted a reduction in the levels of PGE_2 in deep pockets during the follow-up; however, there were no significant differences between the groups for PGE_2, IFN-γ, and IL-6 levels.

PGE_2 has been implicated as a key inflammatory mediator of periodontitis. In periodontal disease, macrophages stimulated by lipopolysaccharides may represent an important source of PGE_2,[20,37] which can act as a vasodilator, a cofactor involved in the increased vascular permeability, and a mediator of osteoclastic bone resorption.[18,38-40] In fact, the levels of PGE_2 in GCF seem to increase with the severity of periodontal disease.[40,41] Thus, one of the results of periodontal therapy should be the reduction in the levels of this mediator.[20] In the present study, the levels of PGE_2 were reduced only in deep pockets at 3 and 6 months in the experimental group, without differences between experimental and control groups at any time evaluated. A similar finding was reported by Ribeiro Edel et al,[21] who also observed significant reduction for the PGE_2 levels at 6 months (1083.5 ± 1322.3 pg/mL at baseline to 356.5 ± 305.3 pg/mL at 6 months) only in the amoxicillin/metronidazole group in chronic periodontitis patients. Also, Machtei and Younis[42] reported a decrease in amounts of PGE_2 after treatment of aggressive periodontitis patients with nonsurgical therapy and amoxicillin/metronidazole.

In a longitudinal study, a higher GCF level of IFN-γ was found in actives sites than in inactive sites of periodontal disease.[22] Furthermore, higher levels of IFN-γ were found in sites with severe chronic and aggressive periodontitis.[23] Therefore, after periodontal therapy, a reduction in its levels is desired. However, the present results showed that IFN-γ remained unchanged at the 3- and 6-month follow-ups. These results are in agreement with Oliveira et al,[15] who reported that changes in GCF IFN-γ levels in GAgP patients could not be detected after scaling and root planing associated with systemic amoxicillin and metronidazole (0.7 ± 1.1 pg/mL at baseline and 0.4 ± 0.3 pg/mL at 6 months) and scaling and root planing alone (0.8 ± 1.3 pg/mL at baseline and 0.5±0.3 pg/mL at 6 months). Similar findings were reported by Rosalem et al[43] for deep sites. Different findings have been also published by Del Peloso Ribeiro et al,[44] who reported a trend for an increase in GCF levels of IFN-γ after full-mouth ultrasonic debridement in chronic periodontitis patients. On the other hand, Thunell et al[45] reported statistically significant reductions as a result of periodontal mechanical treatment in GCF IFN-γ levels of chronic periodontitis. These contradictory findings might have been influenced by methodologic differences among studies such as sample size, therapy used, and number of sites sampled.[45]

In inflamed periodontal tissue, IL-6 induces B-cell maturation and immunoglobulin secretion, activates T cells, activates the complement cascade, and induces bone resorption.[46,47] Therefore, reduction in IL-6 levels is expected after periodontal therapy. However, in the present study, IL-6 levels in GCF in both groups remained stable during the follow-up. This finding differs from the result reported by Oliveira et al,[15] who found an increase in GCF levels of IL-6 in GAgP patients after periodontal therapy (2.0 ± 1.9 pg/mL at baseline to 6.6 ± 7.4 at 6 months), and Goutoudi et al,[48] who reported that periodontal treatment resulted in a significant increase of GCF IL-6 in diseased sites of chronic periodontitis patients. In contrast, Thunell et al,[45] after initial periodontal therapy, observed a decrease in IL-6 levels in chronic periodontitis subjects.

This contradictory result, observed in the present study, may be explained by the fact that GCF content might not fully reflect the levels of certain biomarkers within the inflamed periodontal tissue.[15] Indeed, Guillot et al[49] reported that IL-6 was localized within the inflamed tissues, resulting from the creation of an increased number of IL-6 receptors and subsequent IL-6 binding in diseased tissues. This could explain the lower levels of IL-6 in GCF at inflamed sites. Another possible explanation is that IL-6 might also act as an anti-inflammatory cytokine through suppression IL-1 expression, synthesis of IL-1 receptor antagonist (IL-1Ra), and increased production of tissue inhibitors of metalloproteinases (TIMPs).[48-50] Therefore, future studies should assess the IL-6 levels together with large numbers of cytokines, and in sites with different levels of attachment loss, in order to understand the changes that occur in sites with inflammation, and also to evaluate its relationship with other inflammatory markers.

One limitation of the present study is that only three pro-inflammatory cytokines were assessed, which although important to the inflammatory process that occurs in periodontal disease, cannot represent the multipath mechanisms involved in the pathogenesis of aggressive periodontitis. These immuno-inflammatory responses involve a complex group of protective and damaging reactions, with the presence of several other pro-inflammatory cytokines, anti-inflammatory cytokines, chemokines, and MMPs, which can modulate or be modulated by them in multiple ways until an overall outcome is established.[51,52]

Similar to the present report, a recent study could not demonstrate an additional impact of amoxicillin and metronidazole on GCF biomarkers when compared to mechanical therapy alone in GAgP treatment.[15] This may be explained by the hyperinflammatory response of GAgP subjects, which may increase host susceptibility to tissue destruction. Indeed, several studies have demonstrated that individuals with GAgP seem to have a preexisting susceptibility for systemic as well as local inflammation compared to healthy subjects and subjects with generalized chronic periodontitis, which is possibly unrelated to the periodontal infection and

probably related to the hyperreactivity of inflammatory cells.[53-55]

The results of the present study should be interpreted with caution, because although a modest additional clinical benefit was observed, the prescription of antibiotics must be done rationally to limit the adverse effects associated with the use of antibiotics. The consensus statement of the sixth European Workshop on Periodontology indicates the administration of systemic antibiotics in aggressive periodontitis,[56] and in addition there is evidence that additional clinical benefits of antibiotics remain stable in the long term.[33] However, there are still no established protocols for the treatment of aggressive periodontitis, and new forms of therapy should be tested in future studies, including different dosages, other antibiotics, or modulation of host inflammatory response, to obtain better clinical and immunologic results.[26]

CONCLUSION

It can be concluded that amoxicillin/metronidazole associated with nonsurgical therapy promotes an additional PPD reduction in the treatment of GAgP; however, this therapy did not promote additional benefits in the evaluated immunologic parameters (PGE_2, IFN-γ, and IL-6).

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3.8 Salivary carriage of periodontal pathogens in generalized aggressive periodontitis families*

Abstract

BACKGROUND:

Generalized aggressive periodontitis (GAP) is a multifactorial disease that shows a specific microbial profile and a familial aggregation.

AIM:

This study evaluated the salivary microbial profile of families with a history of GAP and compared them with healthy families.

DESIGN:

Fifteen families with parents presenting periodontal health and 15 with parents with a history of GAP were selected. Each family had a child aged 6-12 years. Stimulated saliva was collected from all subjects, and *Porphyromonas gingivalis* (Pg), *Tannerella forsythia* (Tf), and *Aggregatibacter actinomycetemcomitans* (Aa) amounts were determined.

RESULTS:

Children of GAP families showed higher detection of Aa (90%) than children of healthy families (45%) ($P < 0.05$). Parents with GAP showed a Pg salivary concentration statistically higher than that of healthy parents ($P < 0.05$).Children of GAP families, however, exhibited similar Pg concentration than healthy children ($P > 0.05$). Tf amounts did not differ either in parents or in children ($P > 0.05$) The infection risk calculation indicates that children who have one parent who is positive for Aa have 16.3 times (95% CI 3.1-87.2) more risk of being infected with Aa ($P < 0.05$) than children from an Aa-negative family.

CONCLUSION:

It may be concluded that children of parents with aggressive periodontitis have higher levels and higher risk of Aa infection.

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Salivary carriage of periodontal pathogens in generalized aggressive periodontitis families

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Background. Generalized aggressive periodontitis (GAP) is a multifactorial disease that shows a specific microbial profile and a familial aggregation.
Aim. This study evaluated the salivary microbial profile of families with a history of GAP and compared them with healthy families.
Design. Fifteen families with parents presenting periodontal health and 15 with parents with a history of GAP were selected. Each family had a child aged 6–12 years. Stimulated saliva was collected from all subjects, and *Porphyromonas gingivalis* (Pg), *Tannerella forsythia* (Tf), and *Aggregatibacter actinomycetemcomitans* (Aa) amounts were determined.
Results. Children of GAP families showed higher detection of Aa (90%) than children of healthy families (45%) ($P < 0.05$). Parents with GAP showed a Pg salivary concentration statistically higher than that of healthy parents ($P < 0.05$).Children of GAP families, however, exhibited similar Pg concentration than healthy children ($P > 0.05$). Tf amounts did not differ either in parents or in children ($P > 0.05$) The infection risk calculation indicates that children who have one parent who is positive for Aa have 16.3 times (95% CI 3.1–87.2) more risk of being infected with Aa ($P < 0.05$) than children from an Aa-negative family.
Conclusion. It may be concluded that children of parents with aggressive periodontitis have higher levels and higher risk of Aa infection.

Introduction

Generalized aggressive periodontitis (GAP) is characterized by a severe and rapid destruction of the periodontal tissues in young, systemically healthy individuals[1]. Although GAP's prevalence is very low in developed countries[2], undeveloped countries show a higher frequency of this disease, with up to 5% of their populations affected[3]. Another primary characteristic of this disease is familial aggregation. Studies have shown an increased risk of GAP occurrence in the siblings of affected probands[4].

The family aggregation may result from a combination of genetic and environmental factors[4], such as oral microbiota, resulting in individuals of the same family sharing susceptibility factors. Although controversies still exist concerning the mode of GAP's genetic aspect and the genes involved in this pathologic system[5], some studies have emphasized the intimate relationship of GAP with specific pathogens. In this vein, *Aggregatibacter actinomycetemcomitans* (Aa) has demonstrated characteristics and abilities that suggest it may be a major player in GAP's occurrence and development. Numerous studies have confirmed the role of this pathogen in GAP pathogenesis[6–8]. Moreover, a recent cross-sectional study observed that the mean clinical attachment loss was higher in Aa-positive individuals[9], indicating that the presence of this pathogen could be indicative of a higher risk of GAP occurrence.

The usual form of oral pathogen acquirement within a family is by vertical transmission, as shown by some studies, and saliva is the primary carrier of this transmission[10]. No

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study, however, has quantitatively assessed Aa, as well as *Porphyromonas gingivalis* and *Tannerella forsythia* (other important periodontal pathogens), in the saliva of GAP probands and their children. In particular, no studies have compared it with periodontally healthy familiar nuclei. Because GAP has an important familial history and because the acquisition of periodontal pathogens usually occurs within the family, the present study quantitatively determined *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* amounts in saliva of parents presenting GAP history and in their children, comparing the results with periodontally healthy families in an age- and gender-matched study.

Material and methods

Study design

This study was designed as an age- and gender-matched study trial to assess the salivary carriage of periodontal pathogens within families (parents and children) in which the parents have been diagnosed with generalized aggressive periodontitis, in comparison with families that have periodontally healthy parents. The study was approved by the Ethics Committee of the School of Dentistry of Piracicaba (11/0046) and was carried out from March 2011 until April 2012.

Population

Potential study participants were selected from patients referred to the Graduate Clinic of School of Dentistry of Piracicaba, University of Campinas, Brazil. The study inclusion criteria were different for each experimental group:

Group healthy families (n = 15): families with both parents (father and mother) presenting periodontal health and with at least one child (age ranging from 6 to 12 years). To be considered a periodontally healthy individual, a subject should not present any site with periodontal probing depth (PPD) >4 mm with bleeding on probing (BoP); additionally, the subject must have at least 20 teeth and must be in good general health.

Group GAP families (n = 15): families in which at least one parent (father and/or mother) present with a diagnosis of generalized aggressive periodontitis (included in supportive periodontal therapy, that is, previously treated by non-surgical therapy, following the diagnosis criteria previously described in Casarin *et al.*[8]) and at least one child (age ranging from 6 to 12 years). Each GAP subject must have at least 20 teeth, good general health, and age ranging from 20 to 35 years.

The study excluded patients (parents and/or children) who[1] were pregnant or lactating[2], were suffering from any other systemic diseases (cardiovascular, diabetes, or other)[3], had received antimicrobials within the previous 3 months[4], were taking long-term anti-inflammatory drugs[5], had received a course of periodontal treatment within the last 6 months, or [6] were smokers or former smokers.

All subjects were clinically examined, and all answered a questionnaire regarding their educational and socio-economic statuses.

Sampling collection

Paraffin-stimulated whole saliva samples were collected by expectoration into calibrated medical cups over 5 min. The subjects were instructed to avoid eating or brushing their teeth (or using any other dental hygiene methods) for 2 h prior to collection. Each saliva sample was divided into two Eppendorf tubes, which were immediately frozen at −70°C[11].

Microbiological evaluation

The presence and concentration of *Porphyromonas gingivalis* (Pg), *Aggregatibacter actinomycetemcomitans* (Aa), and *Tannerella forsythia* (Tf) were evaluated using specific primers, as reported by Casarin *et al.*,[8] using a quantitative PCR (qPCR) technique.

Initially, DNA was extracted from the subgingival biofilm following a previously described protocol[12]. A qPCR was performed using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Mannheim,

Germany). The concentration of the DNA used in each run was 10 $\mu g/\mu L$. The amplification profiles were 95°/10′, 55°/5′, 72°/4′, with 40 cycles for Pg; 95°/10′, 55°/5′, 72°/3′ with 40 cycles for Aa; and 95°/10′, 55°/5′, 72°/3′, with 45 cycles for Tf. Absolute quantification of target bacteria in clinical samples was performed using Pg (ATCC 33277), Aa (JP2), and Tf (ATCC 43037) as controls, based on predetermined standard curves (Aa – 10^3–10^7; Tf – 10^3–10^8; Pg – 10^3–10^8) and performed at the same period. The determination of the DNA genome copies in the controls was based on the genome size of each bacteria and the mean weight of one nucleotide pair[13].

Data management and statistical analysis

This study tested the null hypothesis that GAP families (parents and children) present the same salivary carriage of periodontal pathogens as healthy families. To test this hypothesis, the SAS Release 9.01, 2003 (SAS Institute Inc., Cary, NC, USA) program was used. Prior to the study, we performed a sample size calculation considering Aa amounts as primary variable. The means and standard deviation values were based on a previous study of our group[8]. For calculation, a difference of 1.7 logs in Aa amounts, a 1.5 log of standard deviation, associate with 80% of power level, was used to calculate sample size. The minimum sample size achieved was 14 individuals per group.

The results, referencing frequency of detection (presence/absence), were compared between GAP parents *versus* healthy parents and between GAP children *versus* healthy children, using Fisher's exact test. The same comparisons were conducted using the total amounts of each pathogen, using Mann–Whitney *U*-test. The relative risk calculation of children's infection in relation to parents' infection was also determined (odds ratio). All statistical tests had a significance level of 5%.

Results

Demographical and clinical aspects

Table 1 describes the demographical and clinical parameters of the individuals included in the study. No significant differences were observed between the parents in healthy and GAP families regarding their ages, educational statuses, or monthly incomes ($P > 0.05$). As expected, GAP parents, however, presented a higher number of sites presenting PPD

Table 1. Demographical and clinical characteristics of healthy and GAP parents and children.

	Healthy parents (n = 26)	GAP parents (n = 26)	P
Age (years ± SD)	35.5 ± 2.4	38.1 ± 3.3	0.1*
Educational status			
Basic	30%	35%	0.89†
Secondary	52%	59%	
Higher	18%	6%	
Monthly income (%)			
<2488 BRL	29	60	
2488–4976 BRL	64	40	0.91†
>4976 BRL	7	0	
$ (mean ± SD)	3278.6 ± 2147.7	2070.0 ± 894.5	0.12*
Number of sites presenting PPD >4 mm and BoP (n ± SD)	0.0 ± 0.0	19.5 ± 6.8	<0.0001*

	Healthy children (n = 24)	Aggressive children (n = 24)	P
Age*	8.7 ± 3.2	9.1 ± 4.2	0.7302
Gender†	13 female	13 female	1.0000

BRL, Real (Brazilian currency); PPD, periodontal probing depth; BoP, bleeding on probing; GAP, Generalized aggressive periodontitis.
*Student's *t*-test ($P < 0.05$).
†Chi-squared test ($P < 0.05$).

>4 mm and BoP ($P < 0.0001$) than healthy parents. As also expected, because this was an age- and gender-matched study, no significant differences between the children of the two groups were seen regarding age or gender distribution ($P > 0.05$).

Salivary results

Figure 1 exhibits the detection frequency of three periodontal pathogens in the saliva of both healthy and GAP families, in both parents and children. Although the percentage of parents positive for Aa within the GAP families has not been different for parents from the healthy families (81% and 59%, respectively – $P = 0.07$), children of GAP families presented a statistically higher frequency of Aa detection (90%) than children of healthy families (45%) ($P < 0.05$). Regarding *T. forsythia* and *P. gingivalis* infections, no significant differences were detected between groups, either for the children or for the parents ($P > 0.05$).

The results observed in Fig. 1 could also be observed considering the qPCR analysis (Table 2). Aa was detected in higher amounts in the saliva of GAP children than in healthy children ($P < 0.001$), although the comparison of their parents did not reveal a significant difference ($P > 0.05$).The GAP parents' saliva, however, showed statistically higher concentrations of *P. gingivalis* than the healthy parents' saliva ($P = 0.04$), which was not seen in the comparison of their children ($P > 0.05$). The *T. forsythia* analysis revealed no differences between the groups ($P > 0.05$).

To determine the risk of child contamination if the parent is positive for periodontal pathogens, the odds ratio for the children's infections was calculated (Table 3). According to the data, children who have at least one parent presenting positive for Aa have 16.3 times more risk of infection ($P = 0.0007$). The wide confidence interval, however, should be considered (3.1–87.2). The children's risk for *P. gingivalis* and *T. forsythia*, however, showed no statistically significant value ($P > 0.05$).

Discussion

Despite the dedicated attention of numerous scientific studies over recent decades, the diagnosis, treatment, and pathogenesis of GAP are still considered challenging. According to the American Academy of Periodontology (AAP), this disease is associated with familial aggregation and a higher presence and prevalence of specific pathogens, including *A. actinomycetemcomitans* and, in certain populations, *Porphyromonas gingivalis*[14,15]. Saliva provides the major route of transmission and acquisition of oral microorganisms; hence, the present study evaluates the pres-

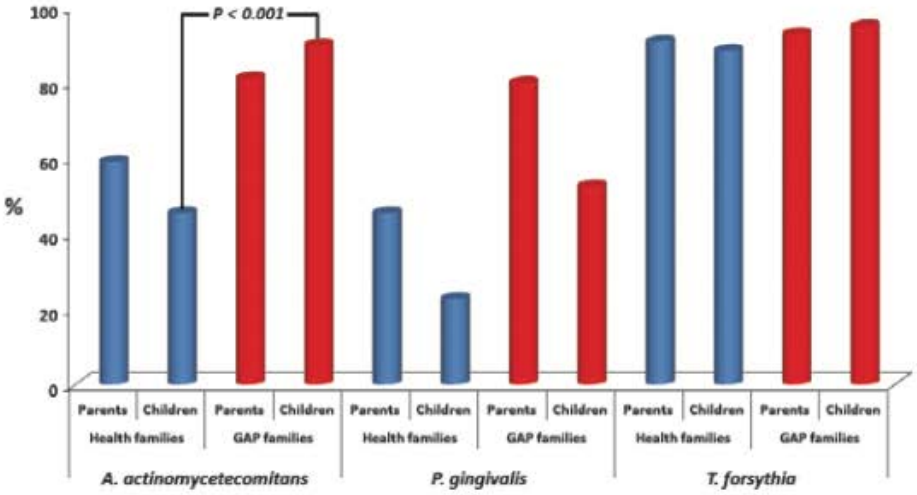

Fig. 1. Frequency detection of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* in the saliva of healthy and generalized aggressive periodontitis (GAP) families (parents and children; Fisher's exact test, $P < 0.05$).

Table 2. Salivary pathogen amounts (mean ± SD) in healthy and GAP families.

Subjects	log [ ]	SD	P*
Aggregatibacter actinomycetemcomitans			
Healthy families (n = 15)			
Parents	1.59	1.52	
Children	0.87	1.32	
GAP families (n = 15)			
Parents	2.58	1.89	0.119
Children	3.01	1.65	0.003
Porphyromonas gingivalis			
Healthy families (n = 15)			
Parents	1.62	1.54	
Children	0.78	1.19	
GAP families (n = 15)			
Parents	3.00	1.54	0.032
Children	0.96	1.06	0.298
Tannerella forsythia			
Healthy families (n = 15)			
Parents	5.47	1.97	
Children	5.51	1.64	
GAP families (n = 15)			
Parents	5.93	0.72	0.887
Children	5.87	0.82	0.402

GAP, Generalized aggressive periodontitis.
*Mann–Whitney *U*-test for comparison between healthy and GAP parents and healthy and GAP children (α = 5%).

Table 3. Relative risk of 'pathogen-infected children' in a 'pathogen-infected family'*.

	Odds ratio	95% Confidence interval	P
Aggregatibacter actinomycetemcomitans	16.3	3.1–87.2	0.0007
Porphyromonas gingivalis	1.5	0.2–11.2	0.91
Tannerella forsythia	23.0	0.6–702.6	0.34

*For 'pathogen-infected families or children', one of the parents or children must present the pathogen.

ence of periodontal pathogens in the saliva of families in which the parents have GAP, comparing them with families that present periodontal health.

The results revealed a higher frequency and quantity of Aa in the saliva of children from families whose parents have a history of GAP, compared with children of periodontally healthy parents. Furthermore, the risk assessment indicated children are 16 times more likely to present Aa in their saliva when their parents have Aa in their saliva (although a wide confidence interval has been detected). Aa is a gram-negative microorganism that has a close relationship with GAP. Several studies have shown a higher frequency [6,16] and concentration [8] of Aa in the subgingival environment of individuals with GAP than in healthy individuals or those suffering from chronic periodontitis. This relationship, however, has not been confirmed in other studies[17,18]. The present study is the first to evaluate the salivary carriage in children from GAP families, compared with the salivary carriage in children from healthy families, through a gender- and age-matched design, revealing a higher prevalence of Aa in the former population.

Interestingly, a comparison of the concentration and frequency of Aa among the parents participating in this study revealed no statistically significant difference. It is noteworthy, however, that the parents with GAP showed higher probing depths than the control group, indicating the presence of some recurrent or refractory sites after periodontal therapy. Furthermore, it indicated that even after the treatment and reduction in oral microorganisms in individuals affected by periodontal disease, relatives are still at risk of being contaminated or maintain a microbiological profile, as observed by Kleinfelder *et al.*[19] Their study found that, even after the individual with a periodontal disease associated with the presence of Aa receives treatment, the microorganism clearly persists in other family members. This explains why the parents in both groups showed similar levels, even though the children of families with GAP exhibited higher levels of Aa infection[19]. Our study design, however, did not allow to identify if or when the vertical transmission occurred. Future studies could track the oral microbiota of GAP children since their birth or before and after their parents' periodontal treatment.

Therefore, the data obtained by the analysis of salivary microbiota have illustrated a clear relationship between parents' contamination and children's contamination. Saliva is the most common transmission vehicle of oral microorganisms[11,20]. Even in periodontally healthy individuals, saliva can be positive for *A. actinomycetemcomitans* and may be a transmission source[20]. Interestingly, studies have shown that the *A. actinomycetemcomitans* found in the saliva may be the same clonal type as

that found in subgingival plaque[20–22]. Furthermore, for interindividual transmission, microorganisms must be viable and sufficiently prevalent in the saliva; the minimum concentration for contamination, however, has not yet been determined[10]. The viability of *A. actinomycetemcomitans* and *P. gingivalis* was confirmed in previous studies, which showed that both bacteria can be cultured from the saliva from periodontitis patients[23,24]. Accordingly, saliva is a strong candidate as a route of transmission for oral bacteria, particularly periodontal pathogens. Moreover, there is no evidence that periodontal bacteria can be transmitted by aerosols[10]. This statement reinforces the idea that saliva and mucosal contact are the routes of transmission of periodontal bacteria[10]. This way, the salivary carriage became an important tool to understanding the colonization of oral environment and the origin of microorganisms.

Studies have confirmed that *A. actinomycetemcomitans* has a clear familial aggregation[25–27]. These observations suggest that the bacteria can be spread among family members and that family members share susceptibility factors for colonization by the bacteria or clonal types of these species. A study of Moroccan families that shared risk habits for bacterial transmission (e.g., sharing food and beverages from the same cups) demonstrated the strong relationship of Aa to families[28]. Asikainen *et al.*[20] presented an important study on the distribution of *A. actinomycetemcomitans* and *P. gingivalis* among family members. They studied the occurrence of *A. actinomycetemcomitans* and *P. gingivalis* in 47 families. Individuals were selected based on the presence of periodontal disease or Aa-positive status, independently if chronic or aggressive disease. Spouses of patients with *A. actinomycetemcomitans*-positive periodontitis, the study indicated, were seven times more likely to be infected with this microorganism than spouses of patients negative for Aa. Moreover, spouses of patients whose cultures tested positive for *P. gingivalis* were twice more likely to test positive than spouses of patients whose cultures tested negative for this specie. Only one of the 19 children with a parent positive for *P. gingivalis*, however, was also positive for it. Most studies have shown that for children who are carriers of Aa, one or two parents are usually carriers of the same genotype.

From these observations, it is assumed that the family is the source of transmission. In a recent, well-conducted review, van Winkelhoff and Boutaga[10] calculated that the transmission of Aa can be estimated to be 30–60%, based on the detection of identical genotypes in children and parents. To date, no study, however, has already included GAP subjects as probands and assessed the Aa contamination of their children. Moreover, this is the first trial to compare this young population, which is hypothetically more susceptible to aggressive periodontal disease, with the children of periodontally healthy probands. Consequently, the children of the present study are a 'focus demand' subset that should be explored fully. As demonstrated by these results and those achieved in the present study, considering higher amounts of Aa in GAP children, it could be acceptable that vertical transmission of *A. actinomycetemcomitans*, but not *P. gingivalis*, occurs in a high frequency between individuals of the same family.

To understand this predilection for Aa salivary colonization, one must consider the characteristics of Aa that can contribute its presence in saliva. Aa is a gram-negative bacilli and facultative, which could facilitate colonization in saliva and in other oral environments that have high levels of oxygen concentration. Differently, Pg and Tf present different characteristics (strictly anaerobic, non-mobile, gram-negative) that favour its presence among periodontal disease but not in healthy sites or saliva.

In spite of pathogens characteristics, the literature lacks information about the salivary presence of other species of periodontal bacteria, especially in children. In a large study on the transmission of *P. gingivalis* in families, Tuite-McDonnell *et al.*[27] used the PCR technique to detect *P. gingivalis* in 564 members of 104 families recruited from a specific population (the same church community). The overall prevalence of *P. gingivalis* was 37.1%. Among the four generations examined, the prevalence ranged from 50% in the great grandparents to 27.1% among children. Children were

significantly less frequently colonized than their grandparents, but no statistically significant difference was observed between either children and their parents or parents and grandparents. Regarding transmission, children and their parents were significantly concordant. When a mother tested positive for *P. gingivalis*, her child's risk of colonization 4.7 times higher than the risk of a child whose mother was not colonized. Moreover, the risk of colonization was higher when the child had two parents colonized with *P. gingivalis*. Based on these observations, Tuite-McDonnell and others concluded that 'contact with an infected family member substantially increased the relative risk of colonization for spouses, children and their parents'.

Other studies, however, found different results and corroborated with the present study. Rotimi *et al.*[29] also identify only few children infected by *P. gingivalis*, although Aa, Tf, *Prevotella intermedia*, and *Prevotella nigrescens* were detected in a higher frequency. Tamura *et al.*[30] evaluated the presence of 10 pathogens in the saliva of mothers and children; they identified a positive relationship between the presence of Aa in mothers and the presence of Aa in children. For *P. gingivalis* and *T. forsythia*, however, the researchers did not detect any vertical transmission. The results of the present study corroborate with those of Tamura and others regarding tested bacteria; there was no difference in the prevalence and concentration of Pg and Tf between children of healthy and GAP families, and there was no significant risk of contamination for those bacteria.

Tannerella forsythia is described in literature as a commensal bacterium of the oral cavity[31]. It is frequently found at both healthy and periodontitis sites[32]. Moreover, previous studies as Cortelli *et al.*,[31] Mayanagi *et al.*,[32] and Riep *et al.*[33] are in agreement with our results, which showed a higher frequency of *T. forsythia* in the oral cavity than did Pg and Aa[31–33]. When comparing parents and children, our study shows a similar prevalence of *T. forsythia* between them, in both GAP and healthy groups. Interestingly, Cortelli *et al.*[31] evaluated the initial colonization by oral pathogens in different ages and showed that 6–12 years were the first period for *T. forsythia* colonization. It is noteworthy that this age is compatible with the age of children selected for the present study. Moreover, they demonstrated that the prevalence of *T. forsythia* in the older age groups (13–18 years, 19–44 years, and >55 years) remains at a similar level to that in those who are 6–12 years of age, indicating that Tf colonization presents a constant load with increasing age[31]. It, however, is important to mention that Tan *et al.*[34] reported that other subtypes of *T. forsythia* exist (prtH genetic subtypes), and the pathogenicity of this bacterium probably varies depending on the strain. Consequently, further studies are necessary to determine whether and which subtypes of *T. forsythia* are associated with periodontitis and healthy conditions[34].

The relatively low frequency of Pg infection is perhaps due to the bacteria's relation with age, as also occurs for Aa, although in a reverse manner: the prevalence of *A. actinomycetemcomitans* appears to decrease with age, whereas the prevalence of *P. gingivalis* increases with age[35]. Kononen *et al.*[35] assessed factors that determine the presence of periodontal pathogens in saliva. Their results indicated a clear relationship between the prevalence of Pg and increasing age, although this result has not been found for other bacteria. This is demonstrated by our data; although GAP parents presented higher concentrations and a greater presence of Pg than the healthy parents, this difference was not seen among GAP children.

Meanwhile, although our results are generally supported by the scientific literature, this study has some limitations and specialized characteristics that should be considered in the analysis and planning of future studies. This study, for example, did not conduct pheno- or genotypic testing to confirm whether the parents and children within each family had the same bacteria types, confirming the vertical transmission occurrence. Different technologies could be applied to obtain these results, such as AP-PCR or genotyping. Moreover, our protocol for DNA extraction included a total DNA and not only viable cells, which could impact evaluation of pathogens detection and quantification. The

study's design, however, ensures the reliability of our results, once a gender- and age-matched study in a relatively expressive population of GAP families (a hard-to-reach population) was delineated. Furthermore, to the best of our knowledge, this is the first study to compare entire families, including families with and without a history of periodontal disease, especially in families presenting generalized aggressive periodontitis. Moreover, no previous studies have evaluated and compared GAP transmission through a quantitative analysis by qPCR.

If the children of parents with aggressive periodontitis have a higher prevalence and amount of *A. actinomycetemcomitans* in their saliva, then this population may be at higher risk of subgingival colonization of this pathogen. The relationship between salivary and subgingival microbiota was confirmed in recent studies. Saygun *et al.*[36] correlated the number of copies of each pathogen (Aa, Pg, Tf, *Prevotella intermedia*, and *Campylobacter rectus*) in individuals' saliva samples with their periodontal statuses. The Pg, Tf, and Pi results showed a positive relationship between the number of copies and the presence of periodontitis. Interestingly, and highlighting the results of this study, Aa only showed higher salivary copy counts in subjects with aggressive periodontitis compared with subjects with healthy periodontium. Furthermore, because GAP presents a familiar aggregation and probably individuals share susceptibility factors, the presence of this pathogen can be a marker for future attachment loss and periodontal disease[7,9,37]. In pathogen carriers, the increased proportion of pathogenic microorganisms should be considered an important tool for predicting current and future periodontal conditions and developing a method to address them in preventive strategies, in an attempt to control the acquisition of the less beneficial members of the human oral microbiota.

Thus, within the limitations of the present study, it could be concluded that children from families with generalized aggressive periodontitis tend to have a higher colonization by *A. actinomycetemcomitans*. This pattern, however, was not identified for other periodontal pathogens.

Disclosure

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Conflict of interest

The authors declare no conflict of interest.

Why this study is important for paediatric dentists?

- The study of precocious colonization, especially by periodontal pathogens, could represent a tool for identifying subjects with potential risk of developing periodontal disease.
- Also important is the study of families in which parents are affected by aggressive periodontitis once this disease presents a familial aggregation, and children of these families could represent a higher-risk population.
- Moreover, as saliva represents the most important via for vertical transmission, it could be considered a useful and non-invasive form to evaluate oral microbiota and its possible relationship to oral health.

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3.9 Periodontal clinical and microbiological characteristics in healthy versus generalized aggressive periodontitis families.*

Abstract

AIM:

Generalized aggressive periodontitis (GAP) is a severe and multifactorial disease in which a familial aggregation and a specific microbiological profile have been suggested. Thus, this case-control study evaluated the clinical and subgingival microbial profile of GAP subjects and their families compared to healthy families.

METHODS:

Fifteen families with parents presenting periodontal health and 15 with parents with a history of GAP were selected. Each family should have at least one child between 6 and 12 years old. Plaque index (PI), gingival index (GI), and periodontal probing depth (PPD), as well as *Porphyromonas gingivalis, Tannerella forsythia*, and *Aggregatibacter actinomycetemcomitans* (Aa) amounts (by qPCR), were assessed from all subjects.

RESULTS:

Children of GAP families showed a higher PI, GI, and PPD when compared to children of healthy families ($p \leq 0.05$). A higher frequency of detection and amounts of Aa was observed in GAP children compared to children of healthy families ($p \leq 0.05$). Moreover, a significant association between Aa amounts and gingival bleeding was observed in children ($p \leq 0.05$, $r = 0.37$).

CONCLUSION:

Children from GAP families have worst clinical conditions, i.e. higher levels of PI, GI, and PPD, a more pathogenic microbiological profile, and the amount of Aa are associated with a higher marginal inflammation.

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Periodontal clinical and microbiological characteristics in healthy versus generalized aggressive periodontitis families

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Key words: *Aggregatibacter actinomycetemcomitans*; generalized aggressive periodontitis; hereditarily; prevention; susceptibility factors

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Although relatively rare, aggressive periodontitis (AgP) has played a major role in periodontal research. However, no clear conclusion regarding its initiation and progression has been drawn yet. AgP presents a severe and rapidly progressive destruction of periodontal tissues in systemically healthy youngsters, usually from the same familial nuclei (Tonetti & Mombelli 1999). Susin et al. (2014) reported a prevalence of AgP of 0.1–0.5% in Europe and 0.28–7.6% in Africa. In Brazil, this disease was detected in 0.3–5.5% of young individuals (Susin et al. 2014). Given its rare occurrence and the difficulties in gathering large populations for study, knowledge of AgP is still limited (Shaddox et al. 2012). This hereditary characterization has been proven in previous studies; one study even showed an increased risk of

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generalized aggressive periodontitis (GAP) occurring in the siblings of affected probands (Nibali et al. 2008). This phenomenon probably occurs because these individuals share susceptibility factors – genetic, environmental, or both – such as specific oral microbiota.

Some studies have indicated a relatively constant microbiota profile in GAP subjects, in contrast to chronic disease in which *Aggregatibacter actinomycetemcomitans* (Aa) has been suggested as the principal pathogen related to its occurrence and progression (Haubek 2010). Other studies, however, have found that *Porphyromonas gingivalis* (Pg) is also more prevalent and concentrated in AgP (Gajardo et al. 2005, Mayorga-Fayad et al. 2007, Casarin et al. 2010). Furthermore, other pathogens (*Parvimonas micra* and *Filifactor alocis*) have been recently reported as common colonizers in diseased sites of children presenting periodontal destruction (Shaddox et al. 2012), and a consortium of bacteria (*A. actinomycetemcomitans, Streptococcus parasanguinis*, and *F. alocis*) was associated with localized AgP and indicated future alveolar bone loss (Fine et al. 2013). In contrast, another study did not confirm the predominance of *A. actinomycetemcomitans* in GAP patients (Faveri et al. 2008) and, in spite of the efforts to determine the importance of each bacterium, the role of specific microorganisms has not been completely understood (Könönen & Müller 2014).

The acquisition of oral pathogens usually occurs through vertical transmission, especially by saliva, during childhood (Van Winkelhoff & Boutaga 2005). In relation to *A. actinomycetemcomitans*, children of Aa-positive parents presented a higher proportion of positive sites than children from Aa-negative parents (Tinoco et al. 1998), indicating a precocious transmission of this pathogen within families. Once transmitted, *A. actinomycetemcomitans* could colonize subgingival areas and lead to attachment loss (Haubek 2010); thus, for some groups, it is considered as an attachment loss predictor (Fine et al. 2007, Åberg et al. 2014). In this vein, based on the impact of subgingival microbiota on periodontal status over time, the detection of specific bacteria associated with this disease in children at a young age – before disease breakdown occurs – would be desirable for the successful prevention and treatment of this disease (Shaddox et al. 2012).

Moreover, considering its hereditary pattern, children whose parents have a history of GAP (GAP children) could be considered a high-risk population for developing this disease. However, no study compared children of GAP parents and children of periodontally healthy parents prior to disease establishment. Thus, the present study tested the hypothesis that these children of GAP families would present dissimilarities in subgingival microbiota when compared to children of healthy families in spite of clinical conditions.

Material and Methods

Study design

This study was designed as an age- and gender-matched, case–control study trial to assess the clinical and microbiological characteristics in families (parents and children) in which at least one parent had been diagnosed with GAP as compared to families that had periodontally healthy parents. The study was approved by the Ethics Committee of the University of Campinas (11/0046) and was carried out between March 2011 and April 2012.

Population and clinical examination

Potential study participants were selected from patients referred to the Graduate Clinic of Piracicaba Dental School. The study inclusion criteria were different for each experimental group:

- *Group GAP families (n = 15)*: One parent (father or mother) with diagnosis of GAP, following the diagnosis criteria previously described in Casarin et al. (2010), and at least one child (age ranging from 6 to 12 years old). All GAP parents were treated by non-surgical therapy and, after disease was controlled, they were included in a supportive periodontal therapy every 6 months. To get included to the group, the patient could not receive subgingival treatment 6 months previously to the clinical evaluation on this study. Moreover, the GAP parent had to have at least 20 teeth, be in good general health, and be between 20 and 35 years of age at the time of diagnosis.
- *Group healthy families (n = 15)*: Families with both parents (father and mother) presenting periodontal health and with at least one child (age ranging from 6 to 12 years old). To be considered a periodontally healthy individual, a subject should not present any site with periodontal probing depth (PPD) > 4 mm with bleeding on probing (BoP); additionally, the subject had to have at least 20 teeth and had to be in good general health.
- All children needed to present permanent first molars, either semi- or totally erupted.

The study excluded patients (parents and their children) who (1) were pregnant or lactating, (2) were suffering from any other systemic diseases (cardiovascular, diabetes, or other), (3) had received antimicrobials within the previous 3 months, (4) were taking long-term anti-inflammatory drugs, (5) had received a course of periodontal treatment within the last 6 months, or (6) were smokers or former smokers.

Teeth in the initial stage of eruption that impede the clinical examination were excluded. All subjects were clinically assessed by a calibrated examiner (MFM – intra-class correlation = 92% for PPD). The clinical parameters evaluated were plaque index (PI; Ainamo & Bay 1975), gingival index (GI; Ainamo & Bay 1975), and PPD (distance of gingival margin to bottom of the periodontal pocket or sulcus) and they were evaluated at six sites per tooth in all permanent teeth (primary teeth were not evaluated). Moreover, all parents of both groups answered a questionnaire regarding their educational and socioeconomic status.

Microbiological evaluation

Following supragingival biofilm removal and relative isolation with cotton rolls, a sterile paper point (N°35) was inserted into the bottom of the periodontal pocket/gingival

sulci for 30 s. The paper points were placed into sterile tubes containing 300 μl of Tris-EDTA 0.5 mM. Subgingival biofilm samples were collected from mesial and distal surfaces of first molars of all subjects. In case of the 1st molar's absence, the 2nd molar was selected. All children had to present permanent 1st molars, either semi- or totally erupted.

The presence and concentration of Pg, Aa, and *Tannerella forsythia* (Tf) were evaluated using specific primers and using a quantitative PCR technique, as reported by Casarin et al. (2012). Briefly, DNA was extracted from the subgingival biofilm. A real-time PCR was performed using the FastStart DNA Master SYBR Green kit (Roche Diagnostics GmbH, Mannheim, Germany). The concentration of the DNA used in each run was 10 μg/ml. The determination of the DNA genome copies in the controls was based on the genome size of each bacteria and the mean weight of one nucleotide pair.

Data management and statistical analysis

Sample size was based on the most similar study that assessed microbiological conditions of families (Kleinfelder et al. 1999) since, to the best of our knowledge, this is the first study that evaluated microbiological patterns in families and did not allow us to determine previous sample variability or significant differences between groups. The results of the clinical data and the total amounts of each pathogen were evaluated using a non-paired Student's *t*-test for comparison between GAP parents *versus* healthy parents and GAP children *versus* healthy children. Each family was composed of only one parent and at least one child between 6 and 12 years old. For GAP families, the parent diagnosed with GAP was chosen. For healthy families, the parent was chosen based on age- and gender-matched after selection of the GAP parents.

A linear correlation test was used for identifying significant relationships between clinical parameters (PI, GI, and PPD) and pathogen amounts. All of the analyses were done using the SAS program (release 9.1 2003; SAS Institute Inc., Cary, NC, USA) and considering the patient as an experimental unit with $\alpha = 5\%$.

Results

Population screening

Figure 1 describes the selection of families. During population screening, 41 GAP subjects were examined and interviewed regarding the age of their children and the exclusion criteria. From them, 21 were preselected and analysed regarding the inclusion criteria, and 15 parents and their children were selected. According to age and gender of selected GAP patients, the healthy subjects and their children were selected to respect the age- and gender-matched design. The healthy patients were assessed in the clinic of Piracicaba Dental School (from the same database of GAP subjects) and the periodontal history was investigated in previous anamneses and examinations, if available. Thus, 28 healthy parents and their families were invited to participate of this study. A new anamnesis and clinical examination was performed for all family members to evaluate the inclusion and exclusion criteria, and 13 families were excluded. Thus, 15 "GAP families" and 15 "healthy families" with similar demographic conditions were selected. Parents of both groups were in treatment in Piracicaba Dental School and they had received hygiene instruction and professional care at least one time in the year before the study.

Table 1 illustrates that no significant differences were observed between the parents in healthy families and GAP families regarding

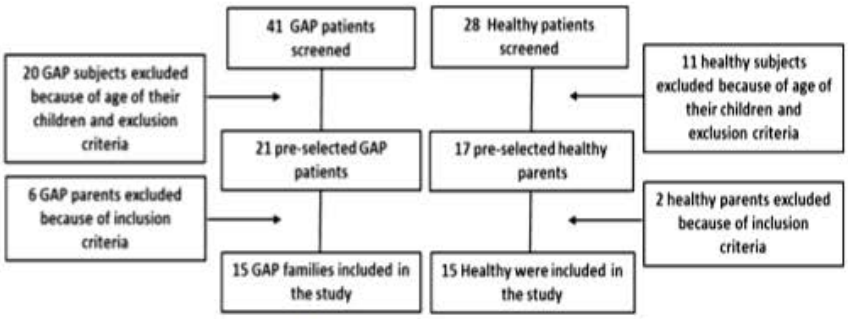

Fig. 1. Flow chart of included families.

Table 1. Demographic and clinical characteristics of healthy and GAP parents and children

	Healthy parents (n = 15)	GAP parents (n = 15)	p
Age (years ± SD)	35.7 ± 0.51	35.4 + 5.1	0.7*
Monthly income (%)			
Low	40	20	0.49#
Middle	47	67	
High	13	13	
BRL (mean ± SD)	3278.6 ± 2147.7	2070.0 ± 894.5	0.12*
Educational status (%)			
Basic	27	53	0.89#
Secondary	60	47	
Higher	13	0	
Number of sites presenting PPD > 4 mm and BoP	0.0 ± 0.0	19.5 ± 6.8	< 0.0001*

	Child of healthy parents (n = 24)	Child of GAP parents (n = 24)	p
Age*	8.7 ± 3.2	9.1 ± 4.2	0.73
Gender#	13 Female	13 Female	1.00

BoP, bleeding on probing; BRL, Brazilian real; GAP, generalized aggressive periodontitis; SD, standard deviation; PPD, periodontal probing depth.
*Student's *t*-test ($p \leq 0.05$).
#Chi-square test ($p \leq 0.05$).

age and demographic aspects ($p > 0.05$). No significant differences between the children were seen regarding age or gender distribution ($p > 0.05$). Moreover, GAP parents presented a higher number of sites measuring PPD > 4 mm and BoP ($p < 0.0001$) than healthy parents.

Clinical aspects

In this study, the clinical parameters evaluated in all subjects were PI, GI, and PPD. As shown in Table 2, statistical differences were observed for all parameters when comparisons between children of healthy parents and children of GAP parents were made. For parents, differences were observed in GI and PPD, while the PI showed similar values when the groups were compared. Children of GAP parents had a higher PI (43.1 ± 22.2%), GI (12.0 ± 10.1%), and PPD (2.4 ± 0.4 mm) when compared to children of healthy parents (24.1 ± 11.1%, 1.7 ± 2.5%, and 1.6 ± 0.3 mm, respectively) ($p \leq 0.05$). GAP parents also had worse clinical conditions than healthy parents ($p \leq 0.05$).

Microbiological results

Regarding *A. actinomycetemcomitans* presence and concentration (Table 3), children of GAP parents presented a higher frequency of detection (63.2%) and a superior quantity (1.8 ± 1.5 log[ ]) than children of healthy parents (28.6% and 0.7 ± 0.9 log[ ], $p \leq 0.05$). For GAP parents, a greater amount of *A. actinomycetemcomitans* in comparison to healthy parents (2.2 ± 2.1 and 0.8 ± 1.3 log[ ], respectively, $p \leq 0.05$), was noted. *P. gingivalis* was more highly detected and concentrated in GAP parents than healthy ones ($p \leq 0.05$), but no differences were observed regarding *T. forsythia* ($p > 0.05$). Tables 4 and 5 display the significant correlation ($p \leq 0.05$) between *A. actinomycetemcomitans* amounts and all clinical parameters (as well as between them) in children. However, when adjustments were made for the presence of plaque, only GI could still be associated with *A. actinomycetemcomitans* amounts ($p \leq 0.05$, $r = 0.37$) but not PPD ($p > 0.05$, $r = 0.20$), indicating that there were higher *A. actinomycetemcomitans* amounts in children, as well as a higher GI, irrespective of the presence of plaque.

Table 3. Frequency of detection and subgingival amounts of periodontal pathogens (mean ± SD) in healthy and GAP families

	Frequency		Quantification	
	%	$p^{\#}$	log[ ]	p^*
A. actinomycetemcomitans				
Healthy families				
Parents	33		0.8 ± 1.3	
Children	29		0.7 ± 0.9	
GAP families				
Parents	73	0.06	2.2 ± 2.1	0.04
Children	63	0.05	1.8 ± 1.5	0.04
P. gingivalis				
Healthy families				
Parents	20		0.6 ± 1.3	
Children	24		0.6 ± 1.1	
GAP families				
Parents	73	0.03	3.2 ± 2.3	0.002
Children	53	0.1	1.6 ± 1.8	0.11
T. forsythia				
Healthy families				
Parents	33		2.5 ± 3.3	
Children	23.8		1.8 ± 2.7	
GAP families				
Parents	71	0.89	5.2 ± 3.0	0.07
Children	52.6	0.1	3.5 ± 3.2	0.07

GAP, generalized aggressive periodontitis.
#Chi-square test and *non-paired Student's *t*-test for comparison between healthy and GAP parents and healthy and GAP children ($p \leq 0.05$).

Table 2. Clinical characteristics of healthy and GAP parents and healthy and GAP children

	Healthy parents ($n = 15$)	GAP parents ($n = 15$)	p^*
PI (%)	23.8 + 17.6	37.9 + 26.6	0.321
GI (%)	2.0 + 3.1	23.3 + 17.2	<0.0001
PPD (mm ± SD)	1.9 + 0.2	2.7 + 0.4	<0.0001
	Child of healthy parents ($n = 24$)	Child of GAP parents ($n = 24$)	p^*
PI (%)	24.1 ± 11.1	43.1 ± 22.2	0.001
GI (%)	1.7 ± 2.5	12.0 ± 10.1	0.0001
PPD (mm ± SD)	1.6 ± 0.3	2.4 ± 0.4	0.04

GAP, generalized aggressive periodontitis; GI, gingival index; PI, plaque index; PPD, periodontal probing depth; SD, standard deviation.
*Student's *t*-test ($p \leq 0.05$).

Discussion

Although the prevalence of AgP is relatively low, and it is apparently confined to susceptible groups, early stages of this disease could easily be underdiagnosed until clear signs of alveolar bone loss are detected in radiographic examinations. Therefore, the detection of specific bacteria associated with this disease in children at a young age could be important for the successful prevention and treatment of this disease (Shaddox et al. 2012), especially for children of GAP parents. Thus, this study assessed children from GAP families and compared them to children of healthy ones, showing worse clinical and microbiological conditions in GAP children.

Children from families with a history of GAP were selected in the present study once they could be considered as having a higher risk of

Table 4. Correlation (*r* [*p* value]) between *A. actinomycetemcomitans* amounts and clinical parameters in GAP and healthy children

	Aa amounts	PI	GI	PPD
Aa amounts	–	0.61 (0.003)	0.62 (0.002)	0.49 (0.004)
PI		–	0.66 (<0.0001)	0.59 (0.005)
GI			–	0.71 (<0.0001)
PPD				–

Aa, *Aggregatibacter actinomycetemcomitans*; GAP, generalized aggressive periodontitis; GI, gingival index; PI, plaque index; PPD, periodontal probing depth.

Table 5. Adjusted *r* value (PI control) of correlation between Aa amounts and clinical parameters in GAP and healthy children

Parameters	GI		PPD	
	r (*p*)	Adjusted *r* (*p*)	*r* (*p*)	Adjusted *r* (*p*)
Aa amounts	0.62 (0.002)	0.37 (0.05)	0.49 (0.004)	0.20 (0.30)

Aa, *Aggregatibacter actinomycetemcomitans*; GI, gingival index; PI, plaque index; PPD, periodontal probing depth.

developing this disease. Familial aggregation is one of the clearest characteristics of aggressive periodontitis (Meng et al. 2011) and it is the result of shared susceptibilities factors, such as genetic conditions, behavioural factors, and environmental factors, including a precocious exposure to specific microorganisms (Van Winkelhoff & Boutaga 2005). Thus, these children could have some conditions that could facilitate the emergence of GAP and the initiation of periodontal breakdown. However, to the best of our knowledge, no study has been designed to assess and compare GAP families to healthy families, allowing us to identify "pre-disease" aspects of GAP and their potential variables.

Generalized aggressive periodontitis subjects, although in supportive periodontal therapy, presented higher amounts and greater frequency of detection of *A. actinomycetemcomitans* subgingivally when compared with healthy subjects, even having a similar PI and no differences in frequency of professional dental care. Interestingly, children who had one parent diagnosed with GAP also have a higher frequency of detection and a greater amount of *A. actinomycetemcomitans*; however, this result is accompanied by a poor oral hygiene and higher PI in these children. The results of the parents reaffirmed the idea that *A. actinomycetemcomitans* could be related to GAP subjects and corroborated some studies that associated *A. actinomycetemcomitans* with disease and bone loss (Haubek et al. 2004, Cortelli et al. 2005). In fact, even after active periodontal therapy, the presence of *A. actinomycetemcomitans* has still been described, despite a reduction in concentration (Kleinfelder et al. 1999). However, although some reports have shown the presence of *A. actinomycetemcomitans* in children of localized aggressive periodontitis families (Tinoco et al. 1998), this is the first study reporting that children of GAP families presented a similar subgingival microbiological pattern to their parents, prior to GAP establishment, and reinforcing the role of *A. actinomycetemcomitans* in this population.

The major route of acquisition of oral microorganisms is vertical transmission, and saliva is the most common vehicle. Van Winkelhoff & Boutaga (2005), in a literature review, estimated the vertical transmission of *A. actinomycetemcomitans* was between 30% and 60% and a recent study (Monteiro et al. 2014) showed that a child having a parent with salivary *A. actinomycetemcomitans* is 16 times more likely to present *A. actinomycetemcomitans* in his or her own saliva. These results suggested that saliva could be the vehicle of contamination in these children by this microorganism, and saliva could be the source for subgingival colonization (von Troil-Lindén et al. 1996, Asikainen et al. 1997). Although no concentration of *A. actinomycetemcomitans* has been established as necessary for the occurrence of transmission, it is probable that as the amounts of microorganisms increase, transmission occurs more easily. This, in part, may justify a higher presence and quantity of *A. actinomycetemcomitans* in the children of GAP families, once their parents present a superior concentration of bacteria in the subgingival biofilm. Another important aspect is the role of individual characteristics on subgingival colonization. According to infectogenomics, host genetic factors interfere with the composition of human microbial biofilms and the response to this microbial challenge (Nibali et al. 2014). Thus, the genotype of the host could determine the susceptibility for colonization, such as an exacerbated inflammatory condition, creating a favourable environment for periodontal pathogen establishment and for disease development. In this way, GAP parents could genetically transmit some characteristics to their children and influence their oral microbiome and subsequent colonization by specific bacteria, such as *A. actinomycetemcomitans*.

Once subgingival colonization occurs, *A. actinomycetemcomitans* could modify the environment and the equilibrium between the host and bacteria, causing damage to the periodontal tissue and the beginning of disease (Haubek 2010). This idea could be linked to our results, which showed a relationship between *A. actinomycetemcomitans* colonization and gingival bleeding in Aa-positive children when adjusted for plaque control. DiRienzo et al. (1994), for example, showed a stronger correlation between the presence of *A. actinomycetemcomitans* and the conversion from periodontal health to disease. Haubek et al., in sequential studies of a Moroccan population, showed a strict relationship between the presence of a specific clone of *A. actinomycetemcomitans* (JP2_clone), and in lower proportion the presence of non-JP2 clone, and the progression of attachment loss and the development of AgP (Haubek

et al. 2004, 2008). A similar association of *A. actinomycetemcomitans* colonization and attachment loss was also observed in a longitudinal evaluation in adolescents from Ghana (Åberg et al. 2014). Furthermore, a longitudinal study in an American population suggested that the presence of *A. actinomycetemcomitans* in periodontally healthy children could serve as a risk marker for the initiation of localized aggressive periodontitis (Fine et al. 2007). This association between *A. actinomycetemcomitans* presence and periodontal breakdown could be due to several virulence factors as exotoxins (leucotoxin and cytolethal distending toxin); those are being considered the major virulence factor of *A. actinomycetemcomitans* and could lead to activation of the host response (Fives-Taylor et al. 1999). Thus, even with no confirmation about the pathogenic importance of *A. actinomycetemcomitans,* in this study, the presence of *A. actinomycetemcomitans* in the children suggests an association with the establishment of poor clinical conditions and GAP development – the follow-up of these children should confirm (or eliminate) this idea.

Meanwhile, it should be highlighted that although *A. actinomycetemcomitans* has been associated with periodontal disease, it could also be detected in healthy subjects. In this study specifically, a high prevalence of *A. actinomycetemcomitans* was observed in the subgingival biofilm of healthy subjects. And, although some results in the literature have appointed a low prevalence of this microorganism in healthy individuals (Cortelli et al. 2008, Lourenço et al. 2014), a high frequency of detection has already been described in a Brazilian population (Silva-Boghossian et al. 2011). Furthermore, despite its prevalence, the concentration in the biofilm is also important, once it could interfere with the intensity of aggression and with host response. In healthy group, *A. actinomycetemcomitans* amounts showed to be significantly lower. Thus, not only the acquisition but also the persistency of the microorganism and an inherent host response to the pathogen are necessary for disease establishment, which should be assessed in future studies. Also, a high prevalence of Tf was demonstrated in healthy subjects. This observation was confirmed by other groups that also showed that Tf is more frequently found in subgingival plaque than Pg and Aa (Mayanagi et al. 2004, Cortelli et al. 2008). This bacterium is considered a commensal pathogen, frequently detected in supragingival and subgingival plaque, and at both healthy and periodontitis sites; thus, it is not individually pathogenic in the oral cavity, but rather may influence the initiation and progression of periodontitis when present together with periodontopathic bacteria (Mayanagi et al. 2004).

Another interesting finding of the present study was the absence of differences in the rate of detection and concentration of Pg in children, especially in spite of the high detection and concentration of Pg in parents with GAP. *P. gingivalis* is a strictly anaerobic, non-mobile, Gram-negative microorganism, and these conditions hamper its presence in healthy sites, as observed in children without signs of the disease. On the other hand, Aa is a facultative, anaerobic microorganism that could easily live in high levels of oxygen. In this vein, the characteristics of *A. actinomycetemcomitans* may contribute to its identification in children and facilitate its colonization in a still healthy environment. Moreover, *A. actinomycetemcomitans* has a predilection in young subjects. The colonization of the oral cavity presumably occurs mainly in the early stages of life, while the colonization with new bacterial species/clonal types later in life may not occur easily (Haubek 2010). Crielaard et al., studying the microbiome of children between 3 and 18 years of age, showed that at these ages, microbiota were already in maturation, and thus, the individual was still susceptible to the transmission of microorganisms. Moreover, the oral microbiome was shown to be relatively stable, despite the significant biological changes that occur during the eruption of teeth (Crielaard et al. 2011). Saarela et al. (1999), for example, showed that children positive for Aa at the time of their first permanent tooth eruption had the persistence of this bacterium 5–11 years later and harboured the same strain as they initially did, suggesting that the oral colonization of Aa is stable and happens between 5 and 7 years old. Regarding this, the initial colonization by this microorganism in children evaluated in our study is a significant event and might be important to the composition of the oral microbiome.

Surprisingly, besides the higher presence of *A. actinomycetemcomitans*, children of GAP families also had worse clinical conditions when compared with children of healthy families. Children of GAP parents had worse oral hygiene than children of healthy parents, as observed by a higher level of plaque accumulation, and this condition could lead directly to a higher gingival inflammation and probing depth observed. Although these children presented higher probing depth, no diagnosis of AgP was observed. Due to the difficulty in detecting the cemento-enamel junction in semi-erupted teeth, CAL was not determined. Children presenting higher PD values were radiographically assessed, confirming the absence of alveolar bone crest resorption. Nevertheless, the higher probing depth in children of GAP parents could represent an initial alteration in clinical condition of this group and could be an important event in the beginning of pathogenic process. Added to this fact, the elevated PI and a subgingival environment could still contribute with the great frequency and quantity of *A. actinomycetemcomitans,* as periodontal pathogens need a complex environment to become established. Tinoco et al. in 1998, found quite similar results in children of localized AgP parents in the Brazilian population. Children presented high plaque, gingival bleeding, and *A. actinomycetemcomitans* colonization. However, in this study, no healthy families were included, which does not allow a comparison or a matched approach (Tinoco et al. 1998). Pahkla et al. in a study that evaluated the clinical and microbiological statuses of mothers with chronic periodontitis and their children, showed that this group had worse clinical conditions, poorer oral hygiene, and a higher rate of detection of periodontal bacteria (including Aa) when compared with healthy mothers and their children. They also detected that 71% of children

with a diseased mother had some alteration in gingiva (gingivitis or periodontitis), while only 15% of children of healthy mothers had these conditions (Pähkla et al. 2010). These results are similar to ours and suggest that the periodontal status of parents could explain the periodontal condition of their children. In addition, the pattern of hygiene appears to be a familial standard, and the sharing of susceptibility factors, not only genetic and microbiological factors but also behavioural factors (hygiene habits), could interfere with healthy periodontal status. Moreover, although clinical and microbiological differences were observed in our study, in children of GAP parents, no children were diagnosed with aggressive periodontitis. This is probably because of the age of the studied group; thus, a longitudinal follow-up is necessary for this kind of observation. Moreover, in addition to longitudinal follow-up, clinical condition of primary teeth and CAL determination should be done once they could be an important tool in diagnosis of aggressive periodontitis in children. Thus, future studies should consider these limitations, planning clinical (including full mouth evaluation and controlling plaque accumulation impact) and a large-scale microbiological evaluation.

Altogether, the results of the present study bring an opportunity to rethink ways to prevent GAP occurrence or to try to detect its establishment prior to periodontal breakdown. Considering that these children are members of a high-risk population and have the worst clinical and microbiological conditions observed in the present study, they should be included in a strict plaque-control regimen and in long-term monitoring. However, future observations will provide more information and confirm the validity of this idea. Although no identification of specific serotypes/genotypes of *A. actinomycetemcomitans* has been made, an important association of *A. actinomycetemcomitans* with GAP and a relationship of this bacterium with marginal inflammation in this population have been described. Thus, different microbiological approaches should be done using an adequate study design (e.g., matching gender and age, as done in the present study).

In conclusion, children who have at least one parent with aggressive periodontitis have worse clinical conditions, a higher frequency of detection, and greater amounts of *A. actinomycetemcomitans* when compared to children of two parents with periodontal health.

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Clinical Relevance

Scientific rationale for the study: As the familial aggregation of generalized aggressive periodontitis (GAP) is a common observation, children from families in which at least one member is affected by disease should be considered as a risk population and the evaluation of clinical and microbiological aspects become necessary.

Principal findings: Children from GAP families have worse clinical condition (higher PI, GI and PPD) and higher Aa amount detection. Furthermore, the concentration of Aa was associated with marginal inflammation.

Practical implications: These findings could guide the preventive activities, such as strict monitoring and plaque control, in a higher risk population for GAP development.

4.CONSIDERAÇÕES FINAIS

A etiopatogenia da Periodontite Agressiva ainda não é completamente compreendida. A forte agregação familiar, característica da PA, ou seja, a tendência dos casos de se desenvolver em uma mesma família, indica que fatores genéticos desempenham um importante papel na sua patogênese (Loos et al. 2015). Este fato vai de encontro com a evidência de que doenças inflamatórias complexas, de início precoce e que apresentam fenótipo severo são fortemente influenciadas por componentes genéticos. De fato, suspeita-se que os fatores genéticos possam ser responsáveis por aproximadamente 50% da variabilidade nos parâmetros clínicos das doenças periodontais, além de estarem associados à presença de microrganismos específicos e maior produção de marcadores inflamatórios (Nibali et al. 2013; Nibali et al. 2007; Vieira and Albandar 2014).

Nas últimas décadas, as pesquisas genéticas na periodontia focaram na identificação de alterações genéticas específicas, principalmente de polimorfismos de nucleotídeo único (SNPs), como fatores de risco para a periodontite (Loos et al. 2015; Schaefer et al. 2010). A despeito desses esforços, os fatores genéticos que contribuem para a patogenese das periodontites, especialmente da PA, ainda não estão totalmente definidos (Loos et al. 2015). Nesse sentido, a evidência atual indica que a PA e a PC são poligênicas, a exemplo de outras doenças complexas, com o envolvimento de multiplos genes de pequeno efeito (geralmente >100 para doenças complexas) (Loos et al. 2015; Vieira and Albandar 2014). Atualmente, apenas uma fração dos genes de susceptibilidade como os genes *GLT6D1*, *ANRIL* (CDKN2B antisense RNA 1), e *COX-2* foram associados a PA em estudos envolvendo algumas populações específicas (europeia, norte-americana e japonesa). Uma vez que cada população apresenta um grau de miscigenação e um perfil genético específico, a validação dessas alterações é necessária. O observado no capítulo 1 desta tese mostra que apenas o polimorfismo no gene da IL-10 mostrou-se significativo para a ocorrência da PA, sendo protetor para a ocorrência desta. Esse resultado confirma a importância de acessar o perfil genético das populações e, em vista do surgimento de novas tecnologias, o estudo mais amplo e profundo torna-se importante e necessário. Como visto no capítulo 2, com a análise por filtros familiares, pode-se identificar novos *locus* candidatos para a ocorrência da PA.

Dentre as alterações observadas, o indel no gene GPRC6A gene (p.Tyr775LeufsTer776) mostrou-se aquela com maior impacto funcional. Análises *in silico* indicaram a perda da cauda

C-terminal, levando a quebra da via de sinalização intra-celular da célula, levando a possíveis impactos fisiológicos. Como discutido no capítulo, essas alterações poderiam indicar novas frentes de estudo. Vale ainda ressaltar a necessidade de estudos funcionais mais profundos, identificando a modulação provocada por essa alteração genética.

Estas alterações genéticas podem ter influência direta na resposta imune dos pacientes afetados por PA (Nibali et al. 2013). Neste sentido, há evidência de que indivíduos com PA apresentam um desequilíbrio da resposta imune-inflamatória, com níveis elevados de mediadores pró-inflamatórios, como a IL-1β e TNF-α, e diminuição nos níveis de citocinas anti-inflamatórias, como a IL-10 e IL-4, no fluído crevicular gengival ou no soro, mesmo após o tratamento periodontal (Bastos et al. 2009; Duarte et al. 2010; Mattuella et al. 2013; Teles et al. 2010). O capítulo 4 demonstrou menor produção local de IL-10 e de anticorpos IgG contra *A. actinomycetemcomitans* em indivíduos com PA, ao passo que níveis elevados desse patógeno foram identificados nos mesmos sítios (Casarin et al. 2010). Interessantemente, o SNP identificado no capítulo 1 no gene da IL-10 está associado a uma menor produção da mesma, corroborando o achado do capítulo 4. Entretanto, outros estudos falharam em detectar quais alterações imunológicas estão associadas a essa condição (Ford et al. 2010). Nesse sentido, uma recente revisão sistemática concluiu que, apesar da evidência de que a PA é caracterizada por um desequilíbrio da resposta inflamatória, os estudos atualmente disponíveis não são suficientes para concluir quais alterações na produção de marcadores inflamatórios explicariam a rápida e severa destruição tecidual da PA (Duarte et al. 2015).

Com o objetivo de compreender melhor a resposta imune associada a PA, alguns estudos empregaram a análise do perfil de expressão gênica. Esses estudos reportaram maior expressão de genes relacionados a resposta imune-inflamatória, produção de anticorpos, e uma pronunciada indução de citotoxicidade mediada por células natural-killer (NK) em lesões de PA, com aumento na expressão de receptores ativadores das células natural killer (CD2-like receptor activating cytotoxic cells - CRACC) (Kebschull et al. 2013; Kramer et al. 2013). Assim, o capítulo 3 avaliou o perfil de expressão gênica da mucosa mastigatória de pacientes com histórico de PA e PC e indivíduos saudáveis, mostrando maior expressão de genes relacionados à resposta imune nos pacientes com histórico de PA, em especial receptores de células NK, o que corrobora com o conceito de que esses pacientes apresentam um quadro hiper-inflamatório (Taiete et al., 2017). Contudo, embora esses estudos identificaram algumas diferenças moleculares entre a PA e a PC, atualmente é impossível estabelecer uma distinção biológica clara entre os padrões de

respostas e vias celulares entre essas duas condições, que expliquem as diferenças fenotípicas entre elas (Armitage et al. 2010; Kramer et al. 2013; Loos and Papantonopoulos 2013; Smith et al. 2010).

Soma-se a falta de definição nos parâmetros imunológicos, a falta de consenso quanto a composição microbiana subgengival da PA. O conhecimento da composição da microbiota associada a PA é essencial para uma melhor compreensão da sua patogênese e para a individualização da terapia periodontal (Nibali 2015). Apesar de existir estudos que reportem alta frequência de detecção e níveis elevados de *A. actinomycetemcomitans*, *P. gingivalis*, *C. rectus, T. forsythia*, *T. denticola, Filifactor alocis*, e espécies de *Selenomonas* no biofilme subgengival de indivíduos com PA, atualmente não há evidência suficiente para discriminar a PA de outras doenças periodontais, como a periodontite crônica, com base em suas características microbiológicas (Faveri et al. 2009; Faveri et al. 2008; Nibali 2015; Schlafer et al. 2010; Teles et al. 2010). Além disso, outros estudos falharam em determinar diferenças na composição microbiana entre os diferentes tipos de periodontite (Armitage 2010; Nibali 2015). Interessantemente, fatores genéticos podem desempenhar um importante papel no perfil de colonização de bactérias comensais e patogênicas, como demonstrado pelos estudos de infectogenômica (Kellam and Weiss 2006; Nibali et al. 2009). Nibali et al. (2011; 2007) demonstraram uma associação moderada entre variantes genéticas da IL-6 e o aumento na detecção de *A. actinomycetemcomitans* em pacientes diagnosticados com PA e CP. Além disso, pacientes positivos para a presença de polimorfismo na IL-6 demonstraram uma tendência para o aumento nos níveis de *A. actinomycetemcomitans* no biofilme subgengival 3 meses após acesso cirúrgico para raspagem, quando comparado com indivíduos sem presença do polimorfismo (Nibali et al. 2013). Essa é uma avaliação que estudos futuros deverão efetivar a fim de estabelecer essa relação.

Entretanto, mesmo que ainda com resultados conflitantes, diversos estudos têm indicado uma relação do Aa com a PA. Dessa forma, a aquisição desse patógeno poderia indicar o início de uma colonização associada a doença, que poderia, no futuro, representar uma perda de inserção e ocorrência em si da PA. Aberg et al, 2014 e Haubek et al, 2008, avaliando jovens marroquinos, mostraram que a presença do Aa-JP-2 foi associada a uma perda de inserção e maior risco de PA. Entretanto, considerando o discutido acima, fatores genéticos intra-familiares podem ser determinantes. Assim, os capítulos 8 e 9 avaliaram filhos de sujeitos diagnosticados com PA quanto a colonização salivar de Aa, Pg e Tf, bem como a colonização subgengival por

esses, associando a situação clínica. Corroborando de certa forma com os estudos de Haubek et al, 2008 e Aberg et al, 2014 citados anteriormente, a presença de Aa foi associada a maior sangramento gengival, independente da presença da placa, além de uma maior prevalência e concentração deste patógeno na saliva e biofilme subgengival. Esse é um novo caminho nos estudos de PA. Indivíduos jovens, já afetados pela doença, foram recentemente estudos pelo grupo da professora Shaddox na Florida (Miller et al, 2017, Shaddox et al, 2016, Shaddox et al, 2012). Contudo, avaliar a condição previamente a doença em indivíduos de maior risco, podem representar, no futuro, a base para o desenvolvimento de terapias preventivas.

A prevenção da PA seria um fato de grande importância em vista do acometimento clínico, psicológico e da dificuldade de tratamento dessa condição. Tradicionalmente, o tratamento da PA tem focado no controle do fator etiológico primário, o biofilme, por meio da associação da terapia mecânica e uso adjunto de antimicrobianos. O capítulo 5 mostrou que o uso de agentes antimicrobianos, no caso iodo povidine, como irrigante no aparelho ultra-sônico, não promove benefícios adicionais. Isso se dá em especial pela dificuldade de ação desses agentes no interior da bolsa por um tempo eficaz (Eakle et al, 1986). Assim, a associação de raspagem e antimicrobianos sistêmicos tem demonstrado a capacidade de promover benefícios clínicos adicionais, com maior redução da profundidade de sondagem e ganho de inserção após 6 meses da terapia, bem como a redução de patógenos e grupos de micro-organismos associados a doença (Aimetti et al. 2012; Mestnik et al. 2012; Silva-Senem et al. 2013). Esses resultados corroboram com o do presente capítulo 6, no qual a combinação de amoxicilina e metronidazol foi associada a terapia de desinfecção de boca toda com ultrassom em 45 minutos, proposta por Wennstrom et al, 2005. Essa proposta converge à sugestão de Slot et al, 2008, o qual em uma revisão da literatura indicou a associação de antimicrobianos com terapias mecânicas de curto prazo para uma maior efetividade do tratamento.

Contudo, mesmo que benefícios adicionais tenham sido observados, essa terapia combinada ainda não se mostrou capaz de reestabelecer completamente a saúde periodontal. Estudos recentes e o mesmo capítulo 6 reportaram a ocorrência de bolsas residuais (6.4% e 10.7% nos estudos de Mestnik et al. (2012) e Casarin et al. (2012), respectivamente), alto risco de recorrência da doença e de perda dental após a fase ativa da terapia periodontal (Teughels et al. 2014). Recentemente, Dopico et al. (2016) mostraram que pacientes de PA apresentam uma significante taxa de recorrência e perda dental após o tratamento e que, a presença de bolsas residuais profundas eleva de maneira significativa a taxa anual de perda dental.

Essa parcela de sítios com resposta parcial e a alta taxa de recorrência da PA pode ser associada não apenas aos fatores microbiológicos abordados pela associação da terapia mecânica e antimicrobianos sistêmicos. Como discutido anteriormente, a PA pode ser caracterizada por uma associação de alterações gênicas, microbiológicas e da resposta inflamatória que, assim como em sua etiologia, poderiam afetar também o resultado da terapia periodontal. Em estudo do nosso grupo, por meio de uma análise de múltiplos fatores, a concentração inicial de IL-10 foi diretamente relacionada ao padrão de resposta a terapia mecânica, enquanto os níveis de certos microrganismos (*Aggregatibacter actinomycetemcomitans* e *Porphyromonas gingivalis*), bem como característica clínicas (PS e NIC) não foram preditivos de resposta (Casarin et al., 2012, Abstract IADR). Esse resultado corrobora outro recente estudo publicado, o qual demonstrou a correlação entre o perfil de resposta inflamatória frente ao estímulo por lipopolissacarídeos com o padrão de resposta clínica após o tratamento periodontal (Allin et al. 2016). Esses resultados, em conjunto com o previamente discutido, indicam que as alterações no perfil imuno-inflamatório, poderiam explicar a pior resposta a terapia periodontal. Assim, alternativas terapêuticas que sejam capazes de reparar o equilíbrio entre os mediadores pró e anti-inflamatórios, chamada de Modulação do hospedeiro, deverão ser foco de estudos para avaliar sua capacidade de adequar a resposta imune e promover benefícios clínicos mais previsíveis.

Assim, em vista do exposto, embora diversos estudos tenham direcionado seus esforços para avaliar os aspectos etiopatogênicos e também o padrão de resposta às terapias periodontais, a PA ainda não apresenta definições claras a respeitos desses fatores, e nem mesmo terapias estabelecidas que promovam melhorias previsíveis ao indivíduo. Além disso, há a necessidade de se integrar os novos conhecimentos, gerados pelas técnicas de última geração, dos fatores genéticos, microbiológicos e imunológicos envolvidos em sua patogênese com a resposta aos tratamentos atualmente empregados (Garcia et al. 2013). Esse conhecimento visa obter a instituição de terapias individualizadas às necessidades específicas de cada paciente. De uma perspectiva científica, essas análises levarão a um melhor entendimento de sua etiologia e patogênese, permitindo uma melhora do manejo clínico do tratamento e, consequentemente, da prevenção da periodontite agressiva.

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