UNESP - Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

Kahena Rodrigues Soldati

Peptídeos antimicrobianos e doença periodontal: influência do tabagismo na expressão de peptídeos antimicrobianos e possíveis efeitos de peptídeos sintéticos sobre biofilmes multi-espécies

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Tese apresentada à Universidade Estadual Paulista (UNESP), Faculdade de Odontologia, Araraquara para obtenção do título de Doutor em Odontologia, na Área de Periodontia.

Orientador: Profa. Dra Daniela Leal Zandim-Barcelos

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Robin Sharma

Soldati KR. Peptídeos antimicrobianos e doença periodontal: influência do tabagismo na expressão de peptídeos antimicrobianos e possíveis efeitos de peptídeos sintéticos sobre biofilmes multi-espécies. [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2020.

RESUMO

Peptídeos antimicrobianos (PAMs) são importantes componentes da resposta do hospedeiro contra patógenos invasores. Além da ação antimicrobiana direta, podem também participar na modulação do sistema imune. No entanto, o papel dos PAMs na etiopatogênese da doença periodontal e os fatores de risco que podem influenciar sua expressão na cavidade bucal ainda não se encontram totalmente elucidados. Dessa forma, os objetivos do presente estudo foram: (1) avaliar a influência do tabagismo nos níveis de LL-37 e HNP 1-3 no fluido crevicular gengival (FCG) de pacientes com periodontite e investigar a associação entre os níveis destes PAMs e de mediadores inflamatórios nos sítios com ausência e presença de doença periodontal; (2) investigar o impacto do tabagismo nos níveis de hBD1 e 2 em pacientes com periodontite e avaliar os efeitos da nicotina e cotinina na expressão destes PAMs por queratinócitos; (3) avaliar a atividade metabólica e enzimática durante a recolonização de biofilmes multi-espécies periodontopatogênicos após tratamento com peptídeos sintéticos derivados da catelicidina humana (LL31 e D-LL31). A quantificação dos PAMs e mediadores inflamatórios no FCG foi realizada por meio de ensaio ELISA sanduiche e Multiplex, repectivamente, enquanto a RT-qPCR em tempo real detectou a expressão gênica dos PAMs por queratinócitos. A produção de ácido láctico foi mensurada para determinar a atividade metabólica nos biofilmes pós-tratamento, enquanto a atividade enzimática foi analisada por meio da dipeptidil peptidase (DPP). Além disso, a viabilidade celular foi avaliada pela contagem de unidades formadoras de colônia e o qPCR identificou a presença de *P. gingivalis* e a concentração de bactérias totais nos biofilmes. Os resultados dos estudos clínicos demonstraram que o tabagismo pode influenciar na expressão dos PAMs, reduzindo os níveis de LL-37, HNP 1-3 e hBD1 e aumentando os níveis de hBD2 no FCG. Nos sítios doentes, foram observados maiores níveis de LL-37 comparados aos sítios sadios, com associação positiva à presença de IL-1β e MMP-8. A HNP 1-3, por sua vez,

apresentou níveis mais baixos, com associação positiva a IL-10. Nos experimentos in vitro, foi constatado que a nicotina reduz significativamente a expressão gênica de hBD1 por queratinócitos, sendo essa redução mais acentuada na presença de *A. actinomycetemcomitans.* Tanto a nicotina como a cotinina promoveram redução na expressão de hBD2 quando associadas à cultura bacteriana. No entanto, esta redução foi significativa apenas para o estímulo simultâneo com cotinina *e P. gingivalis.* Em relação ao efeito dos peptídeos sintéticos sobre a recolonização de biofilmes multi-espécies, os resultados indicam que o D-LL31 possui ação inibitória sobre a atividade metabólica e enzimática durante a recolonização de biofilme multi-espécies periodontopatogênico, podendo seu efeito se prolongar até 5 dias após o tratamento. Assim, podemos concluir que o efeito do tabagismo na expressão dos PAMs pode ser mais um mecanismo associado à maior prevalência e severidade de doença periodontal em fumantes. Além disso, o efeito inibitório do peptídeo sintético D-LL31 torna-o um possível agente terapêutico para utilização no tratamento da doença periodontal.

Palavras chave: Peptídeos catiônicos antimicrobianos. Periodontite. Tabagismo. Biofilmes.

Soldati KR. Antimicrobial peptides and periodontal disease: influence of smoking on the expression of antimicrobial peptides and possible effects of synthetic peptides on multi-species biofilms. [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2020

ABSTRACT

Antimicrobial peptides (AMPs) are important components of the host response against invading pathogens. In addition to the direct antimicrobial activity, they can also participate in the immune system modulation. However, the role of AMPs in the etiopathogenesis of periodontal disease and the risk factors that may influence their expression in the oral cavity are not yet fully understood. Thus, the purposes of the present study were: (1) to evaluate the influence of smoking on the levels of LL-37 and HNP 1-3 in the gingival crevicular fluid (GCF) of patients with periodontitis and to investigate the association between the levels of these AMPs and inflammatory mediators at sites with absence and presence of periodontal disease; (2) to investigate the impact of smoking on hBD1 and 2 levels in patients with periodontitis and to evaluate the effects of nicotine and cotinine on the expression of these AMPs by keratinocytes; (3) evaluate the metabolic and enzymatic activity during the recolonization of multi-species periodontopathogenic biofilms after treatment with synthetic peptides derived from human cathelicidin (LL31 and D-LL31). The quantification of AMPs and inflammatory mediators in the GCF was performed by sandwich ELISAs and Multiplex assay, respectively, while RT-qPCR in real time detected the gene expression of the AMPs by keratinocytes. The production of lactic acid was measured to determine the metabolic activity in post-treatment biofilms, while the enzymatic activity was analyzed using dipeptidyl peptidase (DPP). In addition, cell viability was assessed by counting colony-forming units and qPCR identified the presence of *P. gingivalis* and the concentration of total bacteria in biofilms. The results of clinical studies have shown that smoking can influence the expression of AMPs, reducing the levels of LL-37, HNP 1-3 and hBD1 and increasing the levels of hBD2 in the FCG. Higher levels of LL-37 were observed in diseased sites compared to healthy sites, with a positive association with the presence of IL-1β and MMP-8. Nevertheless, HNP 1-3 presented lower levels with positive association with IL-10. In in vitro experiments, it was found that nicotine significantly reduces the

gene expression of hBD1 by keratinocytes, being this reduction more pronounced in the presence of *A. actinomycetemcomitans*. Both nicotine and cotinine reduced hBD2 expression when associated with bacterial culture. However, this reduction was significant only for the simultaneous stimulation with cotinine and *P. gingivalis*. Regarding the effect of synthetic peptides on the recolonization of multi-species biofilms, the results indicate that D-LL31 has an inhibitory action on the metabolic and enzymatic activity during the recolonization of periodontopathogenic multi-species biofilms, and its effect can be prolonged up to 5 days after treatment. Thus, we can conclude that the effect of smoking on the expression of AMPs may be one of the mechanisms associated with the higher prevalence and severity of periodontal disease in smokers. In addition, the inhibitory effect of the synthetic peptide D-LL31 makes it a possible therapeutic agent for use in the treatment of periodontal disease.

Keywords: Antimicrobial cationic peptides. Periodontitis. Smoking. Biofilms.

SUMÁRIO

1 INTRODUÇÃO

A periodontite é uma doença inflamatória caracterizada pela destruição dos tecidos de suporte do dente[1]. Apesar do biofilme bacteriano ser considerado o fator etiológico primário das doenças periodontais, a severidade e progressão das mesmas depende de um balanço dinâmico entre biofilme, fatores genéticos e fatores ambientais[2]. Portanto, as interações que ocorrem entre os mecanismos de defesa do hospedeiro e o desafio bacteriano são essenciais para a determinação da suscetibilidade individual à periodontite.

Os mecanismos de defesa do hospedeiro são constituídos pela imunidade inata e adaptativa[3]. O sistema imune inato mantém uma barreira física e funcional contra microrganismos, tendo um papel primário na defesa do hospedeiro contra o biofilme bacteriano. O epitélio oral funciona como uma barreira física contra microrganismos patogênicos, e os neutrófilos, que migram através do epitélio juncional, formam uma barreira entre o biofilme bacteriano e o epitélio subjacente[4,5]. Tanto as células epiteliais como os neutrófilos produzem os peptídeos antimicrobianos (PAMs) que possuem atividade antimicrobiana contra bactérias Gram-positivas e Gram-negativas, fungos e vírus [6–8]. Além disso, estes PAMs são quimioatraentes para células fagocitárias e mastócitos, induzem mediadores inflamatórios e degranulação de mastócitos, regulam eventos inflamatórios iniciais, e aumentam a imunidade adaptativa[9,10]. As principais atividades imunomodulatórias dos PAMs estão citadas na Tabela 1[11]. Assim, os PAMs são importantes moléculas do sistema imune inato que contribuem para a manutenção do balanço entre saúde e doença da cavidade bucal. Em relação à sua expressão na cavidade oral, os PAMs podem ser detectados na língua, mucosa bucal, epitélio gengival e glândulas salivares[5,12,13].

Dentre os PAMs mais estudados e que apresentam participação importante na resposta imune, pode-se citar a catelicidina e as defensinas[14]. As defensinas são uma classe de PAMs produzida por muitos organismos eucariotos, incluindo mamíferos, insetos e plantas[15]. Em humanos, são produzidas por células epiteliais de superfície de mucosa e por leucócitos contendo grânulos, incluindo neutrófilos e células natural killer[16], apresentando-se tanto em tecidos sadios como em tecidos inflamados[17]. A ação antimicrobiana das defensinas é baseada em suas propriedades físicas e

depende de sua capacidade em se ligar às membranas microbianas através de interações eletrostáticas e hidrofóbicas, levando à disruptura da membrana celular dos microrganismos. Enquanto sua atividade quimiotática pode ser efetiva para neutrófilos, eosinófilos, monócitos, células T[18,19], mastócitos e células dendríticas[20,21].

Tabela 1 - Atividades imunomodulatórias dos peptídeos antimicrobianos

Peptídeo antimicrobiano	Tipo	Atividade relevante para imunidade	
		In vitro	**In vivo**
Alfa-defensinas	HNP 1-3	Atividade antimicrobiana Atividade antiviral Atividade quimiotática Promoção fagocitose Degranulação de mastócitos Estimula a produção de citocinas e quimiocinas Regula ativação complemento Inibição do receptor ACTH e produção de glucocorticóide	Recrutamento de células imunes Indução de citocinas Aumento da resposta imune humoral e celular (Ag-específica) Confere resistência à invasão bacteriana
Beta-defensinas	hBD 1-3	Atividade antimicrobiana Atividade quimiotática através CCR6 Degranulação de mastócitos Estimula a produção de citocinas e prostaglandinas Indução da maturação de células dendríticas através TLR4 (apenas mBD 2)	Aumento da resposta imune humoral e celular (Ag-específica) Confere resistência à invasão bacteriana
Catelicidina	LL-37	Atividade antimicrobiana Atividade quimiotática Promoção fagocitose Estimula a produção de quimiocinas Degranulação de mastócitos Neutralização endotoxina bacteriana	Efeito antibacteriano Estimula angiogênese Estimula re-epitelização de feridas na pele

Fonte: Tabela adaptada de Yang et al.[11], 2004

As duas principais subfamílias de defensinas humanas, denominadas alfa e beta-defensinas (hBD), são diferenciadas pela localização das pontes de dissulfeto, pelo comprimento dos aminoácidos e também pelas diferentes células que as expressam[22,23]. As alfa-defensinas são subdvididas em seis diferentes tipos, que podem ser sintetizados por neutrófilos, peptídeos neutrofílicos humanos (HNP) 1-4, ou pelas células de Paneth presentes na mucosa do intestino (defensina humana 5 e 6 – HD 5 e HD 6). As HNP 1-4 são sintetizadas como precursores, clivadas proteoliticamente, gerando uma defensina catiônica madura que é estocada em grânulos azurófilos, presentes nos neutrófilos, e depois liberada [24,25]. Estruturalmente

semelhante às HNP, as hBD também são constituídas por três pontes de dissulfeto, porém diferenciam-se pelo espaçamento e emparelhamentos dos resíduos de cisteína [26]. As hBDs, por sua vez, são sintetizadas por células epiteliais. As defensinas podem ser expressas na gengiva, língua, glândulas salivares e mucosa, podendo ser encontradas na saliva e fluido crevicular gengival (FCG).

Embora tenham sido encontradas cerca de 30 tipos de catelicidina em mamíferos, apenas uma foi identificada em humanos até hoje, a LL-37/hCAP18 que primeiramente apresenta-se como uma proteína antibacteriana catiônica humana de 18 kDa (hCAP18). Nos neutrófilos, os hCAP18 são armazenados nos grânulos secundários, outro tipo de grânulo citoplasmático especializado, que contém agentes antimicrobianos. O aminoácido número 37 da alfa hélice da extremidade C-terminal desta proteína precursora é clivado, formando o peptídeo LL-37, que é a forma ativa e madura da catelicidina[27,28]. Na cavidade oral, a LL-37 tem sido encontrada no epitélio gengival, FCG e saliva[29,30].

Na última década, observa-se um aumento do número de publicação sugerindo uma possível implicação dos PAMs na homeostasia periodontal. No sétimo Workshop Europeu de Periodontia foi destacado que os PAMs podem influenciar a homeostasia da cavidade bucal não somente devido à sua atividade antimicrobiana contra patógenos bucais, mas também pelas suas propriedades imunomodulatórias tanto na resposta imune inata como na adaptativa.

Em resposta à condição de saúde ou doença nos tecidos periodontais, a regulação de PAMs pode sofrer as mais diferentes alterações. Estudos clínicos demonstraram que a catelicidina, LL-37/hCAP18, apresentou-se significativamente elevada no FCG de pacientes com periodontite crônica em comparação com pacientes periodontalmente saudáveis[31,32], assim como em pacientes com periodontite agressiva quando comparados à gengivite e a pacientes saudáveis[33]. Foram observadas concentrações mais elevadas de HNP1-3 no FCG de pacientes com periodontite agressiva e crônica do que em pacientes periodontalmente saudáveis[32]. No entanto, biópsias gengivais de pacientes com periodontite apresentaram níveis mais baixos de mRNAs de hBD1, hBD2 e hBD3 em comparação com biópsias de tecido gengival saudável[34,35]. Também foi demonstrado que o nível de hBD3 estava inversamente correlacionado com a severidade da doença e o grau de colonização de espécies bacterianas com elevado potencial periodontopatogênico[36].

Alguns trabalhos têm demonstrado que fatores de risco para a doença periodontal como tabagismo, diabetes melitus e obesidade podem provocar alterações na expressão dos PAMs. Dessa forma, os PAMs poderiam funcionar como um possível mecanismo de ligação entre as doenças periodontais e seus fatores de risco.

Diferentes mecanismos biológicos podem explicar a maior prevalência e severidade da doença periodontal em fumantes. Componentes do cigarro podem alterar mecanismos como: vasoconstrição periférica[37], a qual reduz a distribuição de oxigênio e nutrientes no tecido gengival; alterações nas funções imunológicas[38], podendo afetar a função neutrofílica[39] e fibroblástica[40–42] além de promover a diferenciação de osteoclastos no tecido periodontal[43–46]. Além disso, o tabagismo pode aumentar a degradação periodontal e ainda comprometer a resposta ao tratamento periodontal[47,48]. De maneira geral, o fumo parece inibir a defesa do hospedeiro contra infecções microbianas enquanto promove ou aumenta a reação inflamatória[49]. Quanto à regulação de PAMs, estudos prévios mostraram que os níveis de hBD2 e LL-37 estavam significativamente mais elevados no FCG de pacientes fumantes com periodontite agressiva generalizada em comparação com não fumantes. Da mesma forma, níveis mais altos destes PAMs foram detectados no FCG de fumantes com gengivite quando comparados a não fumantes com gengivite[50]. No entanto, a expressão de hBD1 e 2 no tecido gengival saudável, com ausência de inflamação, de pacientes fumantes foi significativamente menor do que no tecido gengival de pacientes não fumantes[51]. Além disso, pacientes fumantes com periodontite crônica também apresentaram níveis mais baixos de LL-37 na saliva e no FCG quando comparados aos não fumantes[52]. Apesar destas evidências clínicas, o efeito do tabagismo na regulação da expressão dos PAMs ainda não se encontra devidamente elucidado.

Tem sido hipotetizado que uma combinação específica de PAMs poderia preservar a saúde bucal e que qualquer modificação desta combinação poderia refletir em um desequilíbrio da resposta imune inata[53]. Este desequilíbrio poderia representar uma parte dos processos chaves que ainda faltam ser identificados para a compreensão dos mecanismos envolvidos no início e no desenvolvimento das doenças periodontais[54–57]. De fato, um melhor entendimento sobre a regulação dos PAMs nos tecidos periodontais é importante para o desenvolvimento de novas medidas preventivas, diagnósticas e/ou terapêuticas para as doenças periodontais.

2 PROPOSIÇÃO

Proposição geral: Avaliar a influência do tabagismo e de componentes do cigarro sobre a regulação de peptídeos antimicrobianos endógenos; e determinar o efeito de um peptídeo sintético na recolonização de biofilmes multi-espécies.

Publicação 1 – Smoking reduces cathelicidin LL-37 and human neutrophil peptide 1-3 levels in the gingival crevicular fluid of patients with periodontitis

Objetivo: avaliar a influência do tabagismo nos níveis de LL-37 e HNP 1-3 no fluido gengival de pacientes com periodontite e investigar a associação entre os níveis destes peptídeos e de mediadores inflamatórios com ausência e presença de doença periodontal.

Publicação 2 – The impact of smoking on protein levels and gene expression of human beta-defensins: a clinical and in vitro study

Objetivo: investigar o impacto do tabagismo nos níveis de hBD 1 e 2 em pacientes com periodontite e avaliar os efeitos da nicotina e cotinina na expressão destes peptídeos por queratinócitos.

Publicação 3 – Effect of D-enantiomeric peptide treatment on regrowth of periodontopathogenic microcosm biofilm

Objetivo: avaliar atividade metabólica e enzimática durante a recolonização de biofilmes multi-espécies periodontopatogênicos após tratamento com peptídeos sintéticos derivados da catelicidina humana (LL31 e D-LL31).

3 PUBLICAÇÕES

3.1 Publicação 1

Smoking reduces cathelicidin LL-37 and human neutrophil peptide 1-3 levels in the gingival crevicular fluid of patients with periodontitis

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Runing Title:

The influence of smoking on antimicrobial peptides levels

Key findings: Smoking reduces gingival crevicular fluid levels of cathelicidin and alpha-defensins. Higher levels of cathelicidin and lower levels of alpha-defensins associated to the presence of periodontitis.

(Word count: 2909 ; Number of figures: 2; Number of tables: 3; References in the manuscript: 48)

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Abstract

Background: Antimicrobial peptides are components of innate immune response that have a key role on susceptibility and resistance of the oral cavity to diseases. This study aimed to investigate the influence of smoking on cathelicidin LL-37 and human neutrophil peptides 1 through 3 (HNP 1-3) levels in the gingival crevicular fluid (GCF) of patients with periodontitis. The relationship between levels of these peptides with the periodontal status and selected inflammatory mediators levels in smokers and non-smokers was also evaluated.

Methods: Forty patients with periodontitis, 20 smokers and 20 non-smokers were recruited. After a full periodontal clinical assessment, GCF samples were collected from healthy (n=5) and diseased (n=5) sites of each patient. Peptides and selected inflammatory mediators in the GCF were quantitated by sandwich ELISAs and Multiplex assay, respectively.

Results: Diseased sites had significantly ($p<0.05$) higher levels of LL-37 and lower levels of HNP 1-3 than healthy sites in both smokers and non-smokers. Diseased sites of smokers presented significantly lower levels of LL-37 (approximately 25%) and HNP 1-3 (approximately 50%) when compared to diseased sites of non-smokers. Concentration of LL-37 was directly correlated with the presence of pro-inflammatory mediators MMP-8 and IL-1β and inversely correlated with concentration of IL-10. HNP 1-3 concentration was positively correlated with IL-10 and negatively correlated with concentrations of MMP-8 and IL-1β.

Conclusion: Smoking was associated with reduced levels of LL-37 and HNP 1-3 in GCF of patients with periodontitis. LL-37 had a distinct expression pattern from HNP 1-3: LL-37 was upregulated in diseased sites, and HNP 1-3 were increased in periodontally heathy sites.

Keywords: Cationic antimicrobial peptides. Cathelicidins. Alpha-defensins. Periodontitis. Smoking. Nicotine.

Introduction

Periodontitis is an inflammatory disease that involves the disruption of oral homeostasis in the presence of dental plaque, modified and influenced by genetic and environmental factors[1,2]. Smoking is a well-defined risk factor for periodontal disease[3–5]. Smokers have an increased prevalence and severity of periodontal disease as well as a higher prevalence of tooth loss and total edentulism[6]. Although the association between smoking and periodontal disease has been extensively investigated, the mechanisms by which smoking contributes to the progression of periodontal disease are not totally understood. Evidence indicates that smoking may affect the vasculature and the immune system[7,8].

Antimicrobial peptides (AMPs) are soluble molecules participating in the innate immunity of mucosal surfaces against pathogens[9]. Dysregulated expression of AMPs has been considered a possible causal mechanistic link in the microbial dysbiosis associated with periodontal disease and some risk factors, including tobacco smoking, diabetes mellitus and obesity[10]. In the oral cavity, there is a diverse group of AMPs; and the most studied of these peptides are defensins and the cathelicidin LL-37[11]. In addition to the direct antimicrobial activity, AMPs also possess a wide range of immunomodulatory properties[12,13]. These peptides are involved in wound repair process, cell migration and chemotaxis[14–16]. However, scarce information is available on the impact of smoking in the immune response associated with host-microbial interactions in the oral cavity, especially in the expression of AMPs.

A previous in vitro study showed that cigarette smoke extract decreases expression of β-defensin (hBD) 2 and increases production of IL-8 by human epithelial cells[17]. Gene expression of hBD1 and hBD2 was also significantly reduced in the gingival tissues of smokers in comparison with that of non-smokers[18]. Also, lower

levels of LL-37 were detected in the gingival crevicular fluid (GCF) of smokers with chronic periodontitis in comparison with GCF of non-smokers with periodontitis[19] and a negative correlation was observed between LL-37 and cotinine salivary levels[20].

AMPs not only have chemotactic effects but also affect the production of various chemokines and can influence the microenvironment by affecting the net content of immunoregulatory mediators released by immune cells[21] and keratinocytes[22]. LL-37 can stimulate polymorphonuclear cells to release histamine, TNF and IL-6 and also increase mRNA expression of IL-1β, CCL2 and CCL3[23]. The stimulation of keratinocytes with LL-37 resulted in increased secretion of CCL20[22], as well as upregulation of inflammatory cytokine IL-36[24]. Conversely, HNP 1-3 seems dampen inflammation through down-regulation of pro-inflammatory cytokines produced by macrophages, such as TNF-α, IL-8, IL-6 and IL-1β[25].

The up-regulation of some pro-inflammatory mediators as well as down-regulation of anti-inflammatory mediators in GCF is positively associated with periodontitis independently of bacterial load at a given site[26,27], but the association inflammation-associated molecules with the production and levels of AMPs in vivo is still largely unexplored. This study assesses the influence of smoking on GCF levels of LL-37 and HNP 1-3 in patients with periodontitis, the association between AMPs in the GCF and the periodontal clinical condition, as well as the correlation between levels of AMPs and selected inflammatory mediators in the GCF.

Materials and Methods

Study design

The study protocol was approved by the Ethics Committee on Human Research of the School of Dentistry at Araraquara – UNESP (protocol number 01394712.5.0000.5416) and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013.

A total of 40 patients of both genders with periodontitis were enrolled in this study: 20 smokers (S) and 20 non-smokers (NS). The participants were recruited at the Periodontology Clinic, School of Dentistry at Araraquara – UNESP. Written informed consent was obtained from all subjects.

The diagnosis of periodontitis was performed according to the 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions[28]. The inclusion criteria for periodontitis patients were presence of a minimum of 15 natural teeth and at least five sites with probing depth (PD) ≥ 4 mm, bleeding on probing (BOP) and clinical attachment level (CAL) ≥ 4 mm, with the exception of third molars. Smoking patients regularly smoked at least 10 cigarettes/day for a minimum of 5 years and non-smoking patients had never smoked[29,30]. Individuals with a history of systemic diseases or immunological disorders, currently using hormone replacement therapy, as well as pregnant and lactating women were excluded. Individuals that received periodontal treatment within the last 6 months or with a history of systemic antibiotics and anti-inflammatory drugs within the last 3 months were also excluded.

Clinical examination

Full-mouth clinical periodontal parameters, including Plaque Index (PI), Gingival Index (GI) PD, BOP and CAL, were assessed by a single trained and calibrated examiner. The PI and GI were registered as absence or presence of plaque and gingival bleeding at four sites per tooth (mesial, distal, buccal and lingual)[31], while the PD and CAL were assessed in six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual), excluding third molars. Measurements were performed with a manual UNC periodontal probe (Hu-Friedy, Chicago, IL). For calibration, the measurement of PD was made in six teeth (16, 21, 24, 36, 41 and 44) according to the Ramfjord index, in a total of 6 patients (36 teeth/patient) in two different occasions within 7 days. Data were submitted to Kappa concordance analysis (k=0.91).

Gingival crevicular fluid (GCF) sampling

GCF samples were collected from 10 sites from each patient, including 5 healthy and 5 diseased sites. Collection sites were selected from non-adjacent teeth, preferably including at least one healthy and one diseased site per quadrant. Healthy sites had no gingival inflammation, PD ≤ 3 mm, no BOP or clinical attachment loss; and diseased sites had gingival inflammation, PD and CAL ≥ 4 mm with BOP. Diseased sites were selected according to the deepest probing depth in each quadrant. A single examiner performed GCF sampling, one day after periodontal examination.

Before sampling, the teeth were isolated with cotton rolls, supragingival plaque was removed with a sterile curette and the surfaces were gently air-dried. GCF was sampled by inserting absorbent paper strips (Periopaper®, Oraflow Inc., New York,

USA) into the gingival sulcus or periodontal pocket for 30 seconds. Paper strips contaminated with blood and saliva were rejected. The GCF volume absorbed into each strip was determined by an electronic device (Periotron 8000 - Periopaper, ProFlow, Inc., Amityville, NY, USA), which was calibrated based on a protocol described before[32] (calibration of the Periotron 8000 by polynomial regression). Samples of the same category (healthy or diseased sites) in each subject were immediately pooled into a dry sterile polypropylene microcentrifuge tube (5 paper strips per tube) and kept at -80°C until analysis. The readings from the Periotron 8000 were converted to an actual volume (µL) by reference to a standard curve[33].

Quantification of antimicrobial peptides and inflammatory mediators

First, the absorbed GCF was eluted from the paper strips by adding 750 µL (150 µL per strip) of 1x Phosphate-Buffered Saline (PBS) to each tube. The tubes were left on ice in a horizontal shaker for 30 minutes and then centrifuged at 4°C, 13,000 g for 10 minutes. The GCF levels of cathelicidin LL-37 and HNP 1-3 were measured (pg/mL) using sandwich ELISAs according to the manufacturer's guidelines (Hycult Biotechnology, Uden, Netherlands). The absorbance was measured at 450 nm using a plate reader (VersaMax, Molecular Devices, San Jose, CA, USA). The detection range of LL-37 was from 0.1 to 100 ng/mL and of HNP 1-3 was from 156 to 10,000 pg/mL. Results are expressed in pg/mL.

The levels of the immunoinflammatory mediators IL-1β, IL-10, MMP-8, TNF-α and IFN-gamma in the GCF were analyzed by magnetic bead-based multiplex assay according to the instructions provided by the kit manufacturer (R&D Systems, Minneapolis, MN, USA) and the Luminex® 100™ system was used for the reading. The

amount of protein in each sample was extrapolated based on the standard curve, and concentrations are reported in pg/mL.

Statistical analysis

The statistical analysis was performed with the use of GraphPad Prism 7 (San Diego, CA, USA) and differences were considered to be significant at a P value of <0.05. Distribution normality was analyzed using the D'Agostino and Pearson test. Normal distribution was detected only for age and LL-37 levels parameters. Thus, unpaired t test was performed for inter-group comparison and paired t test for intra-group analysis. Clinical parameters and HNP 1-3 levels of non-normal distribution were analyzed by Mann-Whitney tests for inter-group comparisons and Wilcoxon test intra-group analyses. Correlations between the concentrations of inflammatory mediators and AMPs in both healthy and diseased sites for smokers and non-smokers were determined by Spearman rank correlation test.

Results

Demographics and periodontal parameters

Forty patients aged 19-72 years participated in this study. No significant difference in age (S [44.65 ± 11.21]; NS [38,10 ± 12,26]) and gender (S - 10 males/10 females; NS - 5 male/15 females) was detected between smokers and non-smokers. The only significant difference between smokers and non-smokers in the periodontal clinical parameters was a higher GI in non-smokers (NS) ($p<0.05$) (Table 1).

In both smokers (S) and NS, healthy sites had significantly lower PD and CAL ($p <0.05$, Table 2). Interestingly, crevicular fluid volume (CFV) was significantly

reduced in healthy sites in comparison to diseased sites only in NS patients ($p < 0.05$, Table 2).

Antimicrobial peptides and immunoinflammatory mediators

Figure 1 shows the GCF levels of AMPs in each group. Smokers had significantly lower levels of HNP 1-3 in both healthy and diseased sites in comparison to NS. LL-37 was also reduced in the GCF from both healthy and diseased sites of S in comparison to NS, but this difference was statistically significant only for diseased sites.

Regardless of the smoking status, diseased sites had higher levels of LL-37 and lower levels of HNP1-3. In both S and NS patients, the difference in GCF levels of LL-37 and HNP1-3 between healthy and diseased sites was statistically significant.

Three of the 5 inflammatory mediators were detected in GCF, including IL-1β, MMP-8 and IL-10. TNF-α and IFN-gamma were below the detectable limits of the multiplex assay. Therefore, they were excluded from further analysis.

MMP-8 and IL-1β levels were significantly increased in diseased sites when compared to healthy sites in both S and NS patients ($p<0.05$; Figure 2). IL-10 was significantly lower in diseased sites when compared to healthy sites. No significant difference in MMP-8 and IL-1β levels was found between S and NS, but S patients have reduced levels of IL-10 in both healthy and diseased sites in comparison with NS patients.

The correlations between the concentrations of AMPs and immunoinflammatory mediators in the GCF is shown in Table 3. In general, LL-37 was positively correlated with MMP-8 and IL-1β, and a negatively correlated with IL-10. In sharp contrast, HNP1-3 was positively correlated with IL-10 and negatively correlated with IL-1β and MMP-8 (Table 3).

Discussion

The main finding of the present study is the remarkably lower concentration of the peptides LL-37 and HNP 1-3 in the GCF of smokers compared to nonsmokers, irrespective of the inflammatory status of the periodontal tissues. These results indicate that smoking can affect production of AMPs, which may have direct and indirect effects on immune response to the microorganisms in a dysbiotic dental biofilm. It is tempting to speculate that the reduced levels of LL-37 and HNP 1-3 in smokers may be a possible mechanism contributing to the increased periodontal tissue destruction.

In accordance to our results, Turkoglu et al. reported that smokers with chronic periodontitis presented lower levels of LL-37 in the GCF when compared to non-smokers[19]. Moreover, a study conducted by Takeuchi et al. reported that smoking could decrease LL-37 salivary levels and increase the risk of chronic periodontitis[20]. However, there is a report of significantly increased levels of LL-37 in the GCF of smoker patients with either generalized aggressive periodontitis or gingivitis in comparison to non-smokers[34]. It is possible that this contrast is related with clinical diagnosis (‘gingivitis/aggressive periodontitis’ versus ‘periodontitis’) and the related pathological characteristics. To the best of our knowledge, this is the first study to report on HNP 1-3 levels in the GCF of smoker patients. Recently, Grant et al.[11] found no association between smoking and salivary levels of HNP 1 and LL-37 in patients with periodontal disease.

LL-37 and HNP 1-3 are mostly released by neutrophils; however, evidences suggest some neutrophil anomalies caused by smoking, such as impaired chemotaxis or reduced inflammatory response[35], possibly mechanisms responsible for AMPs

reduction in smoker patients. Furthermore, smokers patients showed reduced general transcriptional activity compared to non-smokers[36]. Besides that, smokers also present increased levels of peptidylarginine deiminase, which can be responsible by AMPs citrullination, turning AMPs into potential substrates for proteinases[37]

Few in vitro studies have determined that smoking can decrease the expression of AMPs in gingival epithelial cells or skin keratinocytes[17,18,38]. In contrast, hBD2 secretion by keratinocytes was increased by p38 MAPK activated upon binding of nicotine to activated nicotinic acetylcholine receptors[39]. Similarly, Semlali et al.[40] showed that gingival epithelial cells exposed to cigarette smoke had sustained increased expression of hBD 2, hBD 3 and IL-1β and IL-6. The contrasting information indicates that the influence of smoking on production of AMPs and possible influence on periodontal disease is still not clear.

Regardless of the smoking status, LL-37 was significantly increased in the GCF of sites with clinical signs of periodontitis. Production of LL-37 has been shown to be up-regulated during infection and inflammation[41–43]. Previous studies have demonstrated that hCAP18/LL-37 levels in the GCF were significantly elevated in patients with chronic periodontitis compared to periodontally healthy patients[41,43]. Turkoglu et al.[42] investigated serum and GCF hCAP18/LL-37 levels in patients with aggressive periodontitis. These patients had significantly higher levels of GCF hCAP18/LL-37 than healthy subjects and patients with gingivitis. Turkoglu et al.[41,42] also observed a positive correlation between GCF LL-37 levels and severity of periodontal disease. Taken together, these results suggest that periodontal diseases promote higher production of LL-37 that is proportional to the extent and severity of periodontal destruction. The fact that LL-37 was reduced in smokers suggest an

impaired immune response, which can be associated with reduced resistance to the microorganisms.

The expression patterns of HNP 1-3 in the GCF were in sharp contrast with those of LL-37. In this study, lower levels of HNP 1-3 were detected in GCF of diseased sites than in healthy sites of patients with periodontitis. This suggests that HNP 1-3 may be associated with a homeostatic status. However, contradictory results have been reported in the literature. Puklo et al.[43] recorded higher concentrations of HNP 1-3 in the GCF of patients presenting aggressive and chronic periodontitis than in periodontally healthy subjects. Likewise, Grant et al.[11] reported overabundance of HNP 1 in saliva of patients with periodontal disease compared to patients without periodontal disease. However, other studies showed no significant difference in GCF HNP 1-3 levels between healthy subjects and patients with periodontal disease[33,44]. In fact, the study of Lundy et al.[44] found higher levels of HNP 1-3 in GCF of healthy sites, but without statistical significance. HNP 1-3 was increased in the GCF of chronic periodontitis patients after non-surgical periodontal therapy associated with systemic antibiotic administration[45], which supports a possible role for HNP 1-3 in the repair/resolution process. Further studies are necessary for a better understanding of the role of HNP 1-3 in periodontal disease.

The cathelicidin hCAP18/LL-37 is stored in specific secondary granules of neutrophils as a biologically inactive precursor (hCAP18) and HNP 1-3 are stored in neutrophils azurophilic granules in a biologically active form. However, it is important to consider that these peptides are also produced by other cell types, including epithelial cells, macrophages, dendritic and mast cells[46], while HNP 1-3 are also produced by lymphocytes[47].

In addition to its antimicrobial activity, AMPs have various immunomodulatory properties. LL-37 influence the immune response by regulating expression of pro-inflammatory cytokines and chemokines[22], such as the induction of IL-8 secretion by neutrophils in periodontal diseased sites[48]. LL-37 also enhances cytokine-induced expression of IL-1β by peripheral blood mononuclear cells[21], amplifying the immune response. Our results showed a positive correlation between the levels of LL-37 and IL-1β and MMP-8 in the GCF and a negative correlation with IL-10. This suggests an involvement of LL-37 in inflammation associated with host-microbial interactions in the periodontal microenvironment. Conversely, HNP 1-3 was negatively correlated with levels of IL-1β and MMP-8 in GCF of healthy sites and positively correlated with IL-10, indicating that HNP 1-3 may participate in maintaining homeostasis and/or in repair/resolution processes. In support to this anti-inflammatory/resolution role, HNPs can inhibit the release of nitric oxide and inflammatory cytokines from macrophages[25]. It is important to consider that correlation cannot confirm that production of cytokines is directly influenced by AMPs.

The present study has other limitations. The groups are composed only of patients with chronic periodontitis and the healthy samples are derived from the same patients. Since this is an observational clinical study, the exact nature or possible mechanisms involved in smoking-induced reduction of AMPs and the possible impact on periodontal disease susceptibility and pathogenesis cannot be defined.

Conclusion

Our findings indicate that smoking is associated with reduced GCF levels of LL-37 and HNP 1-3. In comparison with non-smoking patients, reduction of AMPs in smokers is observed in both healthy and diseased periodontal sites. In addition, we observed that LL-37 is associated with inflammation and HNP 1-3 with homeostatic conditions in periodontal sites. These associations are supported by correlations between these peptides and the levels of pro- and anti-inflammatory cytokines.

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Conflicts of interest

There is no conflict of interest.

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Figures

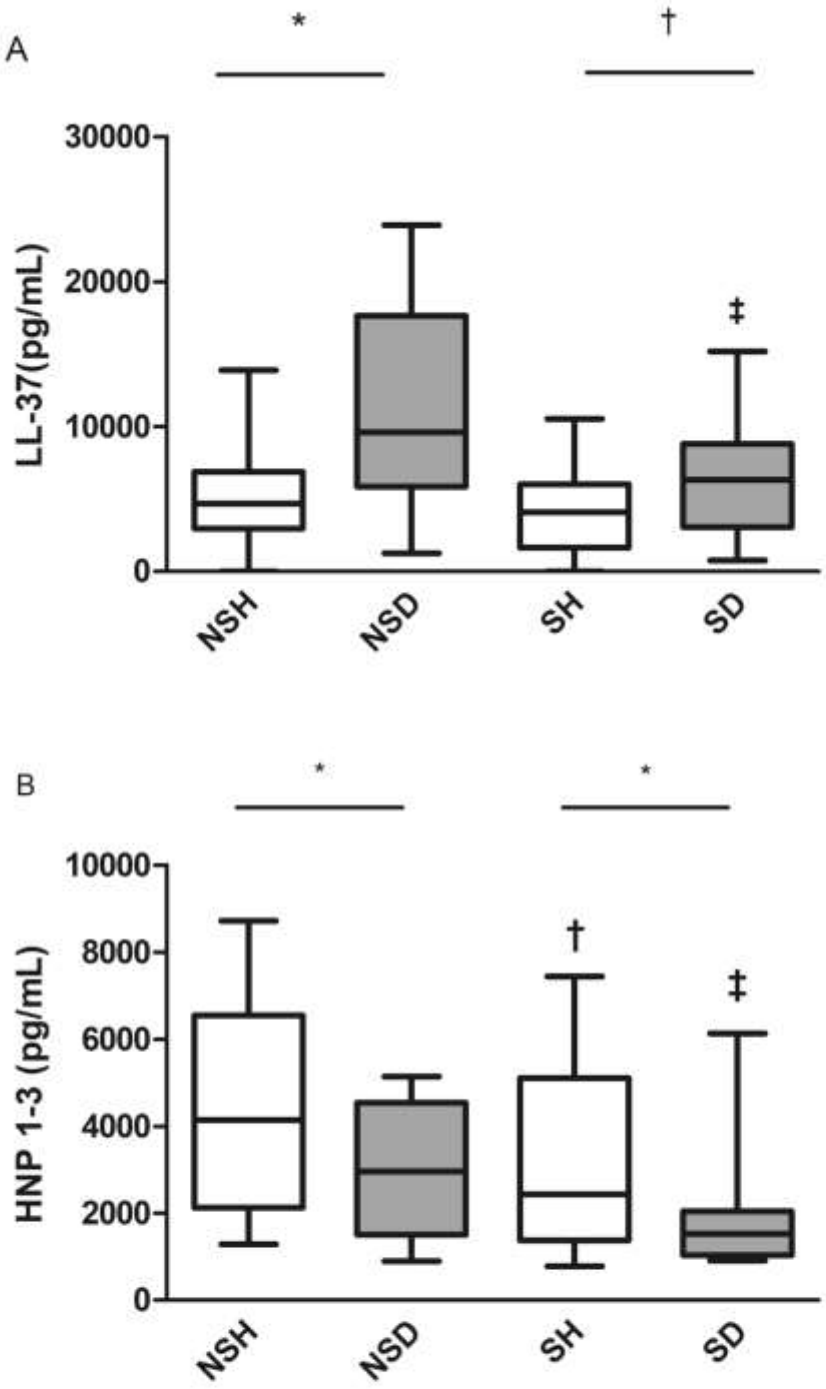

Figure 1. Distribution of antimicrobial peptides at the sites selected for GCF sampling. A) GCF cathelicidin LL-37 levels. *p<0.0005; †p<0.05 - Difference between healthy and diseased sites - paired t test; ‡p<0.05 - Difference from non-smokers diseased - Unpaired t test. B) GCF HNP 1-3 levels. *p<0.005 - Difference between healthy and diseased sites - Wilcoxon test; †p=0.01 - Difference from non-smokers healthy - Mann Whitney test; ‡p<0.05 - Difference from non-smokers diseased - Mann Whitney test. NSH - non-smokers healthy; NSD - non-smokers diseased; SH - smokers healthy; SD - smokers diseased.

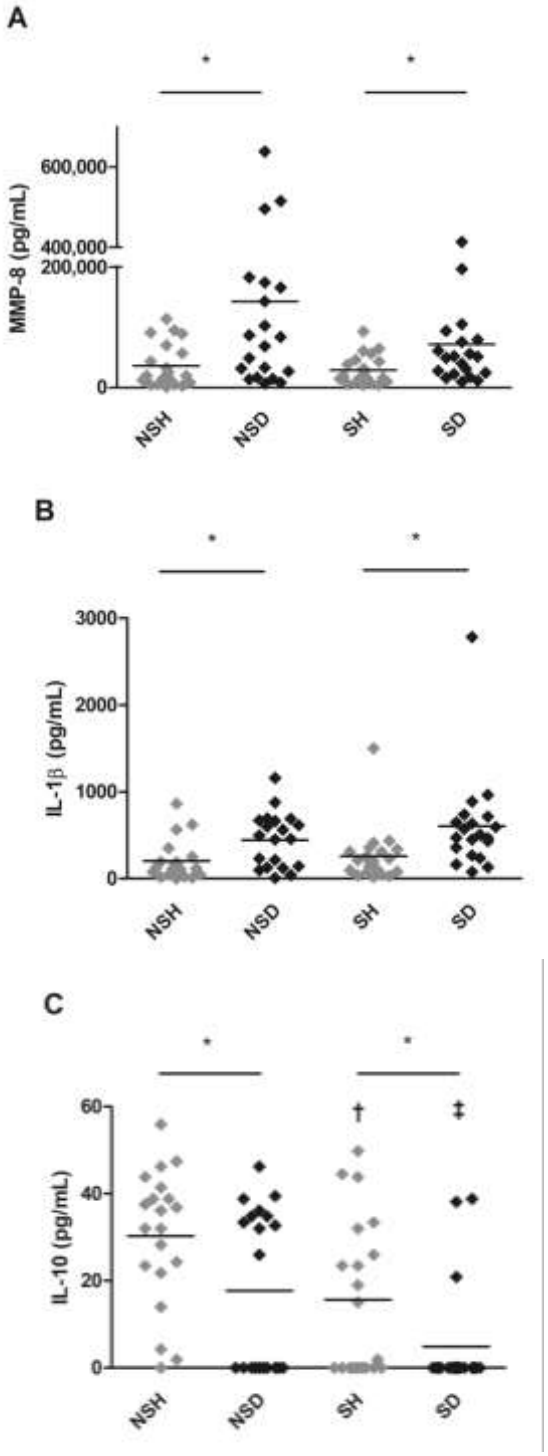

Figure 1 – Distribution of inflammatory mediators at the sites selected for GCF sampling. **A)** GCF MMP-8 levels. *p<0.05; **p<0.005 - Difference between healthy and diseased sites - Wilcoxon test **B)** GCF IL-1β levels *p<0.05; †p<0.005 - Difference between healthy and diseased sites - Wilcoxon test **C)** GCF IL-10 levels *p<0.05 - Difference between healthy and diseased sites - Wilcoxon test; † p<0.05 - Difference from non-smokers healthy sites - Mann Whitney test; ‡ p<0.01 - Difference from non-smokers diseased sites - Mann Whitney test. NSH – non-smokers healthy; NSD – non-smokers diseased; SH – smokers healthy; SD – smokers diseased.

Tables

Table 1. Clinical periodontal parameters of full-mouth of the study groups. Data are expressed as mean (median) ± SD.

Clinical Parameters	Non-smokers (n=20)	Smokers (n=20)
PI (%)	30.50 (29.43) ± 13.69	34.46 (23.19) ± 23.19
GI (%)	14.08 (11.95) ± 11.95*	9.03 (4.90) ± 12.77
BOP (%)	18.51 (13.73) ± 14.56	18.83 (12.17) ± 20.77
PD (mm)	2.98 (2.81) ± 0.63	3.20 (3.18) ± 0.74
PD ≤ 3mm (%)	77.10 (83) ± 18.64	73.00 (78) ± 18.99
PD 4-5mm (%)	19.10 (15) ± 14.99	21.7 (17) ± 13.46
PD ≥ 6mm (%)	4.00 (1) ± 5.90	5.25 (1) ± 8.94
CAL(mm)	3.29 (2.89) ± 1.11	3.51 (3.24) ± 0.99
CAL ≤ 2mm (%)	35.25 (41) ± 23.12	29.65 (28) ± 17.06
CAL 3-4mm (%)	43.05 (40.50) ± 16.69	42.06 (47) ± 14.24
CAL ≥ 5mm (%)	21.80 (13.50) ± 19.72	27.85 (21) ± 20.84

* P <0.05 – Significant difference from smoker group, Mann Whitney test; PI – Plaque index; GI – Gingival index; BOP – Bleeding on probing; PD – Probing depth; CAL – Clinical attachment level.

Table 2. Clinical periodontal parameters of the sampled sites. Data are expressed as mean ± SD.

Clinical Parameters	Non-smokers		Smokers	
	Healthy	Diseased	Healthy	Diseased
PD (mm)	2.13 ± 0.65*	5.29 ± 1.12	2.13 ± 0.46*	5.66 ± 1.17
CAL (mm)	2.20 ± 0.77*	5.62 ± 1.48	2.48 ± 0.82*	5.97 ± 1.61
CFV (μL)	0.50 ± 0.27*	0.82 ± 0.26	0.66 ± 0.34*	0.85 ± 0.30

*p<0.05 – Significant difference from diseased group, Wilcoxon test; PD – Probing depth; CAL – Clinical attachment level; CFV – Crevicular fluid level.

Table 3. Correlation between cathelicidin LL-37 and HNP 1-3 and inflammatory mediators, MMP-8, IL-1β and IL-10, levels at the sites selected for GCF sampling (n = 80).

	Non-smokers		Smokers	
LL-37	**Healthy**	**Diseased**	**Healthy**	**Diseased**
MMP-8	0.703*	0.846*	0.453*	0.669*
IL-1β	0.849*	0.788*	0.287	0.711*
IL-10	0.001	-0.523*	-0.044	-0.232
HNP1-3				
MMP-8	-0.080	-0.212	-0.526*	-0.398
IL-1β	0.087	-0.271	-0.480*	-0.711*
IL-10	0.532*	0.845*	0.728*	0.619*

*p<0.05 – Statistical significance – Spearman's correlation.

3.2 Publicação 2

The impact of smoking on protein levels and gene expression of human beta-defensins: a clinical and in vitro study

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Abstract

Aim: To compare the gingival crevicular fluid (GCF) hBD1 and 2 levels in smokers and non-smokers with periodontitis. In addition, evaluate the effect of nicotine and cotinine in association or not with periodontopathogenic bacteria on hBD1 and 2 expression by human keratinocytes.

Material e methods: In the clinical study seventy patients were recruited, being 20 periodontally healthy and 50 with periodontitis, 25 smokers and 25 non-smokers. After periodontal clinical evaluation, GCF samples were collected from healthy sites (n=5) of periodontally healthy patients and healthy (n=5) and diseased (n=5) sites of patients with periodontitis. Peptides quantification was performed using the sandwich ELISA technique. In vitro, HaCats cells were cultured in 12-well plates in Dulbecco´s minimum essential medium (DMEM) at a density of $1x10^5$ cells per well. Cells were treated with 10 µg/ml of either nicotine or cotinine and in combination with *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*. Total RNA was extracted and analyzed by real-time RT-qPCR for hBD-1, -2 and GAPDH mRNA.

Results: Clinical study showed that smoking reduced the levels of hBD1, but enhanced hBD2 in diseased sites. Periodontally healthy patients presented higher levels of hBD1 than healthy sites of patients with periodontitis as well as higher levels of hBD2 only when compared to non-smokers healthy sites. In non-smokers patients, the levels of hBD1 and 2 were significantly higher in diseased sites than in healthy sites. However, in smokers patients there was no significant difference among healthy and diseased sites. The in vitro study revealed that treatment with nicotine resulted in a significant down-regulation of hBD1 expression and the decrease was accentuated in association to *A. actinomycetemcomitans* culture. Simultaneous treatment with nicotine or cotinine in combination with bacteria culture resulted in down-regulation of hBD2 expression by HaCat cells, but it was only significant when cotinine was associated to *P. gingivalis* culture.

Conclusion: In the presence of periodontal disease, host immune response can be impaired by risk factors, such as smoking. Moreover, cigarettes compounds may modulate hBD expression in keratinocytes, especially associated with periodontopathogens.

Keywords: Cationic antimicrobial peptides. Beta-defensins. Periodontitis. Smoking. Nicotine. Cotinine. Keratinocyte.

Introduction

Periodontal disease is an inflammatory disorder resulted of the oral homeostasis disruption. The imbalance between the pathogenic potential of the biofilm and the host immune response can be specially compromised by some risk factors[1,2]. It has been demonstrated that smokers are about three times more likely to develop periodontitis than non-smokers,present greater attachment loss, rapid progression of periodontal destruction and less gingival bleeding than has been observed in non-smokers[3,4].

Smoking is responsible for suppressing the immune response of periodontal tissue . It can decrease blood flow, impair neutrophil and monocytes activities, alters adhesion molecule expression and antibody production, as well as cytokine and inflammatory mediator release[5,7,8]. Additionally, smoking inhibits oral tissue proliferation, inhibits attachment and migration of fibroblasts and promotes the differentiation of osteoclasts in periodontal tissue[3,9]. Nevertheless, the effect of smoking on antimicrobial peptides (AMPs) production and release by human gingival epithelial cells or keratinocytes is still not completely understood.

Intended to maintain a healthy balance, AMPs, such as human beta-defensins (hBDs), can be synthesized by a variety of epithelial cells. In the oral cavity, they are present in gingival tissue, tongue, salivary glands and mucosa, gingival crevicular fluid (GCF) and saliva[10–12]. AMPs have special properties that allow them to act directly against bacterial, viral and fungal invasion[13–15]. They are able to bind to microbial membranes through electrostatic and hydrophobic interactions, resulting in elimination of the microorganisms by disrupting their cell membranes[16]. Besides its direct killing activity, AMPs can promote immunomodulatory activities, being chemoattractive for effector cells, and stimulating the production and release of various immunoregulatory

mediators by inflammatory cells. Therefore, they are recognized as potent agents in inflammatory processes and modulators of adaptive immunity[17,18].

Few in vitro studies have demonstrated that smoking can affect the expression of AMPs in gingival epithelial cells or skin keratinocytes. In nicotine pre-treated HaCaT culture, followed by TNF-α stimulus, it was reported a reduction of hBD2 expression when compared to control group. Similar result was observed in *P. gingivalis* lipopolysaccharide (LPS)-stimulated epithelial cells treated with cigarette smoke extract[19]. On the other hand, cigarette smoke extract promoted a significant decrease on hBD1 expression, but increased the expression of hBD2 in human epithelial cells. In a previous clinical study, the expression of hBD1 and 2 genes was down-regulated in non-inflamed gingival samples of smokers compared to non-smokers[20]. Nevertheless, higher GCF hBD2 level was observed in smokers than in non-smokers with periodontitis[21]. Therefore, the smoking mechanisms involved in AMPs regulation still need to be more elucidated.

Numerous harmful substances are present in tobacco smoke. Presenting a short half-life, nicotine is one of the major tobacco smoking constituents, but is rapidly metabolized into cotinine[22]. However, cotinine levels appear to remain relatively constant in active smokers over a long time. Furthermore, a positive correlation has been reported between cotinine in body fluids and the presence of severe periodontal attachment loss[23]. Considering that AMPs produced by epithelial cells appears to play an important role in the innate immune defense system of the oral cavity, a clinical and in vitro study was performed to evaluate the possible impact of smoking on protein levels and gene expression of human beta-defensins. We compared the GCF hBD1 and 2 levels in healthy and diseased sites of smokers and non-smokers with periodontitis. In addition, the effect of nicotine and cotinine, associated or not with

periodontopathogenic bacteria, on hBD1 and 2 expression by human keratinocytes was evaluated.

Materials and methods

Clinical Study

Study design

The study protocol was approved by the Ethics Committee on Human Research of the School of Dentistry at Araraquara – UNESP (protocol number 01394712.5.0000.5416) and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. The purpose and procedures were explained to all subjects, and all participants gave written informed consent.

Total of 70 participants were recruited at the Periodontology Clinic, School of Dentistry at Araraquara – UNESP. The diagnosis of periodontitis was performed according to the classification of the 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions [24]. All patients should present a minimum of 15 natural teeth. A total of 20 patients were included in periodontally healthy control group. All subjects were free of systemic and periodontal diseases. They should present probing depth (PD) ≤ 3 mm, no bleeding on probing (BOP), no clinical attachment loss, plaque index (PI) and gingival index ≤ 15%. Fifty patients were included in periodontitis groups. They should present at least five sites with PD ≥ 4 mm, BOP and clinical attachment level (CAL) ≥ 4 mm, with the exception of third molars. From the 50 patients, 25 were non-smokers and 25 were smokers who should smoke at least 10 cigarettes/day for a minimum of 5 years and non-smoking patients had never smoked.

None of the patients had a history of systemic diseases or immunological disorders. Subjects who had received antibiotics and anti-inflammatory drugs within

the last 3 months or received periodontal treatment within the last 6 months were not included in the study. Patients using hormone replacement therapy, pregnant and lactating women were also excluded.

Clinical examination

One calibrated examiner performed all clinical examinations. The intra-examiner reproducibility was assessed by measuring the PD in six teeth (16, 21, 24, 36, 41 and 44), according to the Ramfjord index, in a total of 6 patients (36 teeth/patient) in two different occasion in a time interval of 7 days. Data were submitted to Kappa concordance analysis (k=0.91). The parameters PD, BOP and CAL were assessed at 6 sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, disto-lingual), while PI and GI were assessed at four sites (mesial, distal, buccal and lingual)[25], excluding the third molars. Measurements were performed with a manual periodontal probe (UNC15; Hu-Friedy, Chicago, IL, USA).

Gingival crevicular fluid (GCF) sampling

GCF samples were collected from healthy (n=5) sites of each periodontally healthy patient and from healthy (n=5) and diseased (n=5) sites of each periodontitis patient[26]. Sites at different non-adjacent teeth, preferably one site per quadrant, were selected for GCF collection in each subject, one day after periodontal examination.

Healthy sites: Absence of gingival inflammation, PD ≤ 3 mm, no BOP or CAL.

Diseased sites: Presence of gingival inflammation, PD and CAL ≥ 4mm with BOP. The diseased sites were selected according to the deepest probing depth at each quadrant.

The sites to be sampled were isolated with cotton rolls, supragingival plaque was removed with a sterile curette and the surfaces were gently air-dried. GCF was sampled by inserting absorbent paper strips (Periopaper®, Oraflow Inc., New York, USA) into the gingival sulcus or periodontal pocket for 30 seconds. Paper strips contaminated with blood and saliva were rejected. The GCF volume absorbed into each strip was determined by an electronic device Periotron 8000 (Periopaper, ProFlow, Inc., Amityville, NY, USA), which was calibrated based on a protocol described before[27] (calibration of the Periotron 8000 by polynomial regression). The GCF was obtained as a pooled sample of the same category (healthy or diseased site) in each subject. The papers strips were immediately placed into a dry sterile polypropylene tube (5 paper strips per tube) and kept at -80°C until analysis. The readings from the Periotron 8000 were converted to an actual volume (µL) by reference to a standard curve.

Immunosorbent assay for hBD1 and hBD2 quantification

First the absorbed GCF was eluted from the paper strips by adding 750 µL (150 µL per strip) of 1x Phosphate-Buffered Saline (PBS) to each tube. The tubes were left on ice in a horizontal shaker for 30 minutes and then centrifuged at 4°C, 13,000 g for 10 minutes. The GCF levels of hBD1 and hBD2 were measured using the sandwich ELISA technique according to the manufacturer's guidelines (Peprotech, Rocky Hill, NJ, USA). The absorbance was measured by spectrophotometer at 450 nm wavelength. The levels of AMP in each sample were determined using the concentration values of standards included in the kit contents. The results were expressed in pg/mL.

In vitro Study

Cell culture

HaCaTs cells (HaCaT; Cell Lines Service, 300493) were cultured in Dulbecco`s minimum essential medium (DMEM – Thermofisher Scientifc) supplemented with 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany), 1% glutamine and 1% streptomycin + penicilin. Cells were grown to 80-90% of confluency and then were evenly transferred to 12-well plates in a concentration of $1x10^5$ cells per well. After 48h the respective treatments were executed.

Bacterial culture and conditions of cell stimulation

The *Porphyromonas gingivalis* (Pg) strain ATCC 33277 was grown on Tryptic Soy Agar supplemented with 5% defibrinated sheep blood, 0.5 mg/mL hemin and 1 mg/mL menadione. *Aggregatibacter actinomycetemcomitans* (Aa) strain JP2 was grown on Tryptic Soy Agar containing 0.6% yeast extract. Both were incubated anaerobically at 37 °C in 85% N_2, 5% CO_2 and 10% H_2 for 2–3 days and harvested at the mid-logarithmic phase of growth. The final bacteria concentration was adjusted for the OD of 0.5 (Pg) and 0.2 (Aa) in a wavelength of 495 nm, which correlated to 10^7 CFU/mL. The proportion of 200:1 (bacteria:cells) was used for stimulation.

Treatment of keratinocytes cell

Preliminary experiments were conducted to determine the non-toxic concentrations of nicotine and cotinine, and its solvents DMSO and ethanol, respectively, in order to ensure that any of their observed effects on HaCaT function did not result from cellular toxicity or cell death. Viability of treated and untreated cells were assessed after 24 h of exposure to nicotine or cotinine and compared by Alamar

Blue assay (Figure 1). The 10 μg/mL concentration of nicotine and cotinine was chosen considering the toxicity test and physiological concentration[28,29] (Figure 1). After reaching 80-90% confluency, cells were stimulated with the following treatments per 24h: 1. Sterile DMEM medium (negative control); 2. Nicotine; 3. Cotinine; 4. Nicotine + Aa; 5. Cotinine + Aa; 6. Nicotine + Pg; 7. Cotinine + Pg. After 24 h, the cells were collected for measurement of hBD1 and hBD2 expression. This experiment was performed in triplicates and repeated 3 times.

Quantitative real-time RT-PCR

Total RNA was extracted using affinity column system (RNAqueous kit, Ambion Inc., Austin, TX, USA). Concentration of RNA was determined in a microvolume spectrophotometer (NanoView Plus, GE Healthcare, Munich, Germany) and 200 ng of total RNA were used for cDNA synthesis using random hexamer primers and reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, ThermoFisher Scientific).

Real-time PCR was performed using TaqMan chemistry (TaqMan Fast Advanced Master Mix, Applied Biosystems, ThermoFisher Scientific) and pre-designed and optimized sets of primers and probe (TaqMan gene expression assays, Applied Biosystems, ThermoFisher Scientific) specific for hBD1 (Hs00608345) and hBD2 (Hs00175474). Expression of GAPDH (Hs02758991) was used as the endogenous control for normalization.

Statistical analysis

The statistical analysis was performed using the software GraphPad Prism 7 (San Diego, CA, USA). For clinical and in vitro data, normal distribution was analyzed using the D'Agostino and Pearson test. For normally distributed data, it was used One-way ANOVA followed by Tukey for healthy sites and unpaired t test for diseased sites on inter-group analyses and paired t test for intra-group analyses. For non-normally distributed data, Kruskall-Wallis and Dunn was used for healthy sites and Mann-Whitney tests was used for diseased sites on inter-group analyses, while Wilcoxon test was performed for intra-group comparison. Data from the real-time RT-qPCR were analyzed using the Mann-Whitney test. A p value of less than 0.05 was considered significant.

Results

Clinical Study

Demographics and periodontal parameters

Significant difference in age was found between periodontally healthy subjects (25.79 ± 3.95) and periodontitis patients (NS [40.32 ± 12]; S [43.88 ± 10.64]) ($p<0.05$). The distribution of the male and female participants had no significant difference between study groups, periodontally healthy subjects (5 males/15 females) and periodontitis patients (NS - 9 males/16 females; S - 12 males/13 females) ($p = 0.28$).

The periodontal clinical parameters are presented in table 1. Regarding the whole mouth periodontal clinical parameters, PI, BOP, PD and CAL were significantly lower in periodontally healthy subjects than in periodontitis patients ($p<0.0001$). Only GI showed no difference between periodontally healthy patients and smokers patients ($p=0.0001$). The sampled sites of periodontally healthy subjects presented significant

lower values of PD and CFV than healthy sampled sites of smokers and non-smokers with periodontitis ($p<0.05$), as well as lower CAL than healthy sampled sites of smokers ($p<0.05$) (Table 2). In patients with periodontitis, the intra-group comparison revealed lower levels of PD, CAL and CFV in the healthy sampled sites than in the diseased sites ($p<0.0001$) (Table 2).

hBD1 and hBD2 quantification

The Figure 2 illustrate the results of hBD1 and hBD2 quantification in GCF. The levels of hBD1 was significantly higher in the GCF of periodontally healthy sites of the control group (median 30.14; IC 95% 25.87 - 54.20) than in healthy sites of smokers (median 7.47; CI 95% 5.79 – 11.04) and non-smokers (median 2.32; CI 95% -1.13 – 18.82) with periodontitis ($p<0.0001$). The lowest GCF hBD2 level was observed in the healthy sites of non-smokers patients (median 0; CI 95% -0.25 – 7.75) ($p<0.05$). The comparison between diseased sites of periodontitis patients revealed significantly higher levels of hBD2 and lower levels of hBD1 in the GCF of smokers (hBD2 [median 24.02; CI 95% 24.97 – 84.22]; hBD1 [median 2.12; CI 95% 2.25 – 10.34] compared to non-smokers (hBD2 [median 0; CI 95% 7.64 – 251]; hBD1 [median 14.24; CI 95% 8.44 – 17.93]) ($p<0.05$). In non-smokers, higher levels of hBD1 and 2 were observed in diseased sites than in healthy sites ($p<0.05$)). However, in smokers, no significant difference was found between healthy and diseased sites (hBD1 healthy [median 7.47; CI 95% 5.79 – 11.04] diseased [median 2.12; CI 95% 2.25 – 10.34] and hBD2 healthy [median 6.27; CI 95% 7.69 – 80.15] diseased [median 24.02; CI 95% 24.97 – 84.22]).

HaCat culture

The expression of hBD1 was down-regulated in HaCat cells stimulated with nicotine or cotinine, being the nicotine regulation statistically significant ($p<0.05$) (Figure 3.A). However, no difference was noticed on hBD2 expression by HaCaT cells stimulated only with nicotine or cotinine. The negative effect was evident when these products were associated with bacteria (Figure 4). The *A. actinomycetemcomitans* co-culture accentuated the reduction of hBD1 expression in HaCaT cells stimulated with nicotine ($p<0.05$) (Figure 3.B). On the other hand*, P. gingivalis* caused a significant decrease on hBD2 expression in HaCaT cells when associated with cotinine treatment ($p<0.05$) (Figure 4.C).

Discussion

The clinical arm of the present study showed that smoking has a significant effect on GCF hBD1 and hBD2 levels. Reduced GCF hBD1 levels were observed in diseased sites of smokers compared to the same sites of non-smokers. In healthy sites, no difference was found between hBD1 levels in periodontitis patients regarding smoking status. However, higher levels of hBD2 were detected on healthy and diseased sites of smokers compared to non-smokers. To the best of our knowledge, this is the first study to evaluate hBD1 and 2 simultaneously among smokers patients with periodontitis. Ertugrul et al. also demonstrated that smoker patients either with generalized aggressive periodontitis or with gingivitis presented higher GCF levels of hBD2 than non-smoker patients[21]. On the other hand, a previous study reported significant reduction of hBD1 and 2 gene expression in non-inflamed gingival samples of smokers compared to non-smokers[20]. Evidences indicate that smoking is associated to oral microbiome modification in patients with periodontitis. Smokers

patients showed greater abundance of periodontopathogenic microorganism in subgingival plaque than non-smokers[30]. Generally, hBD-2 are expressed at low levels, possibly being induced in response to pathogens invasion and inflammation[31]. Regarding the inductive character of hBD2, the higher levels present in smokers sites is probably due to the accentuated host immune response against pathogens.

Conversely, higher levels of hBD1 can be found in the GCF in absence of stimulus, which can present a constitutive activity[32]. In the present data, highest concentration of hBD1 was observed in the healthy sites of periodontally healthy subjects than healthy sites of patients with periodontitis. Costa et al.[33] also reported significative higher levels of hBD1 in periodontally healthy patients compared to healthy sites of patients with periodontitis. Previous study suggested that genetic intrinsic properties may regulate the hBD inductive expression by epithelial cells[34]. They reported that gingival epithelial cells presented similar regulatory pathways for hBDs in periodontitis and healthy samples in response to the same inflammatory stimulus. However, different outcomes were registered, periodontitis samples expressed lower levels of hBDs than healthy samples[34]. Reinforcing these findings, Brancatisano et al.[35] reported higher expression of hBD3 in GCF of healthy patients in comparison to diseased sites of periodontitis patients. In the present study, in respect to hBD2 regulation, lowest levels were found in healthy sites of non-smokers patients than periodontally healthy and smokers patients.

The comparison between healthy and diseased sites in the periodontitis patients revealed different results for non-smokers and smokers. Non-smokers group showed significant higher GCF hBD1 and 2 levels in diseased sites compared to healthy sites. Increased hBDs levels may have been released to the GCF from periodontal epithelial cells as a protection mechanism against increasement of specific

microorganism colonization. Moreover, it is already known that hBD2 is stimulated by the presence of pathogenic bacteria as well as inflammatory stimulus[32]. On the other hand, the present data showed no statistical difference on hBD1 and hBD2 levels among healthy and diseased sites of smoker patients. This result was not expected regarding the higher levels of hBD expected in diseased sites, thus, it may support the assumption that cigarette compounds may be responsible by modulation of AMP in periodontal disease. Several toxins present in cigarette and other forms of tobacco smoke have varying immunomodulatory effects, such as neutrophil migration, chemotaxis and phagocytosis and production of chemical mediator[8].

According to our inclusion criteria, the difference between periodontally healthy and periodontitis patients regarding the clinical parameters were already expected. The similar clinical findings between smokers and non-smokers were also expected, once they all had the same disease severity. Only GI of periodontally healthy patients did not have significant difference compared to periodontitis smoker patients. GI is considered a sign of periodontal tissue inflammation. Nevertheless, it seems to be reduced in smokers. It was demonstrated in a human model of experimental gingivitis that the development of inflammation in response to bacterial biofilm accumulation is reduced in smokers when compared to non-smokers[36]. According to Dietrich[37], smoking may have a chronic, dose-dependent effect on gingival bleeding suppression.

The in vitro study showed the hBD regulation mediated by tobacco compounds in stimulated HaCaT cell culture. It was possible to detect a significant reduction of hBD1 expression when the cells were stimulated with nicotine. However, neither nicotine or cotinine had an impact on hBD2 expression. Wolgin et al.[38] reported no effect of nicotine on hBD1 and hBD2 expression in HaCat cells culture. Nevertheless, contradictory results were also presented with nicotine stimulus up-regulating hBD2

expression by HaCaT cells [39]. Wang et al[40]. reported that whole cigarette smoke markedly reduced hBD1 expression and increased hBD2 expression in oral mucosal epithelial cells. Moreover, Semlali et al.[41] suggested increased hBD2 expression by human gingival epithelial cells exposed to whole cigarette extract. The differences in cell lines, culture and stimulus conditions may justify these conflicting findings. In fact, the mechanisms involved in AMP regulation by smoking and its toxic products must be more explored.

In addition, our data revealed that nicotine down regulation effect on hBD1 expression was accentuated in the presence of the pathogen *A. actinomycetemcomitans*, while cotinine decreased hBD2 expression only when associated to *P. gingivalis*. hBD has shown antigen specific immune response[35,42]. Different compositions of oral microbiome may be responsible by distinct regulation of hBD. A previous in vitro study found decreased hBD2 expression in nicotine pretreated HaCaT cells stimulated with TNF-α as well as simultaneously treated[38]. Likewise, cigarette smoke extract down-regulated hBD2 expression by *P. gingivalis* LPS-stimulated epithelial cells[19]. Suggesting a strong synergism between cigarette compounds and bacteria or inflammatory stimulus in hBD expression down-regulation.

In conclusion, the smoking habit may interfere in the immune response by regulating the GCF levels of hBD 1 and 2 . Smokers presented reduced concentration of hBD1 and increased concentration of hBD2 in diseased sites than non-smokers. Moreover, cigarettes compounds, such as nicotine and cotinine, reduced hBD1 and 2 gene expression, especially in the presence of periodontopathogens. Thus, it is conceivable that the increased severity of periodontitis in smokers may also occur as a result of the influence of tobacco products on hBDs expression. The knowledge of

how risk factors, such as smoking, can impair hBDs functions, can bring some insights of how each peptide works, and how important are AMPs for the immune response.

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Conflicts of Interest

The authors declare that there is no conflict of interest

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Tables

Table 1 - Clinical periodontal parameters of the study groups. Parametric data are expressed as mean ± standard deviation (SD) and non-parametric data are expressed as mean (median) ± SD.

Clinical Parameters	Healthy	Non-smokers	Smokers
PI (%)	6.39 ± 4.93*	29.62 ± 14.82	35.01 ± 21.77
GI (%)	6.56 ± 20.03δ	12.45 ± 7.72	10.25 ± 15.03
BOP (%)	3.56 ± 3.36*	19.45 ± 14.73	21.89 ± 23.72
PD (mm)	1.49 ± 0.29λ	2.92 ± 0.54	3.05 ± 0.72
PD ≤ 3mm (%)	100*	78.24 (80) ± 15.78	76.44 (84) ± 18.31
PD 4-5mm (%)	0*	18.28 (17) ± 12.61	18.88 (15) ± 13.15
PD ≥ 6mm (%)	0*	3.48 (1) ± 4.78	4.68 (1) ± 8.10
CAL (mm)	1.49 ± 0.29λ	3.30 ± 0.99	3.47 ± 0.91
CAL ≤ 2mm (%)	95.37 (98) ± 6.38*	35.84 (39) ± 20.42	31.28 (29) ± 15.41
CAL 3-4mm (%)	4.63 (2) ± 6.38*	43.04 (45) ± 15.01	42.64 (46) ± 12.83
CAL ≥ 5mm (%)	0*	21.20 (17) ± 15.67	26.08 (17) ± 19.39

* P <0.0001 – Significant difference from the other groups, Kruskal-Wallis with Dunn test; δ P = 0.0001 – Significant difference from non-smokers, Kruskal-Wallis with Dunn test; λ P <0.0001 - Significant difference from the other groups, One-way ANOVA with Tukey test. PI – Plaque index; GI – Gingival index; BOP – Bleeding on probing; PD – Probing depth; CAL – Clinical attachment level.

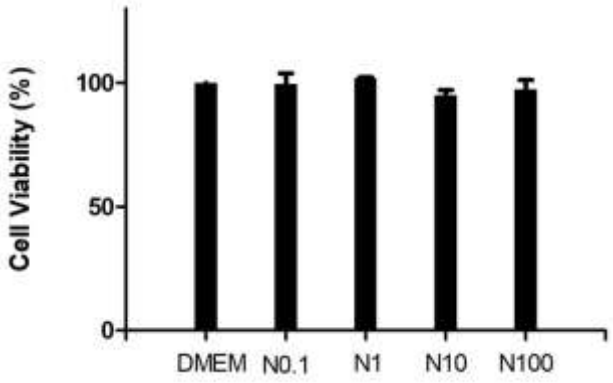
Table 2 - Clinical periodontal parameters of the sampled sites. Data are expressed as mean ± SD.

Clinical Parameters	Periodontally Healthy	Non-smokers		Smokers	
	Healthy	Healthy	Diseased	Healthy	Diseased
PD (mm)	1.55 ± 0.55[a]	2.07 ± 0.65[A]	5.70 ± 1.65	2.11 ± 0.47[A]	5.54 ± 1.51
CAL (mm)	1.55 ± 0.55[b]	2.13 ± 0.72[A]	5.90 ± 1.54	2.51 ± 0.80[A]	6.06 ± 1.70
CFV (μL)	0.20 ± 0.11[a]	0.49 ± 0.26[A]	0.84 ± 0.30	0.64 ± 0.30[A]	0.84 ± 0.33

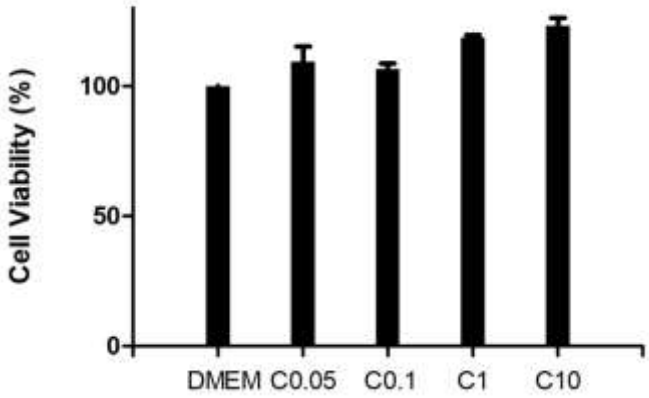
a $p<0.05$ - Significant difference from the other groups, One-way ANOVA with Tukey test; b $P<0.05$ – Significant difference from smokers healthy group, One-way ANOVA with Tukey test; A $P<0.0001$ – Significant difference from diseased group, Paired t test; PD – Probing depth; CAL – Clinical attachment level; CFV – Crevicular fluid volume

Figures

A



B



C

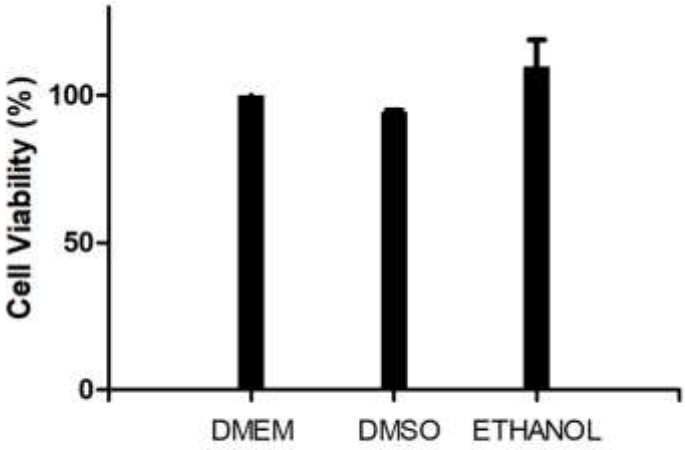
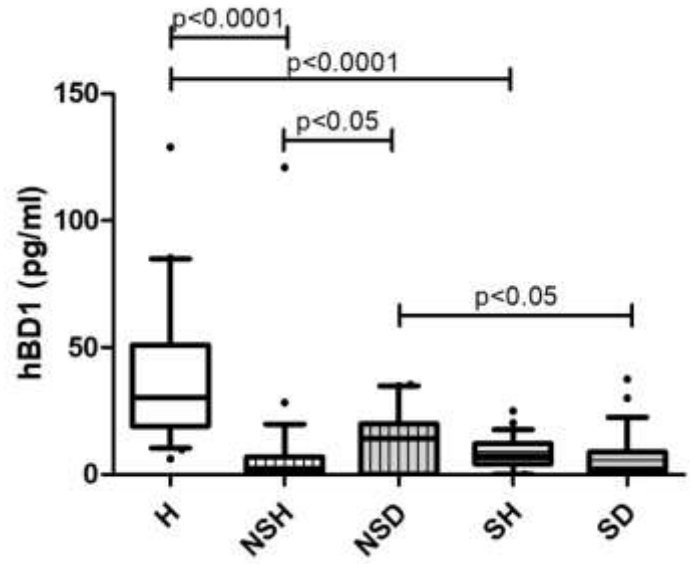


Figure 1 – Alamar Blue assay for cell viability test in different concentrations of stimulus and solvents - A) Comparison between Control (DMEM) and Nicotine concentrations from 0.1μg/ml to 100μg/ml **- B)** Comparison between Control (DMEM) and Cotinine concentrations from 0.05μg/ml to 10μg/ml **C)** Comparison between Control (DMEM) and Solvents - DMSO - 34μl; Ethanol - 17μl

A



B

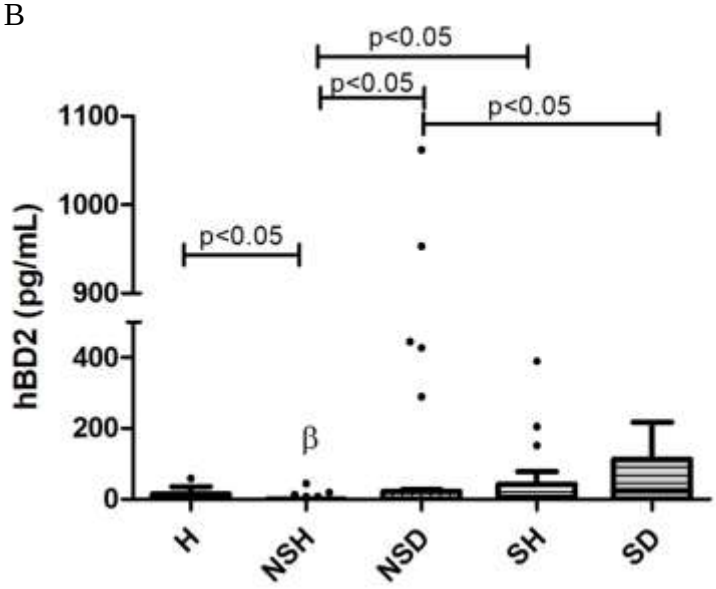


Figure 2 – Distribution of antimicrobial peptides at the sites selected for GCF sampling. **A)** hBD1 levels **B)** hBD2 level. The results are shown as box plots with medians (lines inside boxes), 25th and 75th percentiles (limits of boxes) and the 10th and 90th percentiles (whiskers). H – periodontally healthy NSH – non-smokers healthy; NSD – non-smokers diseased; SH – smokers healthy and SD – smokers diseased

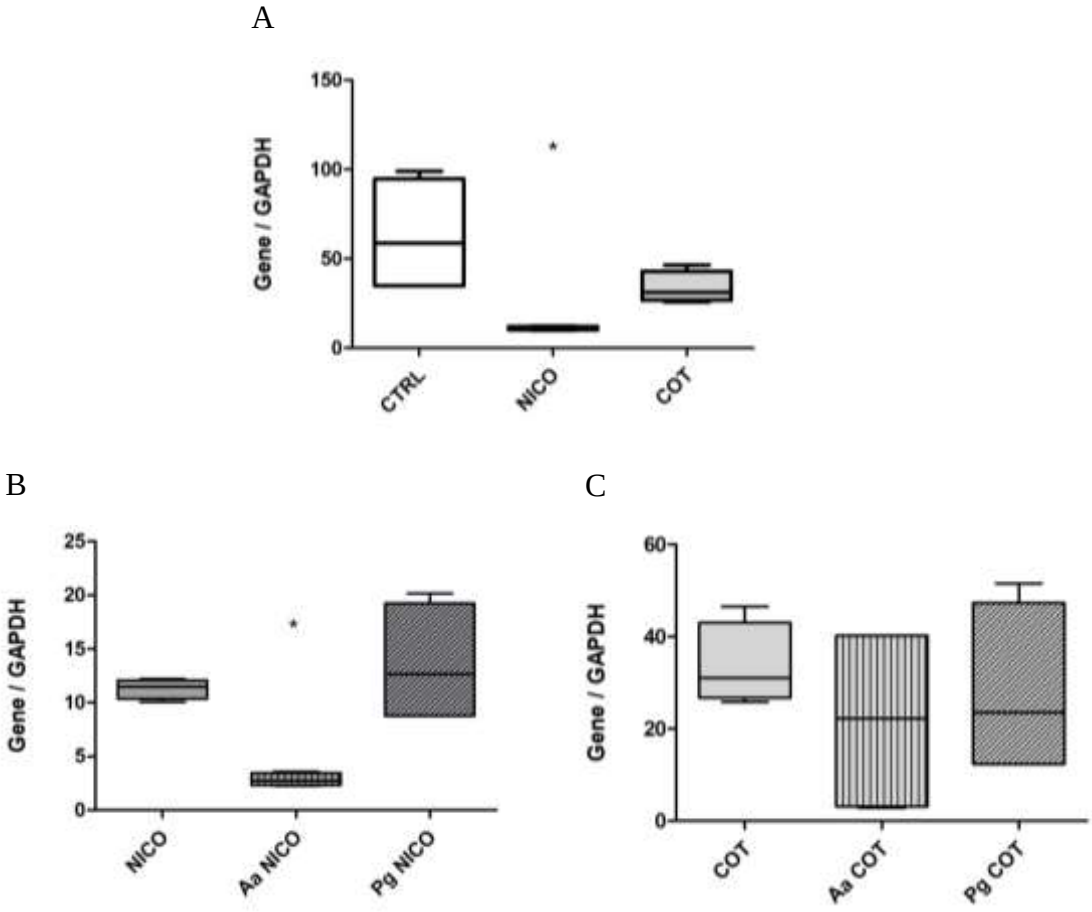


Figure 3 – Normalized expression for hBD1 in stimulated HaCaT cells culture. **A)** Comparison between Control group and Nicotine and Cotinine stimulus; *p<0.05 – Difference from control and cotinine. **B)** Comparison between Nicotine group and Nicotine associated with A. *actinomycetemcomitans* or *P. gingivalis* stimulus ;*p<0.05 – Compared to Nicoine and Pg Nicotine. **C)** Comparison between Cotinine group and Cotinine associated with A. *actinomycetemcomitans* or *P. gingivalis* stimulus. The results are shown as box plots with medians (lines inside boxes), 25th and 75th percentiles (limits of boxes) and the 10th and 90th percentiles (whiskers). CTRL – control group, NICO – nicotine, COT – cotinine, Aa – *Aggregatibacter actinomycetemcomitans* e Pg – *Porphyromonas gingivalis*

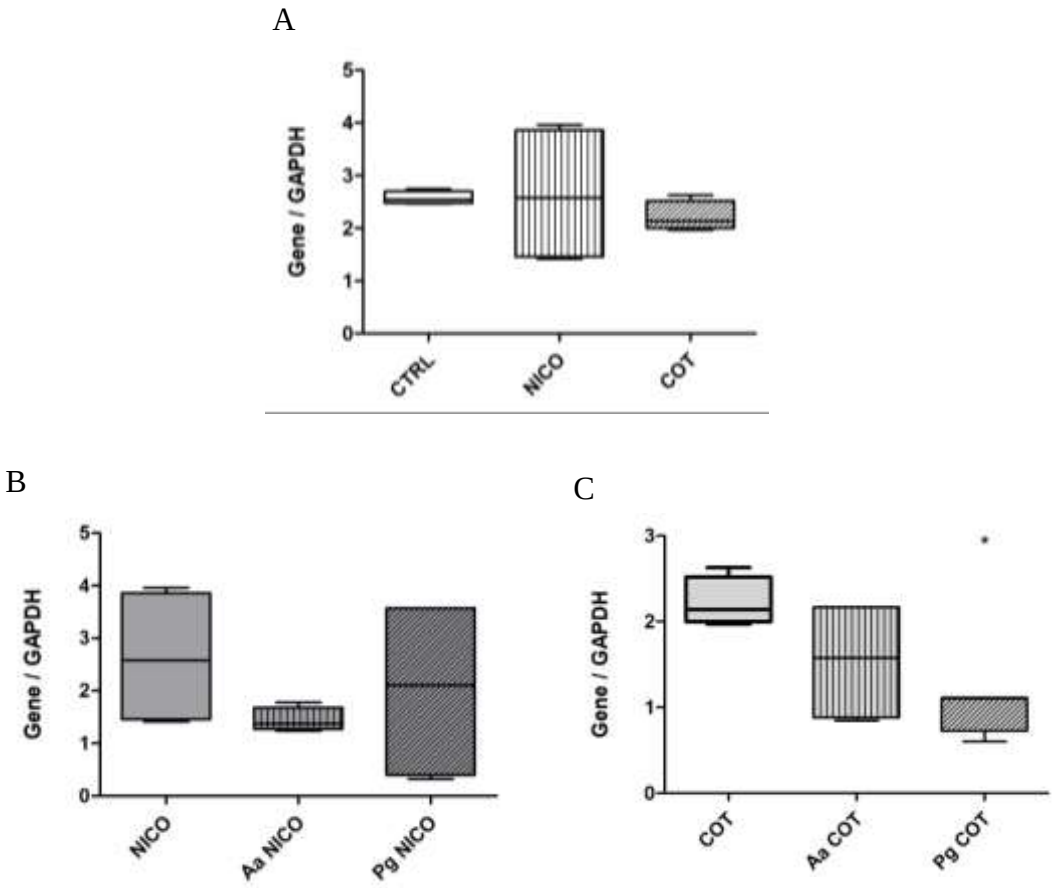


Figure 4 – Normalized expression for hBD2 expressed in stimulated HaCaT cells culture **A)** Comparison between Control group and Nicotine and Cotinine stimulus. **B)** Comparison between Nicotine group and Nicotine associated with A. *actinomycetemcomitans* or *P. gingivalis* stimulus. **C)** Comparison between Cotinine group and Cotinine associated with A. *actinomycetemcomitans* or *P. gingivalis* stimulus. The results are shown as box plots with medians (lines inside boxes), 25th and 75th percentiles (limits of boxes) and the 10th and 90th percentiles (whiskers). CTRL – control group, NICO – nicotine, COT – cotinine, Aa – *Aggregatibacter actinomycetemcomitans* e Pg – *Porphyromonas gingivalis*

3.3 Publicação 3

Effect of D-enantiomeric peptide treatment on regrowth of periodontopathogenic microcosm biofilm

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ARTIGO FORMATADO CONFORME NORMAS DO *ARCHIVES OF ORAL BIOLOGY*

Abstract

Aim: to evaluate the regrowth of periodontopathogenic multi-species biofilm after treatment with synthetic peptides LL31 and D-LL31.

Material and Methods: Saliva-derived microcosm biofilms were inoculated with either saliva (Sal) or saliva enriched with *Porphyromonas gingivalis* (SPg). Twenty-four hours after inoculation, the biofilms were treated with two different antimicrobial peptides, LL-31 and D-LL31, per 24h. Effect of treatments on biofilm regrowth was measured by metabolic activity (lactate acid production), enzymatic activity (dipeptidase), cell viability counts within two different harvesting time points, 1- and 5-days post-treatment.

Results: One-day post-treatment, D-LL31 and LL31 significantly decreased bacteria metabolic activity in SPg regrowth when compared to control ($p<0.005$ and $p<0.05$, respectively). Furthermore, D-LL31 showed higher efficiency than LL31 group ($p<0.05$). On the other hand, Sal group only had the metabolic activity decreased when treated with D-LL31. Five-days post-treatment, SPg treated with D-LL31 maintained biofilm regrowth significantly decreased when compared to control group. The dipeptidase activity was decreased in SPg treated with D-LL31 1- and 5-days post-treatment. However, no difference was found in cell viability between controls and groups treated with peptides.

Conclusion: D-LL31 is efficient decreasing metabolic and enzymatic activity on periodontopathogenic biofilm regrowth.

Keywords: Antimicrobial activity. Cathelicidin peptides. Periodontitis. D-enantiomer peptide. Biofilm regrowth. Microcos

Introduction

Periodontitis is the primary cause of tooth loss in adults worldwide. According to the World Health Organization, 10 to 15% of the world population suffers from severe periodontitis[1]. Periodontitis initiation is due to the dysbiosis between the presence of biofilm and deregulation of host response[2]. Dysbiotic microbiota perpetuate the host response, leading to destruction of soft and hard tissues supporting the tooth[3,4]. Many antimicrobial agents have been used successfully as an adjunct for the elimination of such periodontopathogens, including tetracycline, chlorhexidine, and metronidazole[5–7]. However, the treatment accomplishment is still not stated because of the development of multidrug-resistant microorganisms, interbacterial transfer of resistance determinants, and various side effects[8].

The strict anaerobic, Gram-negative bacterium *Porphyromonas gingivalis* is one of the key pathogens causing periodontitis[9] and lately has been known as a keystone pathogen[10]; when low abundance species have a disproportionate effect on the virulence of the whole community, changing the oral microbiome. The host response can also be obstructed by these microorganisms, *P. gingivalis* may degrade complement cascade, interfere with neutrophil function and prevent phagocytosis[11]. Moreover, beyond oral cavity, several reports detected an association between *P. gingivalis* and systemic diseases, including, cardiovascular diseases, rheumatoid arthritis and Alzheimer´s disease[12–14]. *P. gingivalis* can penetrate gingival tissues and cannot be removed by mechanical debridement[15], possibly resulting in recolonization of periodontal pockets and recurrence of the periodontal disease. Thus, leading to the needs of claiming for adjunct treatment with beneficial effects against periopathogens.

Antimicrobial peptides (AMPs) are substantial components of the innate immunity, and are widely distributed throughout the microbial, animal, and plant kingdoms. They have received special attention as a promising treatment and can be a helpful strategy to fight antibiotic resistance[16,17]. AMPs are attractive candidates for clinical development because of their selectivity, low concentration and reduced potential for resistance development[1218,19]. However, there are limited studies on the use of AMPs against periodontopathogenic bacteria.

The inhibitory effects of the AMP human cathelicidin LL37 on the attachment capability and biofilm formation has been already described for many pathogens, on single specie models, such as *P. gingivalis, Staphylococcus epidermidis, Pseudomonas aeruginosa and Escherichia coli* [18,19]. However, LL37 in its natural form, L-amino acid, is higly susceptible to degradation by bacterial proteases[24,25]. It has been already shown that LL31, a truncated variant of LL37, showed strongest killing effect[26], but still could not be resistant enough to certain proteases. L-amino acids may be substituted by D-amino acids, aiming to improve peptides antimicrobial activity and its protease resistance[27]. The killing activity of LL37 and its shorter variant LL31 against *B. pseudomallei* biofilms has been reported[28], but D-LL31 showed the highest efficacy.

Some oral microbes may contribute to the healthy homeostasis in the oral cavity and it environment should not be disturbed by antimicrobial treatments[29]. Therefore, it is important to investigate antimicrobial agents effects on the oral microbiome composition shift[30], its specificity killing capacity, rather than keep focused only on its total killing. In this study, we purposed to evaluate the metabolic and enzymatic activity, the cell viability and composition of biofilms regrowth after treatment with synthetic antimicrobial peptides LL31 and D-LL31 on hydroxyapatite discs. The two

different biofilm inoculums, saliva and saliva enriched with P. gingivalis, used in this study aimed to mimic non- and periodontopathogenic condition and determinate how the AMPs can interfere on biofilm regrowth.

Materials and Methods

Experimental Design

Saliva was collected from 10 donors, thoroughly mixed and split into aliquots. Saliva was stored at – 80°C until use**.** Biofilms were grown for 24h and then treated with either water, LL31 or D-LL31. After treatment, the growth biofilm medium was refreshed each 24h. The samples were harvested within 3 and 7 days after saliva inoculation. This design was repeated 3 times, resulting in 12 experimental groups with 3 biofilms each (Figure 1).

The study protocol was approved by the Medical Ethicals Committee of the VU University Medical Center Amsterdam (document number, 2011/236) of Amsterdam. The donors were informed about the study purposes and written informed consent was obtained from all subjects.

Peptide synthesis

The peptides LL-31 and the D-LL-31 (Table 1) were synthesized by solid phase peptide synthesis using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry with a Siro II synthesizer (Biotage, Uppsala, Sweden) according to the manufacturer's protocol. Purification by HPLC was performed on a Dionex Ultimate 3000 system (Thermo Scientific, Breda, the Netherlands).

Microcosm biofilm formation

An active attachment biofilm model was used as previously described[31]. Briefly, 24-well polystyrene culture plates (Greiner Bio-One, Alphen a/d. Rijn, the Netherlands) were used. The lid of the plate was replaced by a custom-made stainless-steel lid, onto which 18 nylon clamps were fixed allowing substrates to be inserted, fitting into the wells of the culture plate. By transferring the lid with the substrate to a new plate, the medium could be refreshed. In this study, standardized 9.5mm hydroxyapatite (HAP) disks (Himed, Old Bethpage, USA) were used as substrate. After assembled, the model was autoclaved at 121 °C. The Thompson medium (TpM) was choosing as growth medium[32]. TpM was consisted of bacto proteose peptone (0.7%), trypticase peptone (0.3%), yeast extract (0.5%), KCl (0.25%), L-cysteine hydrochloride (0.05%), pig gastric mucin (0.25%) and PO_4 (0.02 M) in 860ml demi water. After autoclaved, it was supplemented with urea (1 mM), arginine (5 mM), lysine (1 mM), glycine (1 mM), hemin-menadione (1%) and sterile heat-inactivated fetal bovine serum (10%) (FBS, F4135, Sigma-Aldrich, USA).

The inoculation solution was prepared for 2 different groups. First, it was used ratio of 1:50 saliva mixture (0.5ml of saliva in 25ml of TpM) (Sal). Likewise, saliva 1:50 mixture together with 24 h *P. gingivalis* (ATCC33277*)* culture (0.5ml of saliva and *P. gingivalis* final OD = 1 in 25 ml of TpM) (SPg). Subsequently, 1.3 ml of each inoculation solution was added to 9 wells of 2 standard polystyrene 24-well plates (multiwell plates; Greiner Bio One, Alphen aan den Rijn, The Netherlands). The models were incubated anaerobically (10% CO_2, 10% H_2, and 80% N_2) at 37°C.

Treatment of the biofilms

LL31 and D-LL31 were reconstituted in sterile Milli-Q water resulting in concentration of 1mM. After 24 h Sal and SPg inoculation, the biofilms attached in HAP discs were treated by transferring them to a new plate containing the treatment solutions (1.3 ml/well) incubated anaerobically (10% CO_2, 10% H_2, and 80% N_2) at 37°C for another 24 h. Triplicate biofilms were treated with LL31 (40μM) and D-LL31 (40μM) and antimicrobial agent-free wells, containing the same volume of sterile Milli-Q water were used as controls. After the 24 h treatment, the biofilms were transferred to fresh TpM, permiting its regrowth. The 1-day post treatment biofilm was refreshed for 24 h and then harvested, while the 5-day PT biofilm was refreshed each 24h per 5 days until be harvested.

Acid production assay

After 24 h refreshment, the 1-day post-treatment biofilm lid was placed on a plate containing 1.3 ml buffered SDM-Phosphate (SDMP) with 0.4 % sucrose per 2 h at 37 °C. The amount of lactate in SDMP following incubation with sucrose was determined by colorimetric assay, previously described[33]. And the same procedure was repeated 4 days later to 7 days biofilm lactate assay.

Harvesting of the biofilms

After acid production assay, each HAP disk with biofilm was removed using sterile forceps and placed in a sterile vial containing 2 ml of cysteine peptone water (CPW). All samples were kept on ice, vortexed for 30 s and dispersed by sonication for 1 min at 1 s pulsations at the amplitude of 40 W (Vibra Cell; Sonics & Materials Inc., Newtown, CT). After dispersing the biofilms in 2 ml CPW, aliquots of 1,000 μl and

800 µl were centrifuged and the supernatant removed. The samples pellets were stored in -80°C for posterior DNA isolation and peptidase activity assay, respectively.

Viable count determination

Serial dilutions of the dispersed biofilms in CPW were made and plated on anaerobic blood agar (ABA) plates for total viable counts. The plates were incubated anaerobically (10% CO_2, 10% H_2, and 80% N_2) at 37°C for 7 days, and the number of colony-forming unit (CFU) counts was determined.

Measurement of peptidase activity

Dipeptidyl peptidase (DPP) - 4 activity was determined by cleavage rate of AMC from the synthetic substrate Gly-Pro-AMC[34,35]. The biofilm pellet was resuspended in 10mM Tris. The assay was performed in 96-well flat-bottom plate in a total assay volume of 200 µl. It was added 100 µl of Gly-Pro-AMC into 100 µl of the samples distributed in duplicates in the plate and 50 µM Gly-Pro-AMC final concentration was obtained. Fluorescence was measured using a SpectraMax M5 96-well plate spectrophotometer (excitation, 380 nm; emission, 500 nm; Molecular Devices, Sunnyvale, CA) kinetically per 2 h, every 20min in 37°C.

qPCR

DNA was isolated from all samples with the AGOWA® mag Mini DNA Isolation Kit (LGC Genomics) and bacterial loads were determined by real-time PCR using the LightCycler® 480 Instrument (Roche Diagnostics, Mannheim, Germany).

Loads of *P. gingivalis* and total bacteria were determined by real-time PCR. All PCRs were carried out in 20 µl reaction volume containing 10 µl of LightCycler® 480

Probe Master (Roche Diagnostics, Mannheim, Germany). The samples analysis was subjected to pre-incubation cycle of 95°C for 5 min, followed by 45 cycles of quantification at 95°C for 10 s and at 60°C for 20 s.

Ten times serial dilutions of homologous DNA were used as standard curves. For quantification, the results of the samples were calculated using the standard curve of the corresponding bacteria. For quantification of the total bacterial load, a standard curve of *E. coli* was used. PCR water was used as a negative control in every run.

Statistical analysis

The statistical analysis was performed by GraphPad Prism 7 (San Diego, CA, USA). Normal distribution of all assays was tested (Kolmogorov-Smirnov test). Mann-Whitney test was conducted for comparison between samples. All tests were performed at a 5% level of significance.

Results

D-LL31 and LL31 Effect on Lactic Acid Production in Microcosm Biofilms

Biofilm metabolic activity was measured through lactic acid production evaluation. In 1 day post treatment, both D-LL31 and LL31 treatments, in either SPg or Sal groups presented significant reduction compared to control ($p<0.005$ and $p<0.05$, respectively). SPg treated with D-LL31 presented the most expressive reduction with almost half lactic acid production compared to produced by controls, median - 0.36 CI95% (0.53 – 0.67) mM and 0.60 (0.53 – 0.67) mM, respectively ($p<0.005$). D-LL31 presented significant reduction even when compared to LL31 treatment (0.49 [0.43 – 0.55]) ($p<0.05$) (Figure 2A). D-LL31 also showed stronger

effect on Sal, presenting 4 times lactic acid reduction compared to the control, 0.05 (0.02 – 0.07) mM and 0.20 (0.11 – 0.29) mM, respectively ($p<0.005$) (Figure 2A). Five days after treatment, SPg treated with D-LL31 (1.164 [0.75 – 1.67]) maintained the lactic acid production reduced in comparison to the others groups (SPg – 3.94 [2.72 – 4.34] and SPg LL31 - 2.65 [1.60 – 3.40]) (Figure 2B). While all saliva groups, recovered its metabolic activity and presented similar lactic concentration as controls group (3.122 [1.27 – 5.74]).

D-enantiomeric Peptide Reduced Peptidase Activity in Microcosm Biofilms

The results were interpreted according to the fluorescence intensity (FI). D-LL31(median - 2200 CI95% [1776 – 2833]) treatment was able to strongly reduce DPP-4 activity in SPg group 1 day post-treatment compared to control groups (5880 [4605 – 7176]) (Figure 3A) and kept a smaller but significant reduction still in 5 days post-treatment analysis, D-LL31 (8661 [5123 – 10243]) and control (9650 [9283 – 10025]) (Figure 3B). While Sal groups showed no significant difference between treatments in both time points, 1-day and 5-days post-treatment. Moreover, 7 days old Sal biofilms presented higher enzymatic activity than 3 days biofilms, from 240.5 [158.1 - 408.2] to 4376 [1292 – 7454].

***P. gingivalis* and Total Bacteria Load on Microcosm Biofilms After Treatment**

Within 24 h after D-LL31 treatment, *P. gingivalis* (Figure 4) as well as total bacteria load (Figure 5A) were significantly reduced in SPg biofilm compared to the control group. However, 4 days later no more difference was found, the bacteria load was completely recovered for both bacteria investigated (Figure 4 and 5B). For the Sal group samples, it was not found expressive *P. gingivalis* concentration. Besides not presenting significant difference in total bacteria load between Sal groups in 7 days

old biofilm, there was bigger number of samples with higher bacteria concentration in control group than groups treated with antimicrobial peptides (Figure 5B).

Microcosm Biofilms Cell Viability

Surprisingly, divergent results came up from biofilms cells viability compared to the other assays. In 1 day post-treatment, the control groups SPg and Saliva yielded median - 9.12 CI95% (8.83 – 9.42) and 7.02 (6.29 – 7.77) $\log_{10}$ CFU/biofilm, respectively, and no significant difference was found between the controls and groups treated with peptides (Figure 6). Four days later, the counts maintained with no difference between controls and treatment groups, only a small but not significant increase was noticed from 3 days to 7 days for SPg and Sal biofilms, presenting 9.86 (9.78 – 9.94) and 8.55 (8.00 – 9.11) $\log_{10}$ CFU/biofilm (Figure 6).

Discussion

In the present study, we attempted to investigate the effect of the human cathelicidin-derived antimicrobial peptide, LL31 and its D-enantiomer, D-LL31, in two different compositions of microcosm biolfims in HAP surface, either only saliva or saliva enriched with *P. gingivalis* culture inoculums. We demonstrated the difference between a non-periodontopathogenic and a periodontopathogenic biofilm enzymatic activity. Regardless the treatment groups, SPg control group presented higher enzymatic activity than Sal. Taking treatment into account, D-LL31 reduced significantly DPP-4 activity on SPg group in 1-day post-treatment, maintaining the reduction until 4 days later. Nevertheless, no difference was detected between control and treatment groups in Sal biofilm. The small, almost undetectable enzymatic activity in Sal biofilms is in agreement with the non-expressive *P. gingivalis* load found in

qPCR on this group 1-day post-treatment. However, 4 days later it was registered an increase on enzymatic activity, decreasing the difference with SPg groups. Previous interventional clinical study reported an association between the presence of DPP in gingival crevicular fluid and periodontal clinical status[36]. The presence and virulence of Porphyromonas species can be effectively investigated through measurement of some proteinases activity, such as DPPs, which are crucial for both bacterial growth and pathogenicity[37].

The addition of *P. gingivalis* culture into the saliva inoculum was intended to turn the biofilm into periodontopathogenic. It was consistent to the “keystone pathogens” and synergetic pathogenicity concept, where the first defends that the presence of *P. gingivalis* may have the capability of affect the biofilm virulence and modificate the oral microbiome[38,39] and the second suport the idea that the disease is a consequence of an interaction and combined activity between different species[40]. Previously, it has been shown higher biomass in co-culture of *P. gingivalis* and *Treponema denticola*, two components of red complex. It was suggested that P. gingivalis contribute to *T. denticola* growth[41].

The D-enantiomer form of LL-31 peptide, D-LL31, was tested in order to compare and evaluate its protease resistance and consequently antimicrobial activity. Previous studies identified D-enantiomer peptides as presenting superior activity against *P. aeruginosa*[42] and *B. pseudomallei*[43] and our findings were not different. In the present data, D-LL31 showed higher influence on biofilms enzymatic and metabolic activity when compared to LL-31.

The results of this study can demonstrate that both biofilms when exposed to the AMPs presented reduction on its metabolic activity during the regrowth. D-LL31 and LL31 proved to have the ability to inhibit lactic acid production. Only D-LL31 was

able to inhibt lactic acid production until 5 days of regrowth on SPg, but not on Sal biofilm, which was only inhibited until 1-day post-treatment and in 5-days, completely recovered its metabolic activity in comparison to the control group. These results show a particular behave of each biofilm regrowth regarding its different composition and effects of the AMPs on it.

The different mechanisms of metabolisms based on acid or organic bases production, play an important role for both microbe-microbe interactions and host-microbe synergy. In the natural biofilm, as early colonizers, streptococci, a lactic acid producer, set the stage for colonization by later inhabitants such as Porphyromonas species, which utilize amino acids as sole carbon and energy sources. However, in the present study, the periodontopathogenic biofilm was resulted from the inoculation of saliva with high concentration of *P. gingivalis* culture. The higher concentration of lactic acid production found in 1-day post-treatment in the periodontopathogenic biofilm may be due to the higher cell viability compared to Sal group.

Suprinsigly, the biofilm cell viability was not affected by the treatments as enzymatic and metabolic activity. These contradictory results between assays may be due to the model used, an alternative biofilm model, more representative of oral biofilm conditions could tell us further about the antimicrobial peptide activity.

In conclusion, it was possible to certify that D-LL31 is capable to reduce biofilms enzymatic and metabolic activity, being more efficient than LL31, specially 1-day post-treatment. Furthermore, the microcosm biofilm enriched with P. gingivalis seems to be a promissing multi-species periodontopathogenic biobilm model. Thereafter, several studies will be required to asses clinical applicability of D-LL31 and LL-31.

Acknowledgment

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Conflicts of interest

There is no conflict of interest.

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Tables

Table 1- Characteristics of the peptides.

Peptide	*Sequence*	*Mol. Wt.*
LL31	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL	3,824
D-LL31	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL	3,824

Figures

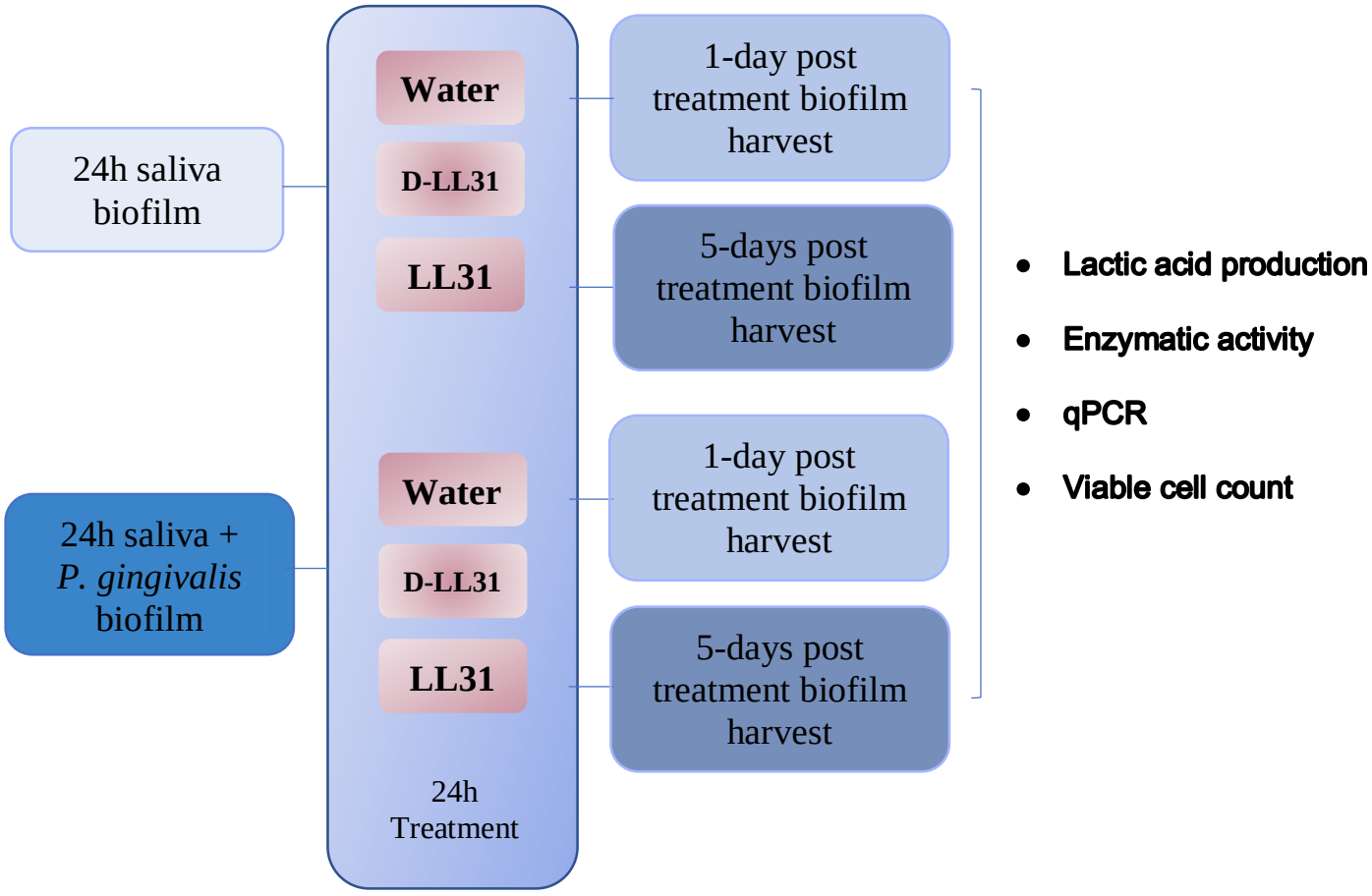
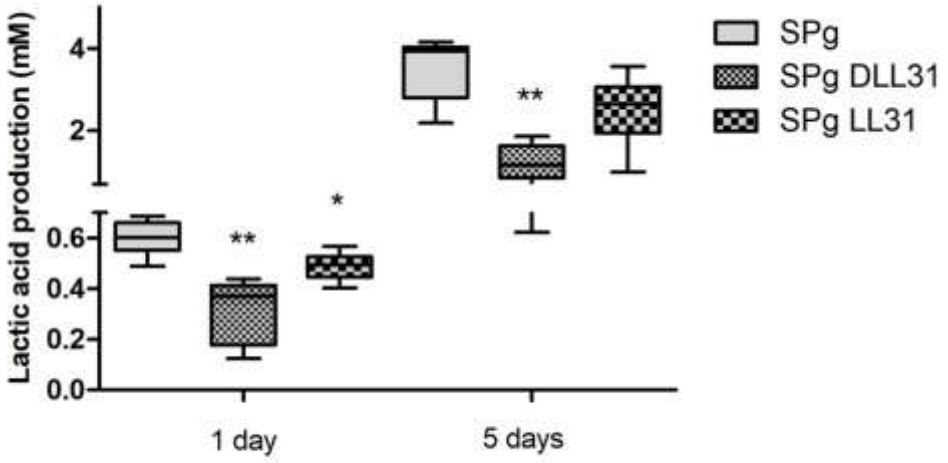


Figure 1- Scheme of experimental design.

A



B

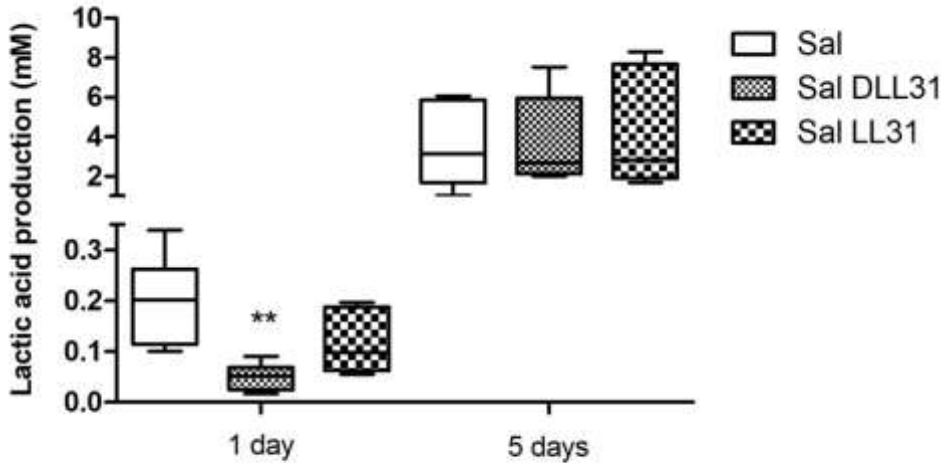
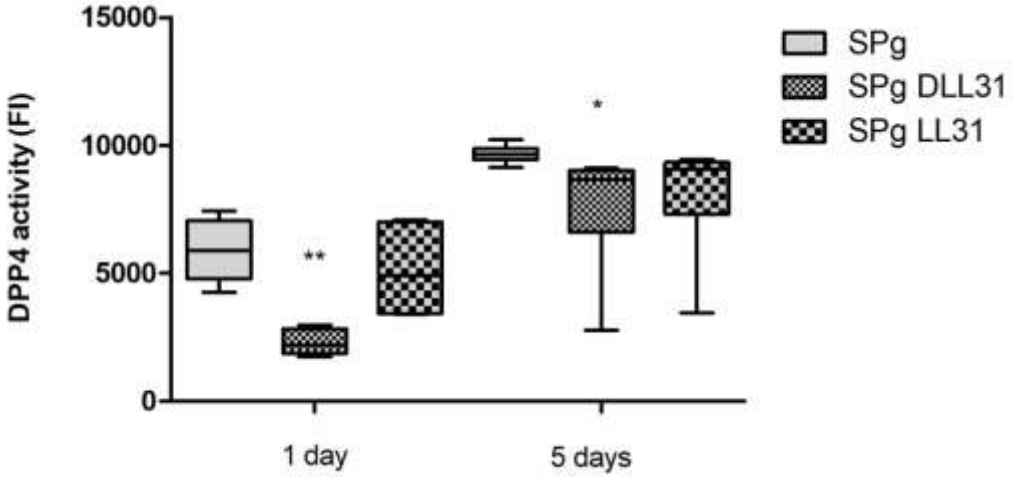


Figure 2 - Lactic acid production on biofilms regrowth in 1-day and 5-days post-treatment with D-LL31 and LL31. Data is represented by box plots with medians (lines inside boxes), 25th and 75th percentiles (limits of boxes) and the 10th and 90th percentiles (whiskers).
* and ** indicate statistically significant differences between treatments ($p<0.05$ and $p< 0.005$, respectively, Mann-Whitney test).

A



B

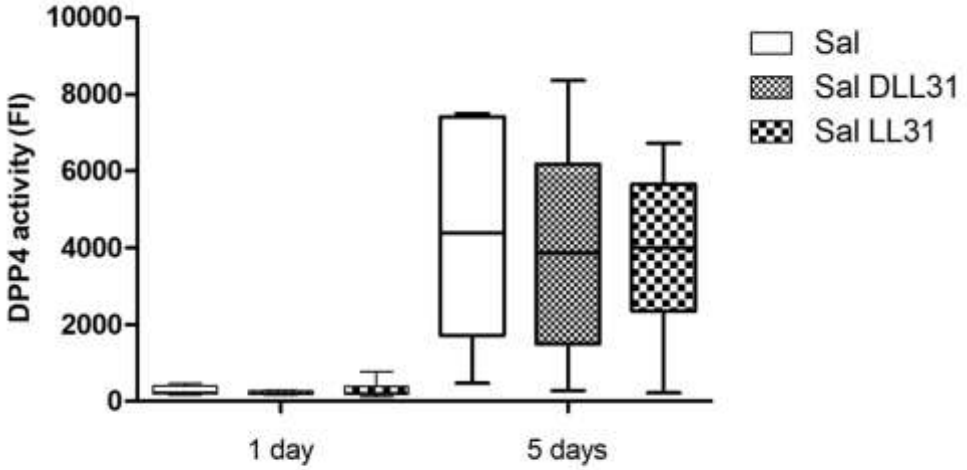


Figure 3 – Dipeptidyl peptidase activiy on biofilms regrowth in 1-day and 5-days post-treatment with D-LL31 and LL31. Data is represented by box plots with medians (lines inside boxes), 25th and 75th percentiles (limits of boxes) and the 10th and 90th percentiles (whiskers). * and ** indicate statistically significant differences between treatments ($p<0.05$ and $p< 0.005$, respectively, Mann-Whitney test).

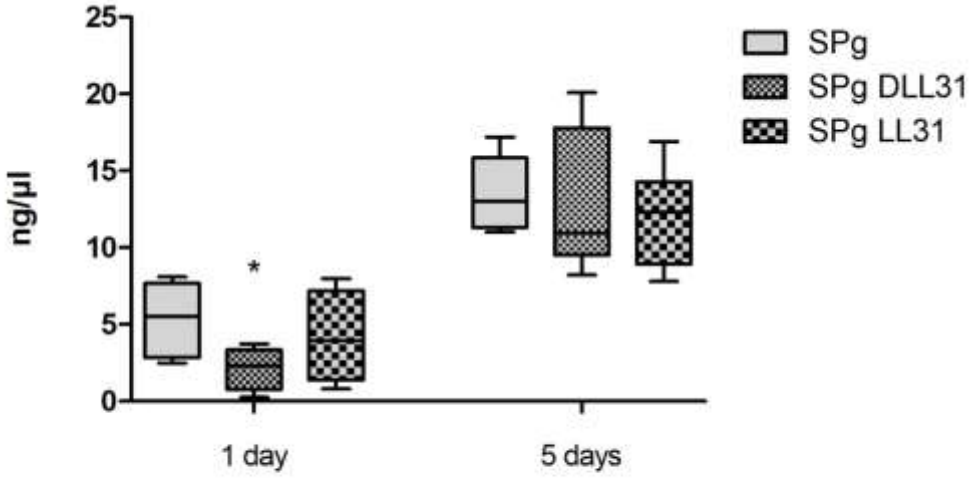


Figure 4 - *P. gingivalis* DNA concentration on *P. gingivalis* enriched microcosm biofilm and treatments effect on its regrowth 1-day and 5-days post-treatment. Data is represented by box plots with medians (lines inside boxes), 25th and 75th percentiles (limits of boxes) and the 10th and 90th percentiles (whiskers). The genomic DNA is expressed in ng/µl. * indicate statistically significant differences between treatments ($p<0.05$, Mann-Whitney test).

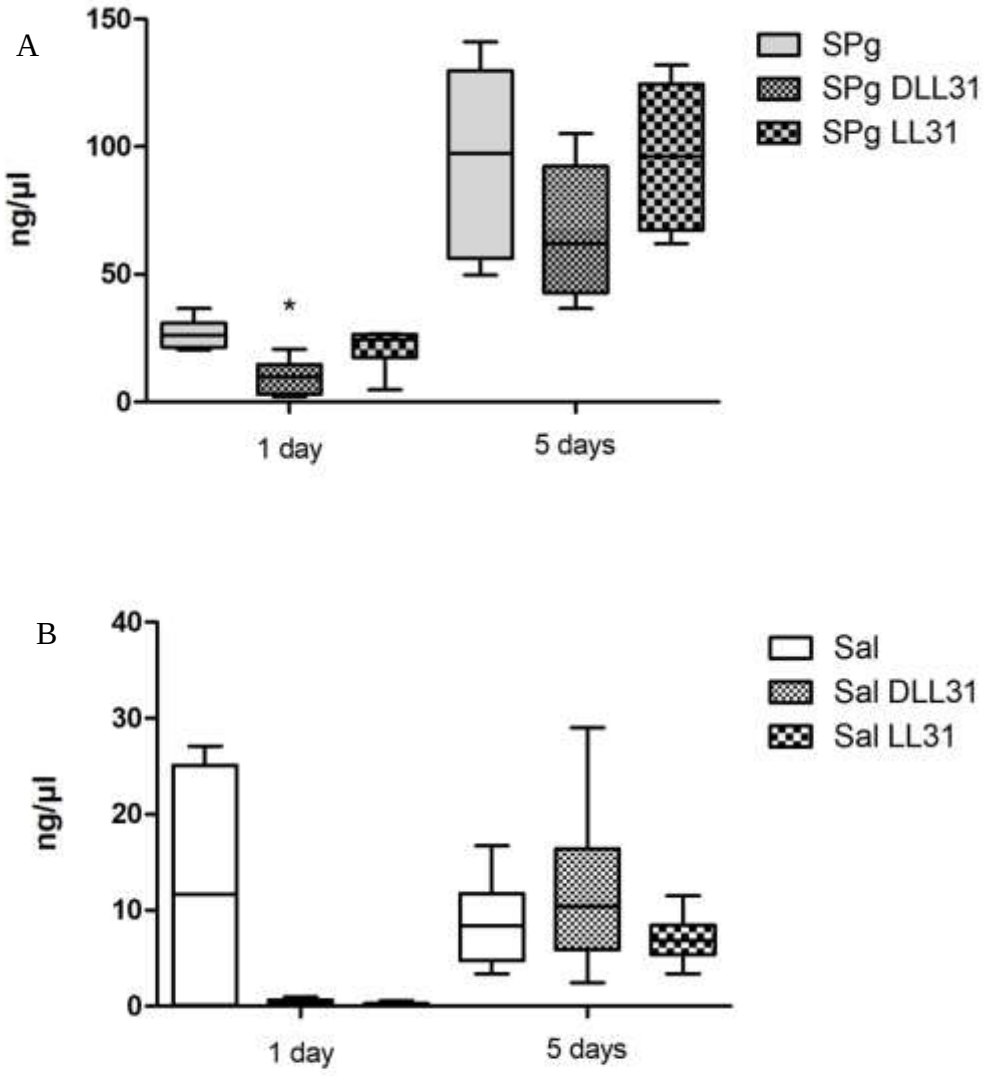


Figure 5 - Total bacteria DNA concentration. Data is represented by box plots with medians (lines inside boxes), 25th and 75th percentiles (limits of boxes) and the 10th and 90th percentiles (whiskers). The genomic DNA is expressed in ng/µl. * indicate statistically significant differences between treatments ($p<0.05$, Mann-Whitney test)

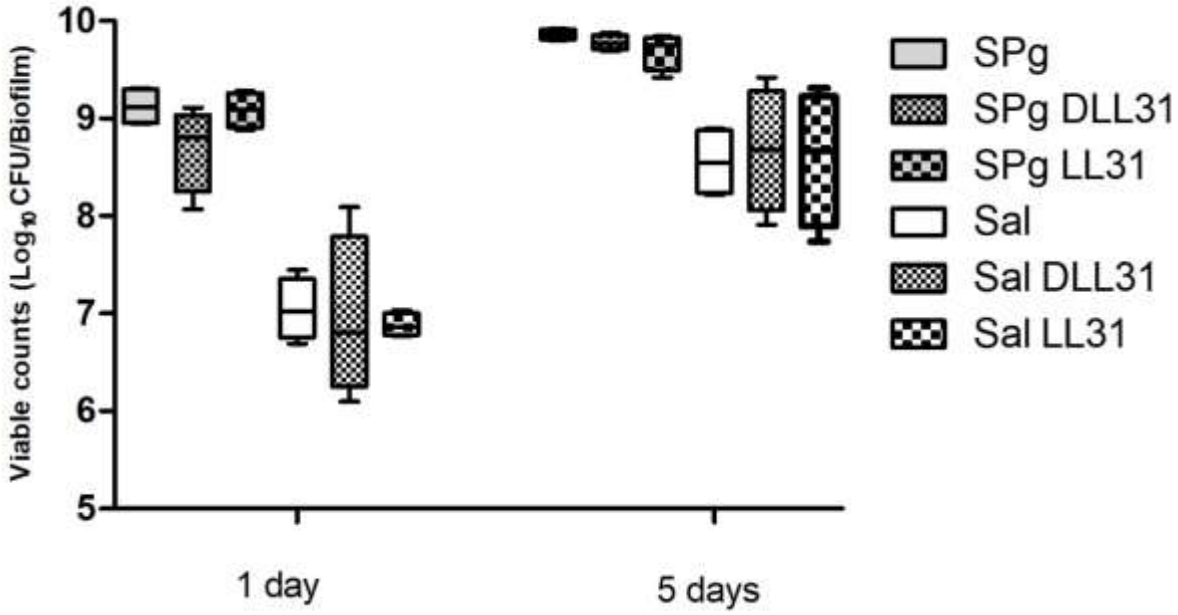


Figure 6 – Microcosm biofilm cell viability. as box plots with medians (lines inside boxes), 25th and 75th percentiles (limits of boxes) and the 10th and 90th percentiles (whiskers). in Log_{10} CFU/biofilm.

4 CONSIDERAÇÕES FINAIS

Os PAMs são importantes componentes da resposta imune inata e adaptativa. No entanto, alguns fatores de risco para a doença periodontal podem interferir na expressão destes PAMs na cavidade bucal, podendo ser considerado mais um mecanismo associado a maior incidência e severidade da doença periodontal nos pacientes expostos a estes fatores. Contudo, o mecanismo exato responsável pela regulação dos PAMs ainda não foi totalmente elucidado . Dessa forma, investigamos a influência de um destes fatores de risco, o tabagismo, sobre os níveis de PAMs antimicrobianos sintetizados, sobretudo, por neutrófilos, retratado no capítulo 1, LL-37 e HNP 1-3, bem como por células epiteliais, retratado no capítulo 2, hBD1 e 2, em pacientes com periodontite. Constatamos uma redução nos níveis dos PAMs de origem neutrofílica, tanto em sítios sadios como doentes, de pacientes fumantes em relação aos não fumantes, sendo que apenas para a LL-37 em sítios sadios essa diferença não foi significativa. Dentre os mecanismos responsáveis por essa redução, podemos sugerir as alterações causadas em neutrófilos pelo fumo, como variação na quimiotaxia e redução da resposta inflamatória[59]. Já nos PAMs de origem epitelial, o fumo reduziu significativamente os níveis de hBD1 e elevou os níveis de hBD2 na presença de doença periodontal. Uma possível explicação para o efeito distinto do fumo sobre os níveis de hBD1 e 2, seriam as regulações diversas causadas pela nicotina sobre os mecanismos responsáveis pela liberação destes PAMs, podendo causar aumento ou diminuição na regulação[60–62].

Também verificamos alterações nos níveis de PAMs influenciadas pela condição de saúde ou doença periodontal. Em sítios com sinais clínicos de periodontite, foram encontrados maiores níveis de LL-37, associados a maiores níveis de IL-1β e MMP-8 comparados a sítios sadios. Enquanto a HNP 1-3 apresentou-se em maiores níveis em sítios sadios e foi positivamente correlacionada a presença de IL-10. Embora sejam majoritariamente sintetizados por neutrófilos, LL-37 e HNP 1-3 também podem ser sintetizadas por diversas outras células constituintes do processo inflamatório[63], possuindo diferentes mecanismos de regulação[64,65], o que poderia justificar a regulação distinta destes PAMs na presença ou ausência de doença periodontal.

Em nosso estudo in vitro, os estímulos com nicotina e cotinina resultaram na redução da expressão da hBD1 por queratinócitos, sendo apenas a redução pela

nicotina significativa. A associação da nicotina com *A. actinomycetemcomitans* acentuou a redução da expressão de hBD1 bem como influenciou negativamente a expressão de hBD2. Enquanto a cotinina, promoveu uma redução significativa da expressão de hBD2 apenas quando estimulado em associação com a *P. gingivalis*. Muitas associações já foram feitas quanto à presença de PAMs em pacientes com periodontite. No entanto, ainda existem controvérsias na literatura. Alguns estudos demonstram maiores concentrações de PAMs no FCG de pacientes com periodontite [31–33], enquanto outros não apresentam diferença significativa[24,32,66,67], quando comparados a pacientes sem doença periodontal. Acreditamos que certas variações metodológicas encontradas nestes estudos clínicos poderiam justificar os resultados controversos, como a seleção dos sítios para coleta do FCG e critérios de definição referentes à presença e severidade da doença periodontal, bem como o método de coleta utilizado. A mesma consideração pode se estender para os estudos in vitro, onde diferentes células são analisadas frente a diversos estímulos, com variadas concentrações e intervalos de tempo. Vale ressaltar também as variações encontradas na expressão dos diferentes PAMs, que podem estar associadas a mecanismos distintos de regulação[64,65], fatores de transcrição, assim como pela diversidade de células progenitoras dos mesmos[63]. Sendo assim, o papel dos PAMs na patogênese da doença periodontal e os mecanismos reguladores da sua expressão precisam ser melhores investigados.

De modo geral, PAMs, especialmente defensinas e catelicidinas, estão presentes no tecido periodontal sadio e doente e evidências científicas têm demonstrado seu importante papel na resposta imune do hospedeiro. No entanto, as células de biofilme podem expressar mecanismos resistentes contra esses PAMs, tanto passiva quanto ativamente, dificultando o controle de seu crescimento durante a recolonização. A atividade antimicrobiana da LL-37 e sua variante mais curta LL-31 contra biofilmes de *B. pseudomallei* foi relatada. No entanto, já foi demonstrado que o LL-37 em sua forma natural, composta por L-aminoácidos, é altamente sensível à degradação enzimática pelas proteases[68,69]. D-aminoácidos podem ser um ótimo substituto, pois apresentam melhor atividade antimicrobiana e a estabilidade proteolítica dos peptídeos[70]. Foi relatado que essa substituição pode melhorar a atividade antimicrobiana e a estabilidade proteolítica dos peptídeos[70] e pode ser uma opção para o desenvolvimento de peptídeos antimicrobianos resistentes a proteases para uso terapêutico. Assim sendo, no capítulo 3 propusemo-nos a avaliar o efeito do

tratamento com LL31 e D-LL31 na recolonização em biofilmes microcosmos e microcosmos periodontopatogênico. Um dia após tratamento, conseguimos identificar que D-LL31 obteve maior eficiência ao inibir a atividade metabólica sobre ambos os biofilmes multi-espécies, mantendo seu efeito sobre a atividade metabólica no biofilme periodontopatogênico até o quinto dia de recolonização comparado ao grupo controle. Quanto à atividade enzimática, D-LL31 demonstrou significativa redução sobre a atividade de DPP-4, tanto um dia, como cinco dias após tratamento. O peptídeo D-enantiômero avaliado apresentou significativa melhoria comparado ao peptídeo em sua forma natural. Tendo em vista DPP-4 como sendo um possível biomarcador para a presença de periodontopatógenos, entre nossos biofilmes, conseguimos demonstrar a diferença nítida da sua atividade em biofilmes com diferentes composições, sugerindo assim que nosso modelo de microcosmo periodontopatogênico pode ser utilizado para tais objetivos.

Dentre as proposições e resultados dos trabalhos apresentados, devemos ressaltar algumas limitações. Tanto os estudos clínicos como in vitro não são capazes de retratar os possíveis mecanismos envolvidos na regulação dos diferentes PAMs pelo tabagismo. Tão pouco podemos afirmar como ocorre a regulação dos PAMs frente ao processo inflamatório. Sendo assim, vale ressaltar a necessidade de novos estudos para uma melhor compreensão do papel dos PAMs na etiopatogênese da doença periodontal, o que seria importante para estabelecimento de novas medidas preventivas, diagnósticas e terapêuticas para as doenças periodontais.

5 CONCLUSÃO

Com base nos resultados obtidos, podemos concluir que existe uma associação entre o tabagismo e alterações na expressão de PAMs. O tabagismo teve um efeito negativo na expressão de PAMs de origem neutrofílica, como a LL-37 e HNP 1-3, no FCG de sítios sadios e doentes de pacientes com periodontite crônica. Já nos PAMs de origem epitelial, o fumo reduziu os níveis de hBD1, porém aumentou os níveis de hBD2 nos sítios que apresentavam sinais clínicos de periodontite.

Em relação a condição clínica periodontal, PAMs apresentaram modulações distintas. Sítios doentes apresentaram maiores níveis de LL-37, positivamente correlacionados com mediadores pro-inflamatórios, enquanto a HNP 1-3 apresentava-se em menores níveis quando comparados a sítios sadios, sendo positivamente correlacionada a mediadores anti-inflamatórios, independentemente da presença do hábito de fumar.

Além disso, produtos tóxicos do cigarro, como a nicotina e cotinina, promoveram uma redução na expressão gênica de hBD1 por queratinócitos humanos, sendo essa redução mais intensa na presença do patógeno *A. actinomycetemcomitans*. Estes mesmos produtos reduziram a expressão gênica de hBD2 apenas na presença de patógenos periodontais, sendo o efeito da nicotina mais acentuado na presença de *A. actinomycetemcomitans* e da cotinina na presença de *P. gingivalis.*

Em relação aos peptídeos sintéticos testados em biofilmes multi-espécies, foi possível demonstrar que a versão D-aminoácido da LL-31 apresenta maior efeito inibitório, proporcionando maior redução da atividade metabólica e enzimática sobre a recolonização de biofilme multi-espécies periodontopatogênico.

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APÊNDICE

Metodologia detalhada – *Publicação 1 e 2*

Aprovação comitê de ética

O protocolo do estudo foi aprovado pelo Comitê de Ética em Pesquisa Humana da Faculdade de Odontologia de Araraquara - UNESP (protocolo número 01394712.5.0000.5416) (Anexo).

Características dos pacientes selecionados

O diagnóstico de periodontite foi realizado de acordo com o Workshop Mundial de 2017 sobre a classificação de doenças e condições periodontais e peri-implantares[1]. Os critérios de inclusão para pacientes com periodontite foram presença de no mínimo 15 dentes naturais e pelo menos cinco sítios com profundidade de sondagem (PS) ≥ 4 mm, sangramento na sondagem (SS) e nível clínico de inserção (NCI) ≥ 4 mm, com exceção de terceiros molares. Pacientes fumantes fumavam regularmente pelo menos 10 cigarros/dia por um período mínimo de 5 anos e pacientes não fumantes nunca deveriam ter fumado[2,3]. Foram excluídos indivíduos com histórico de doenças sistêmicas ou alterações imunológicas, ou fazendo uso de terapia de reposição hormonal, além de gestantes e lactantes. Também foram excluídos indivíduos que receberam tratamento periodontal nos últimos 6 meses ou que fizeram uso de antibióticos e anti-inflamatórios sistêmicos nos últimos 3 meses.

Exame clínico

Os parâmetros periodontais clínicos da boca inteira, incluindo o Índice de Placa (IP), Índice de Sangramento Gengival (ISG) PS, e NCI, foram avaliados por um único examinador treinado e calibrado. Os IP e ISG foram registrados como ausência ou presença de placa e sangramento gengival em quatro sítios por dente (mesial, distal, bucal e lingual)[4], enquanto a PS e o NCI foram avaliados em seis sítios por dente (mesio-bucal, médio a vestibular, vestibular, mesio-lingual, médio-lingual e isto-lingual), excluindo terceiros molares. A avaliação clínica foi realizada com uma sonda periodontal UNC (Hu-Friedy, Chicago, IL, EUA). Para a calibração, a medida da PS

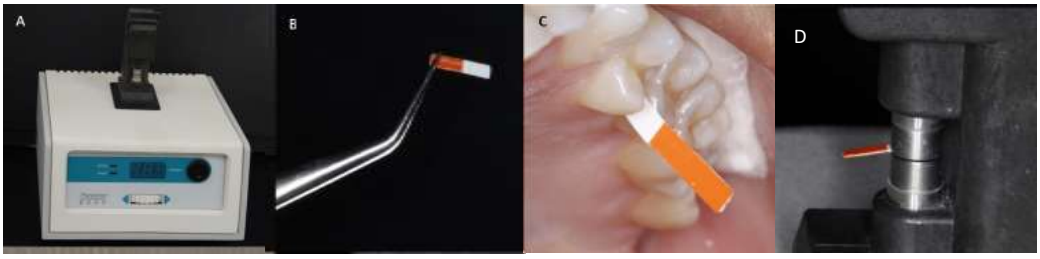
foi realizada em seis dentes (16, 21, 24, 36, 41 e 44) de acordo com o índice de Ramfjord, num total de 6 pacientes (36 dentes / paciente) em duas ocasiões diferentes em 7 dias. Os dados foram submetidos à análise de concordância Kappa (k = 0,91).

Coleta amostras do fluido crevicular gengival (FCG)

O passo a passo da coleta do fluido gengival crevicular está ilustrada na figura A1. As amostras de FCG foram coletadas em 10 sítios de cada paciente, sendo 5 sítios sadios e 5 doentes. Os sítios de coleta foram selecionados a partir de dentes não adjacentes, preferencialmente incluindo pelo menos um sítio e um sítio doente por quadrante. Sítios saudios não apresentavam inflamação gengival, PS ≤ 3 mm, ausência de SS ou perda de inserção clínica; e sítios doentes apresentaram inflamação gengival, PS e NCI ≥ 4 mm com SS. Os sítios afetados foram selecionados de acordo com a profundidade de sondagem mais profunda em cada quadrante. Um único examinador realizou a coleta das amostras do FCG, um dia após o exame periodontal.

Previamente à coleta das amostras, os dentes foram isolados com rolos de algodão, a placa supragengival foi removida com uma cureta estéril e as superfícies foram suavemente secas com jato de ar. O FCG foi coletado inserindo tiras de papel absorventes (Periopaper®, Oraflow Inc., Nova York, EUA) no sulco gengival ou na bolsa periodontal por 30 segundos. Tiras de papel contaminadas com sangue e saliva foram rejeitadas. O volume de FCG absorvido em cada tira foi determinado por um dispositivo eletrônico (Periotron 8000 - Periopaper, ProFlow, Inc., Amityville, NY, EUA), que foi calibrado com base no protocolo descrito anteriormente[5] (calibração do Periotron 8000 por regressão polinomial). Amostras da mesma categoria (sítios sadios ou doentes) em cada indivíduo foram imediatamente reunidas em um tubo de microcentrífuga de polipropileno estéril e seco (5 tiras de papel por tubo) e mantidas a -80°C até a análise. As leituras do Periotron 8000 foram convertidas para um volume real (µL) por referência a uma curva padrão(Tabela A1 e Figura A2)[6].

Figura A1 – Coleta do FCG com utilização de tiras de papel absorvente. **A)**Periotron 8000®; **B)** Filtro de papel antes da coleta; **C)** Filtro de papel em posição durante a coleta; **D)** Mensuração do volume coletado de FCG.



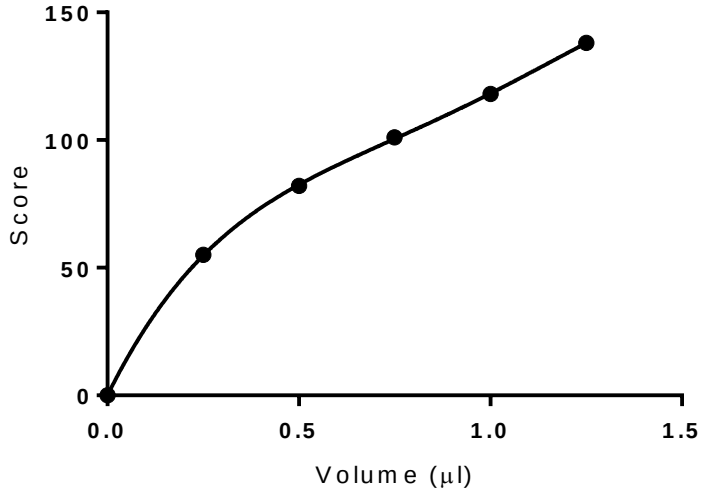
Fonte: Elaboração própria

Tabela A1 - Valores de referência utilizados para construção da curva padrão.

Volume (μl)	**Leitura Periotron**
0.00	0
0.25	55
0.50	82
0.75	101
1.00	118
1.25	138

Fonte: Elaboração própria

Figura A2 - Curva padrão do Periotron (r = 0.9999 - Regressão Polinomial de 4ª ordem).



Fonte: Elaboração própria

Metodologia – *Publicação 1*

Foram incluídos no estudo 40 pacientes de ambos os sexos com periodontite: 20 fumantes e 20 não fumantes. Os participantes foram recrutados na Clínica de Periodontia da Faculdade de Odontologia de Araraquara - UNESP. Consentimento informado por escrito foi obtido de todos os assuntos.

Quantificação de peptídeos antimicrobianos e mediadores inflamatórios

Primeiramente, o FCG absorvido foi eluído das tiras de papel adicionando 750 µL (150 µL por tira) de 1x solução salina tamponada com fosfato (PBS) a cada tubo. Os tubos foram deixados em gelo em um agitador horizontal por 30 minutos e depois centrifugados a 4 °C, 13.000 g por 10 minutos. Os níveis de LL-37 e HNP 1-3 no FCG foram medidos usando ELISAs sanduíche de acordo com o manual do fabricante (Hycult Biotechnology, Uden, Holanda).

Previamente ao ensaio ELISA foi realizado teste sobre os valores presentes em 3 amostras e sua relação com a curva padrão. Frente aos resultados, optamos por utilizar amostras diluídas, na proporção de 75% amostra e 25% água deionizada estéril. Posteriormente, os valores foram convertidos de forma proporcional. A

absorbância foi medida a 450 nm de comprimento de onda pelo espectrofotômetro (VersaMax, Molecular Devices, San Jose, CA, EUA). O intervalo de detecção de LL-37 foi de 0,1 a 100 ng / mL e de HNP 1-3 foi de 156 a 10.000 pg / mL. Os resultados são expressos em pg / mL.

Os níveis dos mediadores imunoinflamatórios IL-1β, IL-10, MMP-8, TNF-α e IFN-gama no GCF foram analisados por ensaio multiplex com base em esferas magnéticas, de acordo com as instruções fornecidas pelo fabricante do kit (R&D Systems , Minneapolis, MN, EUA) e o sistema Luminex® 100TM foi utilizado para a leitura. A quantidade de proteína em cada amostra foi extrapolada com base na curva padrão e as concentrações são relatadas em pg / mL.

Análise estatística

A análise estatística foi realizada através do GraphPad Prism 7 (San Diego, CA, EUA) e as diferenças foram consideradas significativas com um valor de $P < 0,05$. A normalidade da distribuição foi analisada pelo teste D'Agostino e Pearson.

A distribuição normal foi detectada apenas para os parâmetros de idade e níveis de LL-37. Assim, o teste t não pareado foi realizado para comparação intergrupos e o teste t pareado para análise intragrupo. Os dados que não seguem a distribuição normal, como parâmetros clínicos e os níveis de HNP 1-3, foram analisados pelos testes de Mann-Whitney para comparações intergrupos e análises intragrupo pelo teste de Wilcoxon. As correlações entre as concentrações de mediadores inflamatórios e AMPs em sítios sadios e doentes para fumantes e não fumantes foram determinadas pelo teste de correlação de Spearman.

Metodologia – *Publicação 2*

Foram incluídos no estudo 20 pacientes sem doença periodontal e 50 pacientes de ambos os sexos com periodontite: 25 fumantes e 25 não fumantes. Os participantes foram recrutados na Clínica de Periodontia da Faculdade de Odontologia de Araraquara - UNESP. Consentimento informado por escrito foi obtido de todos os assuntos.

Ensaio imunoenzimático para quantificação de hBD1 e hBD2

Primeiramente, foi realizada a eluição do FCG absorvido pelas tiras de papel, adicionando 750µL (150µL por tira) de 1x solução salina tamponada com fosfato (PBS) a cada tubo. Os tubos foram deixados em gelo em um agitador horizontal por 30 minutos e depois centrifugados à 4 ° C, 13.000 g por 10 minutos.

Previamente ao ensaio ELISA foi realizado teste sobre os valores presentes em 3 amostras e sua relação com a curva padrão. Frente aos resultados, optamos por utilizar amostras diluídas sendo 50% amostra e 50% água deionizada estéril. Posteriormente, os valores foram convertidos proporcionalmente.

Os níveis de hBD1 e hBD2 no FCG foram mensurados usando a técnica ELISA sanduíche de acordo com o manual do fabricante (Peprotech, Rocky Hill, NJ, EUA). A absorbância foi mensurada por espectrofotômetro (VersaMax, Molecular Devices, San Jose, CA, EUA) no comprimento de onda de 450 nm. Os níveis de hBD1 e hBD2 em cada amostra foram determinados usando os valores de concentração dos padrões incluídos no kit. Os resultados foram expressos em pg / mL.

Estudo HaCat

Cultura de células

As células HaCaTs (HaCaT; Cell Lines Service, 300493) foram cultivadas em DMEM (Dulbecco modification of Minimum Essential Media - Thermofisher Scientifc) suplementado com soro fetal bovino a 10% (Invitrogen, Karlsruhe, Alemanha), glutamina a 1% e estreptomicina + penicilina a 1% . As células foram cultivadas até 80-90% de confluência e depois foram transferidas uniformemente para placas de 12 poços, numa concentração de $1x10^5$ células por poço. Após 48h, os respectivos tratamentos foram executados .

Cultura bacteriana e condições de estimulação cellular

As cepas estavam armazenadas em caldo BHI (brain heart infusion) contendo 20% de glicerol a -80 ^{o}C. As amostras de *P. gingivalis* foram cultivadas em placas de ágar sangue (sangue desfibrinado de carneiro) suplementado com hemina (0,5 mg/ml) e menadione (1 mg/ml) (Sigma Chemical Co, St Louis, EUA) e em jarra de anaerobiose (GasPack^{TM} EZ Anaerobe Container System with Indicator, BD) a 37^{o} C

durante 7 dias. Com auxílio de uma alça de transfrência foi realizada a passagem da P. gingivalis da placa para o caldo triptone soja (TSB) suplementado com menadiona (1mg/ml) e baixa concentração de hemina (1 µg/ml), até atingir sua fase exponenecial, em torno de 24 h de crescimento, quando então foi realizada a diluição no mesmo meio de incubação até atingir a densidade óptica $(DO)_{495\ nm}$ ~ 0,5.

As amostras de *A. actinomycetemcomitans* foram cultivadas em placas de ágar com meio TSB e em jarra de anaerobiose (GasPack^{TM} EZ Anaerobe Container System with Indicator, BD) em estufa a 37º C durante 48 horas. Para obter culturas de *A. actinomycetemcomitans* em fase exponencial de crescimento, a amostra foi cultivada em caldo TSB e incubada durante 24 horas em jarra com atmosfera de 10% de CO_2 dada pelo envelope GasPak^{TM}. Após este intervalo de tempo, a concentração da suspensão bacteriana foi ajustada até atingir a DO para ~0,2.

As concentrações finais de bactérias foram ajustadas para a DO de 0,5 (Pg) e 0,2 (Aa), correlacionando-se a $2x10^7$ UFC / mL. Para a expressão gênica, foram utilizadas bactérias vivas na proporção de 200:1 (bactérias: células)

Tratamento de células queratinócitos

Experimentos preliminares foram conduzidos para determinar as concentrações não-tóxicas de nicotina e cotinina e seus solventes DMSO e etanol, respectivamente, a fim de garantir que qualquer um de seus efeitos observados na função HaCaT não resultassem em toxicidade celular ou morte celular. A viabilidade das células tratadas e não tratadas foi avaliada após 24 h de exposição à nicotina e cotinina e comparada pelo ensaio Alamar Blue (Figura A3). A concentração de 10µg/mL de nicotina e cotinina foi escolhida em relação ao teste de toxicidade e concentração fisiológica[7,8] (Figura A1). Após atingir 80-90% de confluência, as células foram estimuladas com os tratamentos da figura A4. O experimento foi realizado em duplicata e repetido 3 vezes.

Figura A3 – Ensaio de Alamar Blue para teste de viabilidade celular em diferentes concentrações de estímulos e solventes - **A)** Comparação entre as concentrações de Controle (DMEM) e Nicotina de 0,1 µg/ml a 100 µg/ml - **B)** Comparação entre as concentrações de Controle (DMEM) e Cotinina 0,05 µg/ml a 10 µg/ml - **C)** Comparação entre Controle (DMEM) e Solventes - DMSO - 34 µl; Etanol – 17 µl

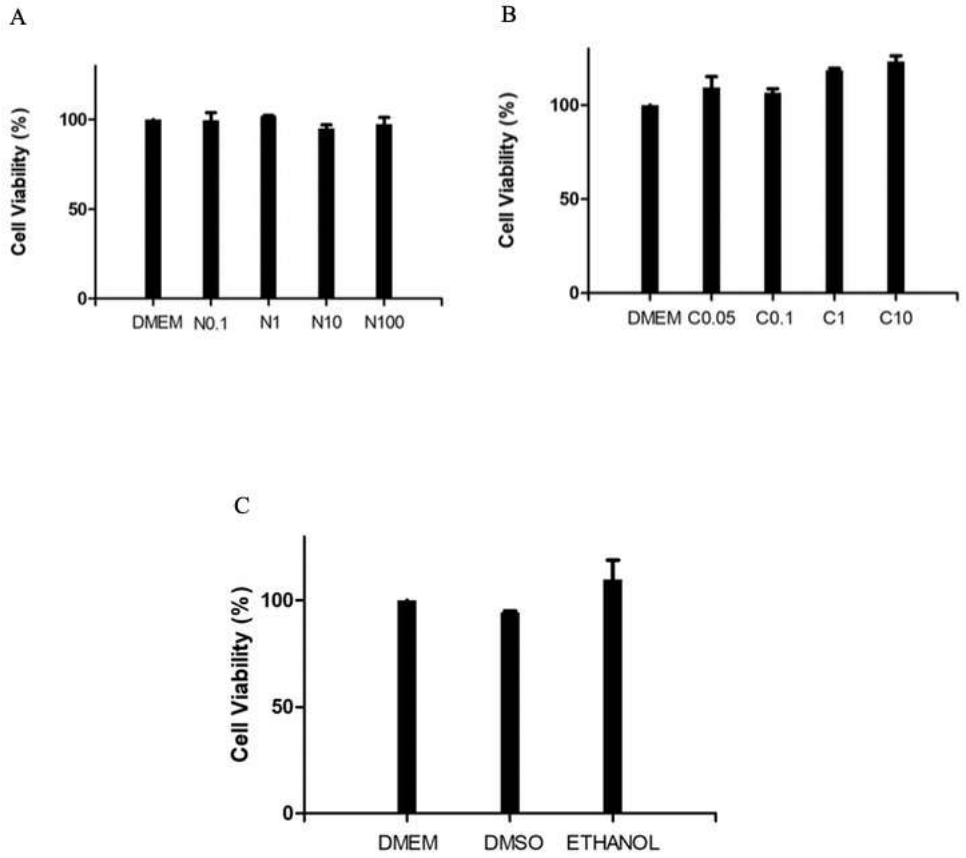
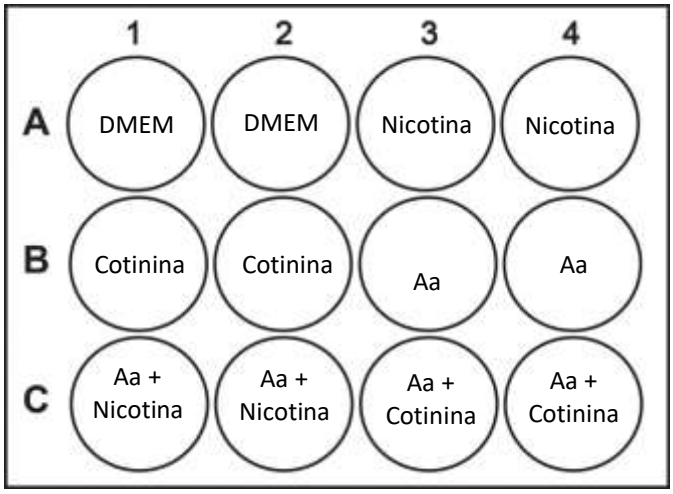
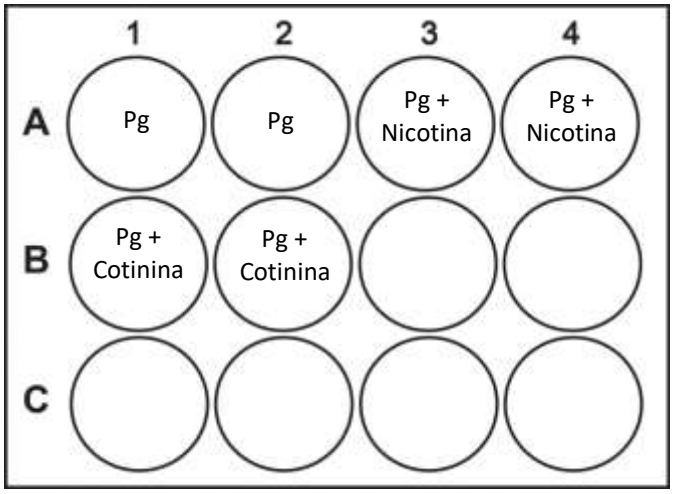


Figure A4 - Esquema representando placas de cultivo celular contendo células HaCat (duplicata) estimuladas com: DMEM; Nicotina; Cotinina; A. Actinomycetemcomitans; A. Actinomycetemcomitans + Nicotina; *P. gingivalis* + Nicotina; *P. gingivalis; P. gingivalis* + Nicotina*; P. gingivalis* + Cotinina.





RT-PCR quantitativo em tempo real

O RNA total foi extraído pelo sistema de coluna de afinidade (kit RNAqueous, Ambion Inc., Austin, TX, EUA). A concentração de RNA foi determinada através do espectrofotômetro de microvolume (NanoView Plus, GE Healthcare, Munique, Alemanha) e 200ng de RNA total foram utilizados para síntese de cDNA usando

síntese hexâmetros aleatoriamente e transcriptase reversa (Kit de Transcrição Reversa de cDNA de alta capacidade, Applied Biosystems, ThermoFisher Científico). PCR em tempo real foi realizado usando TaqMan (TaqMan Fast Advanced Master Mix, Applied Biosystems, ThermoFisher Scientific) e conjuntos pré-projetados e otimizados de iniciadores e sonda (ensaios de expressão do gene TaqMan, Applied Biosystems, ThermoFisher Scientific) específicos para hBD1 (Hs00608345) e hBD2 (Hs00175474). A expressão de GAPDH (Hs02758991) foi utilizada como controle endógeno.

Análise estatística

A análise estatística foi realizada no software GraphPad Prism 7 (San Diego, CA, EUA). Para dados in vivo e in vitro, a distribuição de normalidade foi analisada usando o teste D'Agostino e Pearson. Para distribuição normal, utilizou-se ANOVA de uma via com Tukey (sítios sadios) e teste t não pareado (sítios doentes) intergrupos e teste t para intragrupo. Para dados sem distribuição normal, o teste de Wilcoxon foi realizado para comparação intragrupo, enquanto o teste de Kruskall-Wallis com Dunn (sítios sadios) Mann-Whitney (sítios doentes) foi realizado para intergrupos. Os dados da PCR em tempo real foram analisados pelo teste de Mann-Whitney. Um valor de p menor que 0.05 foi considerado significativo e, abaixo de 0.001, como altamente significativo.

Metodologia - *Publicação 3*

Este trabalho foi realizado em colaboração com integrantes (técnicos, pesquisadores e alunos de doutorado) do laboratório de Odontologia Preventiva da ACTA (Academisch Centrum Tandheelkunde Amsterdam) sob supervisão da Dra. Dongmei Deng, em parceria com Dr. Floris J. Bikker do departamento de Bioquímica Oral da ACTA, quem nos forneceu os peptídeos antimicrobianos.

Desenho do estudo

Saliva de 10 doadores saudáveis foi coletada, misturada, posteriormente aliquotada e armazenadas a -80 ° C até o uso. Os biofilmes foram cultivados por 24

horas e, logo em seguida, tratados com água, LL31 ou D-LL31. Após o tratamento, o meio de cultura para o biofilme foi renovado a cada 24 horas. As amostras foram colhidas dentro de 1 e 5 dias após o tratamento. Essas etapas foram repetidos 3 vezes, resultando em 12 grupos experimentais com 3 biofilmes cada.

O protocolo do estudo foi aprovado pelo Comitê de Ética Médica do VU Centro Médico de Amsterdã (número do documento, 2011/236) Centro Médico Acadêmico (AMC) de Amsterdã. Os doadores foram informados sobre os objetivos e todos os procedimentos que envolviam no estudo e o consentimento informado por escrito foi obtido de todos os participantes.

Síntese dos peptídios

Os peptídeos derivados da catelicidina humana, LL37, LL31 e o enantiômero D-LL31 (Tabela 2) foram sintetizados por síntese de peptídeos em fase sólida usando química de fluoren-9-ilmetoxicarbonil (Fmoc) com o Sintetizador Siro II (Biotage, Uppsala, Suécia) de acordo com o protocolo do fabricante. A purificação por HPLC foi realizada no sistema Dionex Ultimate 3000 (Thermo Scientific, Breda, Holanda). A autenticidade foi confirmada por espectrometria de massa (Bruker Daltonik GmbH, Bremen, Alemanha) exatamente como descrito anteriormente[9,10]. As concentrações molares foram calculadas com base no seu peso.

Tabela A2 - Características dos peptídeos.

*Peptídeo**	*Sequência*	Peso Molecular	Carga**
LL31	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL	3,824	6 +
D-LL31	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL	3,824	6 +

*A pureza dos peptídeos era de pelo menos 95% e a autenticidade dos peptídeos foi confirmada por espectrometria de massa MALDI-TOF.

**Carga calculada em pH neutro.

Cultura *P. gingivalis*

As cepas de *P. gingivalis* encontravam-se armazenadas a -80ºC. Uma alíquota de *P. gingivalis* foi descongelada e plaqueada em placas de ágar sangue anaeróbico

e mantidas anaeorobicamente, (10% CO2, 10% H2 e 80% N2) a 37 ° C por 4 dias. A atmosfera de anaerobiose foi preparada pelo Anoxomat (Advanced Instruments – Norwood, MA, EUA) em jarras anaeróbicas. Logo em seguida, com o auxílio de uma alça de transferência, foi realizada a passagem para o meio BHI enriquecido com hemina e menadiona a 1% e mantido em meio anaeróbico (10% CO2, 10% H2 e 80% N2) a 37 ° C por 24h, até atingir sua fase exponencial.

Formação de biofilme de microcosmo

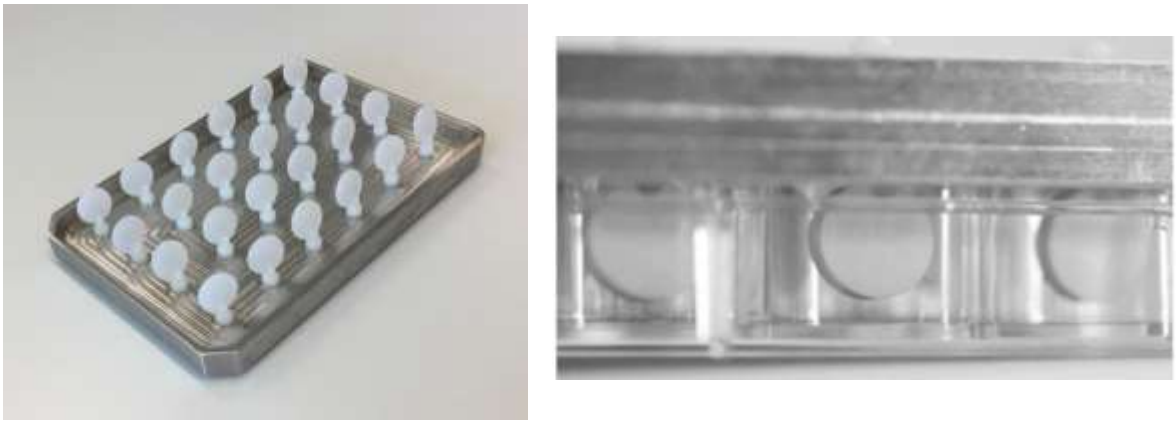
O modelo de biofilme (AAA – Amsterdam Active Attachment model) foi utilizado como descrito anteriormente (Figura A5)[11]. Resumidamente, foram utilizadas placas de cultura de poliestireno de 24 poços (Greiner Bio-One, Alphen a / d. Rijn, Países Baixos). A tampa da placa foi substituída por uma tampa de aço inoxidável personalizada, na qual foram fixados 18 grampos de nylon, permitindo a inserção de substratos, encaixando-se nas cavidades da placa de cultura. Ao transferir a tampa com o substrato para uma nova placa, o meio seria renovado. Neste estudo, discos padronizados de hidroxiapatita de 9,5 mm (HAP) (Himed, Old Bethpage, EUA) foram utilizados como substrato. Depois de montado, o modelo foi autoclavado a 121°C. O meio de cultura Thompson (TpM) foi escolhido como meio para cultivo[11]. Os ingredientes do TpM foram: peptona de bacto protease (0,7%), peptona de tripticase (0,3%), extrato de levedura (0,5%), KCl (0,25%), cloridrato de L-cisteína (0,05%), mucina gástrica de porco (0,25%) e PO4 (0,02 M) em 860 ml de água desmineralizada. Após autoclavado, foi suplementado com uréia (1 mM), arginina (5 mM), lisina (1 mM), glicina (1 mM), hemin-menadiona (1%) e soro fetal bovino estéril inativado pelo calor (10%) (FBS, F4135, Sigma-Aldrich, EUA).

O inóculo foi preparado para 2 grupos diferentes. Primeiramente, foi utilizada a proporção de saliva e meio 1:50 (0,5ml de saliva em 25ml de TpM) (Sal). Da mesma forma, a saliva 1:50 juntamente com cultura de *P. gingivalis* (ATCC33277) (0,5 ml de saliva e DO_{600} final de *P. gingivalis* = 1 em 25 ml de TpM) (SPg). Subsequentemente, 1,3 ml de cada solução de inoculação foram adicionados à 9 poços de 2 placas de 24 poços de poliestireno padrão (placas de múltiplos poços; Greiner Bio One, Alphen aan den Rijn, Países Baixos). Os modelos foram incubados anaerobicamente (10% de CO2, 10% de H2 e 80% de N2) a 37 ° C.

Tratamento dos biofilmes

LL31 e D-LL31 foram reconstituídos em água Milli-Q estéril, resultando em concentração de 1 mM. Após 24 h de inoculação com Sal e SPg, os biofilmes fixados nos discos de HAP foram transferidos para uma nova placa contendo os tratamentos (1,3 ml/poço) e assim, incubada anaerobicamente (10% CO2, 10% H2 e 80% N2) a 37 °C por mais 24 h. Biofilmes em triplicatas foram tratados com LL31 (40μM), D-LL31 (40μM) e água Milli-Q estéril como controle. Após o tratamento de 24 horas, os biofilmes foram transferidos para novo TpM e foram mantidos por mais 24 h para que ocorresse assim sua recolonização. Após 24h, foi realizada a extração do biofilme para avaliação com um 1 dia pós-tratamento, enquanto no biofilme de 5 dias, o meio foi trocado a cada 24 horas por 5 dias até ser realizada uma análise.

Figura A5 – Modelo de biofilme – AAA (Amsterdam Active Attachment model) preparado com discos de hidroxiapatita.



Produção de ácido láctico

Passadas 24 h em novo meio de cultura, a tampa de biofilme pós-tratamento de 1 dia foi colocada em uma placa contendo 1,3 ml de SDM-fosfato tamponado (SDMP) com 0,4% de sacarose por 2 h a 37 °C. A quantidade de lactato no SDMP após a incubação com sacarose foi determinada por ensaio colorimétrico, descrito anteriormente[12]. E o mesmo procedimento foi repetido 4 dias mais tarde para 7 dias de ensaio de lactato de biofilme.

Sonicação do biofilme

Após o ensaio de produção de ácido, cada disco de HAP com biofilme foi removido usando uma pinça estéril e colocado em um frasco estéril contendo 2 ml de meio água de cisteína-peptona (CPW). Todas as amostras foram mantidas em gelo, agitadas por 30 s e dispersas por sonicação por 1 min a 1 s de pulsações na amplitude de 40 W (Vibra Cell; Sonics & Materials Inc., Newtown, CT). Após dispersão dos biofilmes em 2 ml de CPW, alíquotas de 1ml e 0,800 ml foram centrifugadas e o sobrenadante removido. Os pellets das amostras foram armazenados em -80 ° C para posterior isolamento do DNA e ensaio de atividade da peptidase, respectivamente.

Determinação viabilidade celular

Diluições em série dos biofilmes dispersos em CPW foram feitas e plaqueadas em placas de ágar sangue anaeróbico (ABA) para contagens viáveis totais. As placas foram incubadas anaerobicamente (10% CO2, 10% H2 e 80% N2) a 37 ° C por 7 dias, e foi determinado o número de contagens de unidades formadoras de colônias (UFC).

Ensaio de atividade da peptidase

A atividade da dipeptidil peptidase (DPP) - 4 foi determinada pela taxa de clivagem de AMC do substrato sintético Gly-Pro-AMC[13,14]. Os sedimentos de biofilme foram ressuspensos em Tris 10 mM. O ensaio foi realizado em placa de fundo plano de 96 poços em um volume total de 200 µl. Foram adicionados 100 µl de Gly-Pro-AMC em 100 µl das amostras distribuídas em duplicatas na placa, resultando em concentração final de 50 µM de Gly-Pro-AMC. A fluorescência foi medida usando um espectrofotômetro de placa SpectraMax M5 de 96 poços (excitação, 380 nm; emissão, 500 nm; Molecular Devices, Sunnyvale, CA) cineticamente por 2 h, a cada 20 minutos em 37 ° C.

qPCR

O DNA foi isolado de todas as amostras com o Kit de Isolamento Mini DNA AGOWA® mag (LGC Genomics) e as concentrações bacterianas foram determinadas por PCR em tempo real usando o LightCycler® 480 Instrument (Roche Diagnostics, Mannheim, Alemanha).

Concentrações de *P. gingivalis* e bactérias totais foram determinadas por PCR em tempo real. Todos os PCRs foram realizados com 20 µl de volume de reação contendo 10 µl de LightCycler® 480 Probe Master (Roche Diagnostics, Mannheim, Alemanha). A análise das amostras foi submetida ao ciclo de pré-incubação de 95°C por 5 min, seguido de 45 ciclos de quantificação a 95°C por 10 s e a 60 ° C por 20 s. Foram utilizadas diluições em série de dez vezes de DNA homólogo como curvas padrão. Para quantificação, os resultados das amostras foram calculados usando a curva padrão das bactérias correspondentes. Para quantificação da carga bacteriana total, foi utilizada uma curva padrão de E. coli. Água de PCR foi usada como controle negativo em todas as etapas.

Análise estatística

A análise estatística foi realizada pelo GraphPad Prism 7 (San Diego, CA, EUA). A distribuição normal de todos os ensaios foi testada (teste de Kolmogorov-Smirnov). O teste de Mann-Whitney foi realizado para comparação entre amostras. Todos os testes foram realizados com nível de significância de 5%

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ANEXOS

APROVAÇÃO COMITÊ DE ÉTICA

FACULDADE DE ODONTOLOGIA DE ARARAQUARA - UNESP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Comparação da expressão de peptídeos antimicrobianos no fluido gengival crevicular e no tecido gengival de pacientes fumantes e não fumantes com periodontite crônica

Pesquisador: Daniela Leal Zandim-Barcelos
Área Temática:
Versão: 2
CAAE: 01394712.5.0000.5416
Instituição Proponente: Faculdade de Odontologia de Araraquara - UNESP

DADOS DO PARECER

Número do Parecer: 140.369
Data da Relatoria: 07/11/2012

Apresentação do Projeto:
Trata-se de uma solicitação de emenda a um projeto de pesquisa que foi recentemente aprovado pelo CEP.

Objetivo da Pesquisa:
as análises que foram acrescentadas não modificaram o objetivo

Avaliação dos Riscos e Benefícios:
não foram alterados

Comentários e Considerações sobre a Pesquisa:
a emenda foi adequadamente justificada

Considerações sobre os Termos de apresentação obrigatória:
adequados

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:
considero que a emenda deve ser aprovada

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:

Endereço: HUMAITA 1680
Bairro: CENTRO **CEP:** 14.801-903
UF: SP **Município:** ARARAQUARA
Telefone: 1633-0164 **Fax:** 1633-0164 **E-mail:** cep@foar.unesp.br; mnagle@foar.unesp.br

FACULDADE DE ODONTOLOGIA DE ARARAQUARA - UNESP

Não

Considerações Finais a critério do CEP:

As alterações solicitadas foram APROVADAS pelo CEP em sessão de 07/11/2012. Encaminhe-se ao pesquisador para continuidade da pesquisa.

ARARAQUARA, 07 de Novembro de 2012

Assinador por:
Maurício Meirelles Nagle
(Coordenador)

Endereço: HUMAITA 1680
Bairro: CENTRO **CEP:** 14.801-903
UF: SP **Município:** ARARAQUARA
Telefone: 1633-0164 **Fax:** 1633-0164 **E-mail:** cep@foar.unesp.br; mnagle@foar.unesp.br

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

UNESP UNIVERSIDADE ESTADUAL PAULISTA
CÂMPUS DE ARARAQUARA FACULDADE DE ODONTOLOGIA

Pesquisa: "COMPARAÇÃO DA EXPRESSÃO DE PEPTÍDEOS ANTIMICROBIANOS NO FLUIDO GENGIVAL CREVICULAR E NO TECIDO GENGIVAL DE PACIENTES FUMANTES E NÃO FUMANTES COM PERIODONTITE CRÔNICA".

Pesquisador responsável: Dra. Daniela Leal Zandim-Barcelos

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Por este instrumento particular, declaro, para os devidos fins éticos e legais, que eu (nome) _____,

(nacionalidade)_____, (profissão)_____,

portador do R.G. _____, C.I.C. _____,

residente à Rua/ Av. _____, na cidade

de _____, Estado de _____, concordo em participar da pesquisa intitulada: "Comparação da expressão de beta defensinas humanas no fluido gengival crevicular e no tecido gengival de pacientes fumantes e não fumantes com periodontite crônica". A doença periodontal é uma doença que ocorre nos dentes, gengiva e osso, levando a uma inflamação da gengiva, sangramento e perda progressiva do osso em volta dos dentes. O objetivo desta pesquisa é identificar a presença de alterações no tecido gengival de pacientes fumantes que podem estar relacionadas com a maior ocorrência e severidade da doença periodontal em fumantes comparado com não fumantes.

Fui esclarecido que deverei responder a um questionário de saúde, serei submetido a exame clínico de rotina dos dentes e da gengiva, exame radiográfico de todos os dentes e coleta de fluido do sulco gengival (líquido presente entre a gengiva e a superfície do dente). Estes procedimentos serão realizados antes do início do tratamento na Clínica de Periodontia desta faculdade.

Estou ciente de que o procedimento de coleta de fluido gengival não apresenta riscos e não causa prejuízo para o tratamento periodontal. A coleta será realizada pelos pesquisadores que deverão utilizar todos os equipamentos de proteção individual (máscara, gorro, avental, óculos e luvas). Para a realização das tomadas radiográficas, usarei avental de chumbo e colar de tireóide, como medida de proteção. As radiografias ficarão armazenadas juntamente com a ficha clínica no prontuário da Clínica de Periodontia.

Caso seja necessária a realização de algum procedimento cirúrgico durante o meu tratamento periodontal, autorizo a coleta dos fragmentos de tecido gengival que forem removidos durante a cirurgia para utilização nesta pesquisa. Fui informado que este procedimento não irá provocar nenhum dano ou prejuízo para o paciente pois o tecido seria descartado após a cirurgia. Os

cuidados pós-operatórios são de responsabilidade dos alunos da Clínica de Periodontia e dos professores da disciplina. Porém, os pacientes poderão entrar em contato com o pesquisador a qualquer momento, inclusive se houver algum problema após a cirurgia.

Torno ciente que recebi todas as informações sobre a minha participação nesta pesquisa e esclareço que minha adesão ao presente trabalho foi voluntária, sem qualquer tipo de pressão ou coação por parte dos pesquisadores. Fui informado que não terei gastos extras, e que a minha desistência ou não aceitação em fazer parte desta pesquisa não irá interferir no meu tratamento na Clínica de Periodontia. Além disso, tenho plena liberdade para desistir da referida pesquisa, retirando o meu consentimento a qualquer momento, sem sofrer nenhum tipo de penalização.

Autorizo, para os devidos fins, o uso, a divulgação e publicação em revistas científicas, brasileiras ou estrangeiras, dos dados obtidos na pesquisa. Recebi a garantia do sigilo de minha identidade, assegurando a minha privacidade.

Caso haja qualquer dúvida sobre a minha participação na pesquisa, terei plena liberdade de contactar o pesquisador responsável, Dra. Daniela Leal Zandim-Barcelos, pelo telefone: (16) 3301-6376. Além disso, estou ciente que possuo plena liberdade em consultar o Comitê de Ética em Pesquisa, para qualquer informação adicional em relação à pesquisa da qual participo, pelo telefone (16) 3301-6432 ou (16) 3301-6434.

Desta forma, uma vez tendo lido e entendido tais esclarecimentos e, por estar de pleno acordo com o teor do mesmo, dato e assino esse Termo de Consentimento.

Araraquara, ____ de _____ de 20___.

_____

Assinatura do Paciente

_____

Assinatura do Pesquisador Responsável

Dra. Daniela Leal Zandim-Barcelos

Não autorizo a publicação deste trabalho

pelo prazo de 2 anos após a data de defesa

Araraquara, 16 de março de 2020

Kahena Rodrigues Soldati