UNIVERSIDADE DE TAUBATÉ
Alexandre Lustosa Pereira

AVALIAÇÃO DA EFICÁCIA TERAPÊUTICA PERIODONTAL POR MEIO DE PARÂMETROS CLÍNICOS, MICROBIANOS E IMUNOLÓGICOS

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Tese apresentada para a obtenção do Título de Doutor pelo curso de Doutorado em Odontologia do Departamento de Odontologia da Universidade de Taubaté.
Área de Concentração: Periodontia
Orientador: Prof. Dr. José Roberto Cortelli

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A Deus, por sua infinita e sempre suficiente graça.

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Amo você, e amarei eternamente!

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Pereira AL. Avaliação da eficácia terapêutica periodontal por meio de parâmetros clínicos, microbianos e imunológicos [Tese de doutorado]. Taubaté: Universidade de Taubaté, Departamento de Odontologia, 2012. 133f.

RESUMO

Objetivo: o presente estudo prospectivo avaliou a presença de microrganismos periodontopatogênicos, os níveis salivares de arginase e de HBD-2 em indivíduos com gengivite e periodontite tendo como controle indivíduos periodontalmente saudáveis, correlacionando-os aos respectivos parâmetros clínicos. Também foi avaliada expressão gênica do PAR_2 crevicular em indivíduos saudáveis e com periodontite. **Método:** Inicialmente, foram avaliados 89 indivíduos sem doenças sistêmicas, nunca fumantes, sendo 31 saudáveis (média de idade 25,06 ± 5,97), 27 com gengivite (média de idade 33,22 ± 12,09) e 31 com periodontite (média de idade 52,16 ± 11,54), todos submetidos à terapia periodontal não cirúrgica. Coleta salivar para avaliação dos níveis de arginase (quantificada por meio de espectrofotometria) foi realizada no início do tratamento em todos os indivíduos, e naqueles com gengivite e periodontite respectivamente em 30 e 50 dias pós-tratamento. Avaliação clínica de profundidade de sondagem, perda de inserção clínica e índices de placa e gengival e microbiana (*Campylobacter rectus, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tanerella forsythia, Treponema denticola* e *Prevotella intermedia)* foram também avaliados nos mesmos tempos. Dentre os 89 indivíduos, amostras do fluido gengival foram coletadas em 10 indivíduos saudáveis e 10 com periodontite para mensuração da atividade do PAR_2 crevicular por meio de RT-PCR. Como esta relação foi positiva, foi verificada a quantidade de HBD-2 salivar — por meio de ELISA — de todos os 89 indivíduos e sua relação com os parâmetros clínicos e microbiológicos. A significância de todas as relações e quantificações foi analisada por meio de testes estatísticos apropriados. **Resultados:** foi observada uma melhora estatisticamente significativa dos parâmetros clínicos e microbianos após o tratamento periodontal. A arginase salivar estava significativamente mais elevada nos indivíduos com periodontite em relação àqueles com gengivite, e nestes em relação aos saudáveis. O tratamento periodontal promoveu melhora dos indivíduos doentes, cujos parâmetros avaliados tornaram-se estatisticamente semelhantes aos dos saudáveis. Houve maior atividade do PAR_2 nos 10 indivíduos com periodontite em relação aos saudáveis e, após o tratamento, houve uma redução estatisticamente significativa deste parâmetro. Por fim, foram observados níveis estatisticamente mais elevados de HBD-2 salivar nos indivíduos com periodontite comparados àqueles com gengivite e aos saudáveis. Não foi possível observar uma correlação entre HBD-2 salivar e os microrganismos analisados. **Conclusões:** com base nos resultados observados, podemos concluir que: a arginase salivar está significativamente aumentada nos indivíduos periodontalmente comprometidos em relação aos saudáveis; o tratamento periodontal promoveu melhora dos indivíduos doentes em relação aos parâmetros avaliados; indivíduos com periodontite têm maior expressão gênica do PAR_2 do que aqueles saudáveis e o tratamento tornou esta expressão semelhante nos dois grupos; indivíduos com periodontite têm níveis estatisticamente mais significativos de HBD-2 salivar do que aqueles saudáveis e aqueles com gengivite; a saliva parece ser uma ferramenta útil para o diagnóstico periodontal e para o monitoramento da eficácia do tratamento periodontal.

Palavras-chave: Periodontite; Gengivite; Bactérias; Arginase; Receptor PAR-2; beta-Defensinas.

Pereira AL. Assessment of periodontal therapeutic efficacy by clinical, microbial and immunological parameters [Tese de doutorado]. Taubaté: Universidade de Taubaté, Departamento de Odontologia, 2012. 133f.

ABSTRACT

Objectives: This prospective study evaluated the presence of periodontopathogenics microorganisms, as it also examined the salivary levels of arginase and HBD-2 from subjects with gingivitis and periodontitis and periodontally healthy subjects as controls, correlating them to relevant clinical parameters. The gene expression of PAR2 crevicular in healthy subjects and periodontitis was also assessed. **Methods:** Initially, 89 individuals without systemic diseases, who were never smokers, were evaluated. Out of the 89, 31 were healthy subjects (average age 25.06 ± 5.97), 27 have gingivitis (average age 33.22 ± 12.09) and 31 were with periodontitis (average age 52.16 ± 11.54), all underwent nonsurgical periodontal therapy. Saliva was collected for assessing levels of arginase at baseline in all subjects (quantified by spectrophotometry), and repeated on those with gingivitis and periodontitis respectively at 30 and 50 days post treatment. Clinical evaluation of probing depth, clinical attachment loss, both plaque and gingival index as well as microbial evaluation (*Campylobacter rectus, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tanerella forsythia, Prevotella intermedia* and *Treponema denticola*) were also assessed at the same time. Among the 89 individuals, gingival fluid samples were collected from 10 healthy ones and from 10 with periodontitis to measure crevicular PAR_2 activity by RT-PCR. As the results came out positive, the amount of HBD-2 salivary was tested by ELISA for all 89 subjects assessing its relationship with clinical and microbiological parameters. The significance of all relationships and quantifications were analyzed using appropriate statistical tests. **Results:** we observed a statistically significant improvement of clinical and microbial parameters after periodontal treatment. Salivary arginase was significantly higher in subjects with periodontitis than in those with gingivitis, and those with gingivitis had higher results than the healthy ones. Periodontal treatment promoted improvement of the non-healthy individuals whose parameters became statistically similar to the healthy ones. There was a greater PAR_2 activity in 10 individuals with periodontal disease compared to healthy ones, and after treatment the results showed a statistically significant reduction in this parameter. Finally, we observed statistically higher levels of salivary HBD-2 in individuals with periodontitis compared to both those with gingivitis and those individuals in the healthy group. It has not been possible to observe a correlation between HBD-2 and salivary microorganisms analyzed. **Conclusion:** Based on the observed results, we can conclude that salivary arginase is significantly increased in periodontally compromised individuals relative to the healthy ones; periodontal treatment promoted improvement of individuals in relation to the assessed parameters; individuals with periodontitis have higher gene expression of PAR_2 than those healthy and the periodontal treatment brought similar results to both groups; individuals with periodontitis have statistically more significant levels of salivary HBD-2 than those with healthy gums and gingivitis; and, finally, saliva, besides being useful for periodontal diagnosis, appears to be also helpful for monitoring efficacy of periodontal treatment.

Keywords: Periodontitis; Gingivitis; Bacteria; Arginase; Receptor, PAR-2; beta-Defensins

LISTA DE ABREVIATURAS

CAL — Nível clínico de inserção

DNA — Ácido desoxirribonucleico

ELISA — Enzyme linked immunosorbent assay

G — indivíduos com gengivite

GAPDH — Gliceraldeído 3-fosfato-desidrogenase

GCF — Fluido gengival crevicular

GI — Índice gengival

H — indivíduos saudáveis

HBD — Beta-defensina humana

PI — Índice de placa

PS — Profundidade de sondagem

NO — Óxido nítrico

NOS — Óxido nítrico sintetase

P — indivíduos com periodontite

PAR_2 — Receptor 2 ativado por protease

PCR — Reação em cadeia da polimerase

PD — Profundidade de sondagem

RNAm — Ácido ribonucleico mensageiro

RT-PCR — Reação em cadeia da polimerase com transcrição reversa

SAA — Atividade de arginase salivar

TNFα — Fator alfa de necrose tumoral

SUMÁRIO

1 INTRODUÇÃO

A doença periodontal é uma das enfermidades mais comuns da cavidade bucal. Sua natureza é infecciosa, essencialmente provocada por bactérias específicas que induzem, inicialmente, a uma inflamação gengival (gengivite), caracterizada clinicamente pela presença de sangramento à sondagem e pela ausência de perda de inserção. A gengivite pode evoluir para uma periodontite em função do hospedeiro e de alguns fatores microbianos. A periodontite, por sua vez, caracteriza-se clinicamente por um aumento da profundidade de sondagem, perda de inserção e destruição do osso alveolar de suporte. Esta progressão pode ocorrer de forma contínua ou por surtos episódicos de atividade destrutiva (Löe et al., 1986; Jeffcoat & Reddy, 1991).

A periodontite pode ser classificada em crônica e em agressiva. A crônica é caracterizada por ter uma evolução lenta, pela quantidade de fatores irritativos locais compatíveis com a severidade da destruição periodontal e por acometer mais frequentemente indivíduos adultos. Já a periodontite agressiva apresenta uma rápida progressão da perda dos tecidos de suporte, caracterizando-se por uma incompatibilidade entre a presença de fatores irritativos locais e o grau de destruição periodontal que ela provoca, por uma tendência familiar e por acometer predominantemente indivíduos mais jovens (Armitage, 1999).

Um dos grandes enfoques da periodontia moderna é a prevenção, que é buscada tanto na perspectiva clínica — tentando estabelecer métodos adequados de controle de biofilme, impedindo, assim, a proliferação do biofilme dentário — quanto na perspectiva diagnóstica, buscando formas apropriadas de se prever não

apenas o surgimento, mas também o momento em que esta doença poderá evoluir (Nomura et al., 2012). Quanto mais precocemente esta previsão acontece — e a terapêutica adequada é instituída — possivelmente, menor destruição tecidual o paciente apresentará (Kirkwood et al., 2007).

Para isso, alguns investigadores têm buscado métodos laboratoriais e marcadores mais eficientes que permitem avaliar, com maior precisão, se a doença está ou não em atividade destrutiva ou o momento em que esta atividade está prestes a ocorrer (Sanz et al., 2007; Nomura et al., 2012).

Outra aplicação destes métodos é o monitoramento de indivíduos tratados, em que é feita a quantificação destes marcadores na pré e na pós-terapia na tentativa de verificar se o indivíduo passou de uma condição de doença para uma condição de saúde periodontal. Diversas substâncias podem ser utilizadas para este fim; elas podem ser encontradas no fluido crevicular presente no sulco gengival ou na bolsa periodontal e na saliva de indivíduos afetados pela doença periodontal, podendo ser derivadas tanto dos microrganismos quanto do hospedeiro (Kaufman & Lamster, 2000; Armitage, 2004; Gheren et al., 2007; Patil & Patil, 2011; Pfaffe et al., 2011; Liu & Duan, 2012; Parwani et al., 2012).

A saliva apresenta muitos componentes que podem ser úteis como marcadores bioquímicos que poderiam fornecer uma abordagem não invasiva e de boa relação custo/benefício para o monitoramento de doenças da cavidade bucal (Kaufman & Lamster, 2000; Cortelli et al., 2009; Patil & Patil, 2011; Pfaffe et al., 2011; Liu & Duan, 2012; Parwani et al., 2012).

Um desses componentes encontrados na saliva é a arginase, uma enzima chave no ciclo da uréia que tem como função essencial eliminar a amônia, que é tóxica ao organismo. Ela atua sobre a arginina produzindo uréia e ornitina. Outra

enzima — a óxido nítrico sintetase — compete pelo mesmo substrato, a arginina, para produzir óxido nítrico.

Nos tecidos periodontais, a arginase é produzida por macrófagos ativados (Salimuddin et al., 1999), podendo essa produção ser estimulada pela presença de bactérias periodontopatogênicas (Sosroseno et al., 2006; Uematsu et al., 2006). Pode ser parte de um mecanismo natural de defesa não específico contra bactérias patogênicas ou, alternativamente, pode contribuir para a destruição tecidual na periodontite — em quantidade excessiva (Ugar-Çankal & Özmeriç, 2006). Desta forma, a arginase tem um importante papel na doença periodontal, pois o aumento de sua atividade causa uma diminuição na síntese de óxido nítrico (NO), o que diminui as propriedades antibacterianas da saliva e torna os tecidos periodontais mais susceptíveis aos periodontopatógenos (Özmeriç et al., 2000). Gheren et al. (2007) concluíram que a arginase encontra-se em níveis mais elevados na saliva de indivíduos com periodontite do que em indivíduos saudáveis, e que o tratamento periodontal básico reduziu significativamente os seus níveis salivares, assim como os níveis clínicos da doença.

Também podem ser encontradas na saliva as chamadas beta-defensinas (HBDs). São pequenos peptídeos catiônicos que criam poros no interior da membrana bacteriana, levando-a à morte. Elas são produzidas no corpo principalmente por células epiteliais, e desempenham um papel importante na cavidade bucal como primeira linha de defesa contra micro-organismos Gram negativos e Gram positivos. O epitélio bucal expressa constitutivamente HBD-1, -2 e -3. (Bissel et al., 2004; Dommisch et al., 2005). No entanto, na presença de inflamação, uma sobre-expressão destes peptídeos pode ocorrer na cavidade bucal (Mizukawa et al., 1999; Dommisch et al., 2005; Vardar-Sengul et al., 2007; Kuula et

al., 2008). Dommisch et al. (2005) mostraram que, em tecidos gengivais saudáveis, há similaridade na expressão de RNAm de HBD-1 e -2. Por outro lado, a expressão de HBD-2 foi estatisticamente maior do que a de HBD-1 nos indivíduos com gengivite e periodontite.

Em um estudo feito por Vardar-Sengul et al. (2007), foi demonstrado que a expressão de RNAm de HBD-1 e -2 foi significativamente maior no grupo com periodontite crônica do que no grupo saudável (controle). Além disso, Kuula et al. (2008) demonstrou que a expressão de HBD-2 foi menor em indivíduos periodontalmente saudáveis do que em indivíduos com os tecidos periodontais e perimplantares inflamados. Analisados em conjunto, esses estudos sugerem um papel potencialmente importante das defensinas na resposta do hospedeiro à infecção por periodontopatógenos. Além disso, recentemente foi demonstrado, in vitro, o papel importante da HBD na regeneração periodontal por meio da promoção da proliferação de fibroblastos (Wang et al., 2011).

A modulação da expressão das HBDs pode ser orquestrada por receptores presentes na membrana celular, que reconhecem certos modelos moleculares associados a certos patógenos, incluindo *Aggregatibacter actinomyctemcomitans, Porphyromonas gingivalis* e *Fusobacterium nucleatum*. Alguns estudos anteriores (Chung et al., 2004; Dommisch et al., 2007) demonstraram que gengipaína — uma protease semelhante à tripsina — produzida por *P. gingivalis* sobrerregulam a expressão do RNAm da HBD-2 por meio do receptor ativado por protease 2 (PAR_2) em células epiteliais gengivais. O PAR_2 faz parte de uma família de proteínas G conjugadas, que são receptores de domínio heptatransmembrânicos, e sua ativação ocorre por clivagem proteolítica da porção N-terminal por proteinases séricas como tripsina, triptase mastocítica, proteinase

neutrofílica 3, fator tecidual/fator VIIa/fator Xa, proteinase sérica tetramembrânica 1 e gengipaína (Lourbakos et al., 2001; Vergnolle et al., 2001). Holzhausen et al. (2010) mostraram que o PAR_2 está sobrerregulado na periodontite crônica humana se comparado a controles saudáveis. Além disso, a presença de *P. gingivalis* na bolsa periodontal está associada à sobrerregulação da expressão gênica do PAR_2 e um maior perfil pró-inflamatório relacionado com uma destruição periodontal avançada (Fagundes et al., 2011).

A identificação de microrganismos em indivíduos com doença periodontal também tem sido recomendada, tanto para fins de diagnóstico quanto para fins de monitoramento. O conhecimento das espécies envolvidas na doença periodontal traz luz sobre a patogênese da doença periodontal, e faz com que ela seja adequadamente tratada (Nishihara & Koseki, 2004).

Dessa forma, os objetivos deste estudo são: (1) comparar a atividade de arginase salivar dos indivíduos saudáveis, à dos com gengivite e à dos com periodontite — antes e após o tratamento periodontal não cirúrgico — com os respectivos perfis clínicos e microbiológicos para as seguintes bactérias: *Campylobacter rectus, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Porphyromonas gingivalis, Tanerella forsythia e Treponema denticola*; (2) avaliar a expressão do RNAm do Receptor 2 Ativado por Protease (PAR_2) no fluido gengival e os níveis salivares de beta-defensina-2 (HBD2) em indivíduos saudáveis e em indivíduos com periodontite crônica; e (3) comparar os níveis de concentração proteica salivar de HBD2 em indivíduos saudáveis, à dos com gengivite e à dos com periodontite, e correlacionar estes níveis com as bactérias *Campylobacter rectus, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Porphyromonas gingivalis, Tanerella forsythia e Treponema denticola.*

2 CAPÍTULOS

2.1 CAPÍTULO A

Reduction of salivary arginine catabolic activity trough periodontal therapy.

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ABSTRACT

Objectives: Salivary enzymes may be used to diagnose periodontal conditions. Salivary arginase activity (SAA) is related to susceptibility to bacterial infection. Therefore, the aim of this controlled interventional study was to determine the SAA before and after non-surgical periodontal therapy. **Materials and Methods:** 89 subjects were selected: 31 periodontal health (H), as control; 27 gingivitis (G), and 31 chronic periodontitis (P). Plaque and gingival indices, probing depth and clinical attachment level were monitored. The presence of *Campylobacter rectus, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tanerella forsythia, Treponema denticola*, and *Prevotella intermedia* was evaluated by PCR. Salivary total protein level and SAA were also established by spectrophotometry. Clinical and arginase data were analyzed using Wilcoxon, Mann-Withney and Kruskal-Wallis tests ($p<0.05$). For microbial data, we used Chi-square. Pearson's correlation was also used between each parameter evaluated. **Results:** After therapy, due to a significant reduction in SAA, the values observed for G and P groups were similar to those found to H group. Interestingly, after therapy, SAA followed the same positive pattern showed by the overall improvement of clinical parameters (G and P mean values: pre > post-therapy) and by the reduction of target pathogens (G group *T.forsythia:* pre > post-therapy; P group *P.gingivalis, T.denticola, P.intermedia* and *T.forsythia:* pre > post-therapy). **Conclusion:** Based on the reduction of SAA after therapy, in accordance to the expected reduction in clinical and microbiological parameters, we concluded that SAA has a potential to serve as a reliable to the therapeutic response of chronic periodontitis subjects treated by non-surgical periodontal therapy.

Keywords: Periodontitis; Gingivitis; Arginase; Therapy.

1 INTRODUCTION

Although gingivitis and periodontitis are infectious diseases mainly associated with oral biofilms showing specific microbial characteristics (such as presence, proportion and levels of selected species), the first is limited to the soft tissue surrounding the teeth while the second also destroys the connective tissue and the alveolar bone that supports the teeth[1]. Microorganisms are clearly related to gingivitis as well as periodontitis. However, clarifying the immunopathogenic mechanisms involved in the disease process can be difficult, mainly due to the complexity of the oral microbiota and the host response that are critical in determining the outcome of this bacterial challenge[2]. Even today, gingivitis is highly prevalent affecting 50% to 90% of adults worldwide[3]. Although numerically lower, the prevalence rate of moderate to advanced periodontitis is also considered high[4].

Although for many years periodontal therapy focused on the reduction and even the eradication of target pathogens, today its target is to provide a host-compatible microbiota through a long-term modified relation between pathogenic and commensal bacterial species[5]. Fortunately, mechanical therapy has been demonstrated to be effective in providing, at least short-term microbial changes for the majority of patients with mild to moderate chronic periodontitis[6]. Regarding clinical parameters, subgingival scaling and root planing may result in resolution of inflammation, reduction in probing pocket depth, and gain of clinical attachment level[7-13].

Currently, in addition to clinical and microbiological examinations, the analysis of salivary components from patients has been widely used as a research

tool for the diagnosis of many oral diseases[14-16]. Due to researchers efforts, salivary diagnosis has been improving and may be more widely used in dental practice in the future since it has shown promising results in academic studies[17-20].

Arginase is an oral peptide present in saliva that plays an important role in periodontal disease as its increased activity causes a decrease in the synthesis of nitric oxide, which reduces the antibacterial properties of saliva making periodontal tissue more susceptible to periodontopathogens infection[21,22]. This enzyme plays an important role in the body's immune response, as well as in tissue healing. Jacobsen et al.[23] demonstrated that it is released from granules of activated neutrophils in areas with infection in order to modulate the immune response and promote tissue regeneration. Considering that arginase is an agent with increasing evidence in the pathogenesis of different diseases, King et al.[24] proposed its role as a marker of respiratory allergic response.

The assumption that arginase activity could represent a predictable tool to evaluate the response to periodontal therapy is based on the previous findings observed by our group[25]. However, scientific evidence is still limited. Also, there is no threshold to determine up to which level arginase activity is beneficial to periodontal tissues and at which level the harmful effects begin. Therefore, the present study used recognized clinical and microbiological parameters to validate arginase activity as a useful indicator of the response to periodontal therapy. Thus, the aim of this controlled interventional study was to compare the salivary arginase activity (SAA) with clinical and microbiological profiles of gingivitis and chronic periodontitis subjects treated by non-surgical periodontal therapy.

2 MATERIALS AND METHODS

2.1 Population

Data and personal information related to medical and dental histories of the subjects were obtained. They signed the informed consent form which was previously approved by the Institutional Committee on Research Involving Human Subjects of the University of Taubate.

A total of 89 subjects were included in this survey. They were allocated in three different groups: 31 subjects (mean age 25.06 ± 5.97) in healthy group (H) (fig. 1), 27 subjects (mean age 33.22 ± 12.09) in gingivitis group (G) (fig. 2) and 31 subjects (mean age 52.16 ± 11.54) in periodontitis group (P) (fig. 3).

Participants included in the present interventional study were recruited from Goiânia City, Brazil. To be included subjects had to be in good general health and have at least 14 natural teeth. Individuals were excluded if they had the following conditions: (a) used antibiotics or anti-inflammatory drugs (steroids or not) in the six months preceding the beginning of the study; (b) had systemic diseases or conditions that could influence the pathway of periodontal disease; (c) were smokers or former smokers; and (d) had undergone periodontal treatment during the six months prior to baseline.

2.2 Periodontal examination and criteria for disease definition

At baseline, subjects received a full-mouth examination to assess the dichotomous Plaque Index (PI) and Gingival Index (GI). In addition, probing depth (PD) and clinical attachament level (CAL) were measured at 6 sites per tooth using a manual periodontal probe (PCPUNC 15 Hu-Friedy Mfg. Co., Inc., Chicago, IL).

We considered subjects to have gingivitis (G-group) when they showed PD < 4 mm, presence of gingival redness and bleeding on probing in more than 25% of sites, according to the criteria established by Lopez et al.[26] We considered subjects to have periodontitis (P-group) when they showed at least four teeth with one or more sites with PD ≥ 4mm and CAL ≥ 3 mm[26]. In addition, individuals showing sites with no CAL, PD ≤ 3mm and absence of inflammatory levels indicating gingivitis[26] were considered periodontally healthy subjects and were defined as control group (H-group).

All clinical periodontal parameters were recorded again on the 30th day after completion of periodontal therapy for G-group and on the 50th day after completion of periodontal therapy for P-group.

2.3 Saliva sampling

Saliva samples were collected from all the study population. Samples from subjects in H-group were collected only once, at baseline, whereas from subjects in

G-group and P-group they were collected twice: at baseline, previous to the beginning of periodontal therapy; on the 30th (G-group) and 50th day (P-group) after the completion of periodontal therapy. No food or drink was permitted for two hours prior to collection. During the sample collection, volunteers remained in a seated position, with their head tilted forward (approximately 45°). The procedure was accomplished in a quiet and well-ventilated room[27]. Immediately before sampling, subjects rinsed their mouth with water and chewed a piece of sterilized rubber tourniquet to stimulate saliva production, which was collected to yield a total 2.0ml. Samples were centrifuged for 10 min at 15,000×g at 4°C, and the supernatants were immediately stored at −80°C.

2.4 Biochemical Procedures

Salivary arginase activity was determined by measuring the formation of L-ornithine from L-arginine, according to the protocol established by Iyamu[28] with modifications. One milliunit (mIU) of arginase was defined as the amount of enzyme that produces 1nmol of L-ornithine per minute at 37°C. The standard reaction mixture (250μl) contained 60mM carbonate buffer pH 9.8, 20mM L-arginine pH 9.8, and 1mM $MnCl_2$, and the reactions were initiated by adding the 20μl of salivary samples. The reaction was stopped with the addition of 250μl of hydrochloric acid 0.75M, followed by centrifugation at 10,000xg for 5 minutes at room temperature. Aliquots of 100μL of the supernatant were transferred to microtube, followed by the addition of 200μL of ninhydrin 6% (w/v) in 2-metoxyethanol. The samples were preheated at 100°C in a

thermo block (Thermomix, Eppendorf, Westbury, NY, USA) for 25 min and allowed to cool to room temperature. Aliquots of these samples were transferred to 96-well polystyrene microplates and were read using a microplate reader (BMG Labtech) at 515µnm. Each sample was analyzed in duplicate.

Salivary protein concentration was determined by a colorimetric protein test based on the method of Bradford[29] with bovine serum albumin as the standard. Arginase activities were normalized to total protein concentration and expressed by mIU/mg protein.

2.5 Microbiological Examinations

Microbial samples were obtained from four periodontal sites, one in each quadrant, based on Cortelli et al.[30] For H-group mesial sites of first molars were sampled, for G-group sites with bleeding on probing and for P-group bleeding sites showing the highest PD values. Each selected tooth was isolated with sterile cotton rolls and the supragingival plaque was removed with sterile curettes. A sterilized #30 paper point (Tanari, Tanariman Industrial Ltda., Manacapuru, Brazil) was carefully inserted to the depth of the sulcus or periodontal pocket, and kept in position for 60 s. The pooled subgingival samples were stored at -80ºC in microtubes containing 1ml of reduced Ringer's solution (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom).

The presence of *C. rectus, P. gingivalis, T. forsythia, P. intermedia, T. denticola* and *A. actinomycetemcomitans* was established by a polymerase chain reaction (PCR) using specific primers [*P. gingivalis*, sense:

5'-AGGCAGCTTGCCATACTGCGG-3', and antisense: 5'-ACTGTTAGCAACTACCGATGT-3' (product size: 404bp); *T. forsythia*, sense: 5'-GCGTATGTAACCTGCCCGCA-3', and antisense: 5'-TGCTTCAGTGTCAGTTATACCT-3' (product size: 641bp); *C. rectus*, sense: 5'-TTTCGGAGCGTAAACTCCTTTTC-3', and antisense: 5'-TTTCTGCAAGCAGACACTCTT-3' (product size: 598bp); *P. intermedia,* sense: 5'-TTTGTTGGGGAGTAAAGCGGG-3', and antisense: 5'-TCAACATCTCTGTATCCTGCGT-3', (product size: 575bp); *T. denticola,* sense: 5'-TAATACCGAATGTGCTCATTTACAT-3', and antisense 5'-TCAAAGAAGCATTCCCTCTTCTTCTTA-3' (product size 316bp); and *A. actinomycetemcomitans*, sense: 5'-AAACCCATCTCTGAGTTCTTCTTC-3' and antisense: 5'-ATGCCAACTTGACGTTAAAT-3' (product size: 550bp)] under standard conditions. The DNA was extracted (InstaGene Bio-Rad Laboratories Inc., Hercules, CA, USA) and the PCR was performed in a thermocycler (Eppendorf, Westbury, NY, USA) as follows: one cycle 94ºC for 5 min, 35 cycles 94ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min, and a final extension of 72ºC for 5 min.

After electrophoresis in 1.5% agarose gel, the DNA fragments were stained with SYBR Safet (Carlsbad, CA, USA) and visualized by UV illumination. The PCR amplicons were compared with both positive and negative controls. A molecular weight marker (Ladder 100) was added in each set. The detection limit of the PCR method was ≤ 50 colony forming units.

2.6 Periodontal Therapy

After the clinical evaluation, bacterial and saliva sampling at baseline, each subject received oral hygiene instructions and a standard kit for mechanical supragingival plaque control. G-group subjects additionally received professional dental prophylaxis. P-group subjects were treated with scaling and root planning, under local anesthesia, in a total of four clinical visits. On the 5th visit they also received professional dental prophylaxis.

Similarly to clinical monitoring, microbial and salivary samples were taken once for H-group and twice for G and P-groups. Thirty days after initial therapy (G0: pre-treatment and G1: post-treatment) for G-group and 60 days for P-group (P0: pre-treatment and P1: post-treatment).

2.7 Statistical Analysis

Statistical analysis was performed using specific software (Bio Estat 5.0 and SPSS 13.0). For clinical and arginase data, intra-group analysis was used Wilcoxon test. For the inter-group analysis, we used Mann-Whitney test for two groups and Kruskal-Wallis test for three or more groups were performed. For microbial data, all comparisons were done using *Chi-square* test. The correlation between each pair of clinical and/or salivary parameters (pre- and post-therapy) was determined by Pearson's Correlation Coefficient.

3 RESULTS

Regarding the clinical parameters evaluated, we observed an intra-group significant reduction in plaque index and gingival index scores for both G- (pre- vs. post-therapy) and P-groups (pre- vs. post-therapy). A significant decrease in mean PD and a significant improvement in CAL were also observed in subjects in P-group after therapy (Table 1).

At baseline, *T. forsythia* was more prevalent in G- and P-groups when compared to H-group ($p < 0.05$), showing significantly higher prevalence in P-group in comparison to G-group ($p < 0.05$). *P. gingivalis, P. intermedia* and *T. denticola* were more highly prevalent in P-group than in G-group. There was no statistical difference between G- and H- group considering these same bacterial species ($p > 0.05$). After treatment, only *T. forsythia* showed a higher prevalence in G- and P-groups than in H-group ($p < 0.05$). All other bacteria showed statistically similar prevalence among groups (Fig. 4).

There was no statistically significant reduction ($p > 0.05$) of microorganisms after treatment in the G-group. Opposite to that, there was a statistically significant reduction ($p < 0.05$) of *P. gingivalis, P. intermedia, T. forsythia* and *T. denticola* after periodontal therapy in the P-group. However, periodontal therapy did not change the prevalence of *C. rectus* and *A. actinomycetemcomitans* in the P-group (Fig. 5).

The levels of arginase activity were statistically higher ($p < 0.05$) for groups G- (88.56mIU/mg) and P- (185.63mIU/mg) when compared to H-group

(48.66mIU/mg) at baseline (Fig. 6A). After treatment, inter-group comparisons revealed no differences among groups (Fig. 6B).

When pre- and post-therapy data was compared a significant reduction ($p < 0.05$) in the levels of arginase activity was observed in P-group (87.14mIU/mg), which did not occur in G-group (Fig. 6C). The total protein was significantly reduced in the P group after treatment ($p < 0.05$), which was not observed on G-group (Fig. 6D).

Finally, we proposed a Pearson correlation between all parameters evaluated in this study in the pre- and post-therapy. The correlations associated with a significant *p* value are shown in Table 2.

4 DISCUSSION

Salivary constituents represent an important tool in the diagnosis of several oral diseases. For periodontal diagnosis, saliva is useful because it is a body fluid rich in locally-produced microbial and host factors. Also, saliva is permanently available and ready for collection. Then, our study evaluated the response to periodontal therapy based on the activity of salivary arginase. Additionally, we compared its pre- and post-therapy activity using as standard clinical and microbiological parameters of gingivitis and chronic periodontitis subjects.

In the present study, as traditionally observed, clinical parameters distinguished gingivitis from periodontitis subjects and diseased from periodontally healthy subjects. However, it is well known that clinical parameters show cumulative effects of periodontal disease[31]. For this reason, for many years, periodontics has been looking for periodontal indicators that better predict the disease activity[32]. In the present study, the differences in prevalence ratio of target periodontal species differed periodontitis from gingivitis and healthy subjects. However, this same parameter failed in differentiating gingivitis from periodontally healthy subjects. It had occurred because we investigated bacteria related to periodontitis and not to gingivitis.

The most important result of our study was that, at baseline, we observed that SAA was statistically higher on periodontitis group than on gingivitis group; and on gingivitis group this activity was statistically higher than on healthy group. Likewise, after periodontal treatment, gingivitis and periodontitis group became similar to healthy group. Therefore, similarly to clinical parameters, SAA was able to

indicate each different periodontal status. This finding may be considered promising because arginase is directly related to disease activity[33]. Due to a competition between arginase and nitric oxide synthetase (NOS) for the same substrate, an increase in arginase production and mainly in its activity is associated with a higher host susceptibility to bacterial infection. The valuable use of SAA as a tool for the initial diagnosis was reinforced by the positive correlation between periodontal pocket depth and arginase observed here at baseline (table 2). Ozmeriç et al.[21] also observed a higher SAA in periodontitis in comparison to periodontal health. Importantly, these differences in SAA between periodontitis and healthy subjects corroborated previous results from a lower number of subjects living in a different geographical area observed by our group[25]. Neither Ozmeriç et al.[21] nor our preliminary study[25] selected a gingivitis group, which makes the results of the present study relevant for the literature that lacked information on the relation between SAA and gingivitis. After determining the role of SAA as an initial periodontal diagnostic tool, the present study analyzed whether periodontal therapy leads to changes in the enzyme activity or not. Also, this study established if these changes were consistent with those showed by the standard clinical and microbial parameters. For the periodontitis group, clinical and microbial parameters followed clear patterns of improvement. Pocket depth and bleeding reductions accompanied by gain of attachment level were previously observed after successful periodontal therapy[7-13]. In addition, reductions of periodontal pathogens have been observed in non progressive periodontal sites treated by non-surgical periodontal therapy[13,34,35]. In the present study, the periodontitis group also showed a beneficial reduction in SAA values (Figure 1D).

Güllü et al.[22] evaluated the levels of arginase and NOS activity in gingival tissue specimens of 13 chronic periodontitis patients before and after both surgical and non-surgical therapies. Although at baseline inflamed periodontal tissues demonstrated a strong inducible NOS expression, the authors showed that this expression decreased after periodontal therapy. Interestingly, this reduction was more evident after surgical than non-surgical periodontal therapy. Arginase showed an opposite pattern. Its initial levels were 0.18 +/- 0.07IU/mg protein in the modified Widman flap group and 0.25 +/- 0.11IU/mg protein in the scaling and root planing group. After periodontal therapy, arginase level increased up to 0.68 +/- 0.14IU/mg protein in the surgical group and up to 1.10 +/- 0.23IU/mg protein in the non-surgical group. Therefore, in patients with greater extent and severity of periodontal disease, these authors observed high levels of arginase after therapy.

Recently, Ozer et al.[36] evaluated salivary arginase activity of patients with gingivitis or periodontitis and age-matched healthy controls. The effects of scaling and root planing on these inflammatory mediators were also measured. In a smaller group of patients they demonstrated an increase of salivary arginase levels after treatment in both gingivitis and periodontitis groups. Moreover, gingivitis group showed the highest salivary arginase levels when compared to periodontitis and healthy groups. This pattern of salivary arginase increase after therapy is in contrast to our present results as well as to their own previous results[22].

In the present study, we measured the product, ornithine, derived from the degradation of the L-arginine substrate by arginase which indicates not merely the presence of the enzyme but its activity. Once again, the results observed in the present study corroborate the data found in the preliminary research conducted by our group[25]. In this context, salivary arginase decrease previously observed by our

group and here confirmed seems to be relevant since other group of researchers failed to sustain their own proposed tendency. In addition, it is important to emphasize that the results here demonstrated derived from a longer follow-up period and a larger group in comparison to our preliminary therapeutic study (30 vs. 60 days and 18 vs. 31 subjects).

It is important to mention that the reduction in the tissue inflammation and in the occurrence of periodontopathic bacteria likely contributed to the decreased SAA observed in the present study[37-39]. Besides the analysis of clinical parameters, the reduction in the total protein level found in saliva could also be considered an indicator of periodontal inflammatory levels after therapy[40].

Although in the periodontitis group SAA followed similar patterns of improvement showed by the standard parameters, in the gingivitis group SAA results were more inconclusive. The amount of plaque and gingival inflammation reduced after therapy. Such reductions seem to be relevant considering that they were previously observed[41,42] by well-maintained gingivitis subjects after treatment. On the other hand, bacterial prevalence did not change after therapy. Although gingivitis group showed reductions in the clinical levels of plaque and inflammation the provided therapy did not lead to changes in the frequency of detection of the searched bacterial species. In periodontitis patients, the supragingival plaque control as the sole therapy failed to provide a microbiota compatible with health[43]. Therefore, we can speculate that the present study population could require a stricter scheme of therapy. Maybe a weekly professional supragingival biofilm control could lead to the desirable changes in bacterial prevalence. Unfortunately, according to microbial parameters, SAA did not decrease when compared to baseline. Considering that arginase production by macrophages[38] can be stimulated by the presence of

periodontal pathogens[39], the maintenance of bacterial prevalence could be partially responsible for this unchanged SAA. Therefore, the low prevalence of periodontopathic bacteria in gingivitis subjects may have determined a lower SAA and, hence, an intra-group reduction that was too minimal to reach statistical significance. Maybe the variable SAA pattern, that followed the inconsistence of the microbial component, actually reflects the transient nature of gingivitis. Thus, gingivitis subjects should be more deeply investigated in further researches.

In the future, fast laboratorial test could be developed to aid clinicians to categorize more correctly healthy, gingivitis and periodontitis patients.

5 CONCLUSION

Based on the reduction of SAA after therapy, in accordance to the expected reduction in clinical and microbiological parameters, we concluded that SAA has a potential to serve as a reliable indicator to the therapeutic response of chronic periodontitis subjects treated by non-surgical periodontal therapy.

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Conflict of Interest:

The authors report no conflicts of interest related to this study.

Tables

Table 1 – Demographic data and clinical parameters according to periodontal diagnosis

Group		PI (%) Mean ± SD	GI (%) Mean ± SD	PD Mean (mm) ± SD	CAL Mean (mm) ± SD
Healthy n=31 (12M:19F)		30.70 ± 16.2	5.98 ± 5.73	1.35 ± 0.37	0.67 ± 0.26
Gingivitis n=27 (9M:18F)	Pre-therapy	70.48 ± 19.73[a]	71.78 ± 22.59[a]	1.78 ± 0.31	0.95 ± 0.28
	Post-therapy	25.33 ± 17.10[b]	23.44 ± 12.28[b]	NM	NM
Periodontitis n=31 (9M:22F)	Pre-therapy	79.00 ± 17.90[a]	75,03 ± 18.27[a]	3.00 ± 0.45[a]	2.25 ± 0.98[a]
	Post-therapy	33.77 ± 15.53[b]	30.23 ± 14.20[b]	2.44 ± 0.29[b]	1.84 ± 1.09[b]

SD – Standard deviation; PI – Plaque Index; GI – Gingival Index; PD – Probing Depth; CAL – Clinical Attachment Level; NM – Not Measured; M – Male; F – Female.
* Different lower-case letters within columns indicate statistically significant intra-group reduction (pre- versus post-therapy; Wilcoxon test– $p < 0.05$)

Table 2 – Significant Pearson Correlation values observed at pre- and post-therapy examinations.

	Gingivitis		Periodontitis		
	Pre-therapy	Post-therapy	Pre-therapy		Post-therapy
	Gingival index X Plaque index	Gingival index X Plaque index	Gingival index X Plaque index	Probing depth X Arginase	Gingival index X Plaque index
Pearson Correlation	0.739	0.855	0.767	0.645	0.782
p value	0.0001	0.0001	0.0001	0.0001	0.0001

Figure legends

Figure 1 – Healty periodontum

Figure 2 – Gingivitis

Figure 3 – Periodontitis

Figure 4 – Bacterial prevalence(%) between groups H (Healthy), G (Gingivitis) and P (Periodontitis) before (A) and after (B) treatment

*, † - Chi-square test

Figure 5 – Bacterial prevalence(%) on G- (A) and P-group (B) pre- and post-therapy

* - Chi-square test

Figure 6 – Inter-groups comparisons of salivary arginase levels (mUI/mg) at pre- (A) and post-therapy (B)

* indicates a statistically significantly higher level of arginase in saliva in the periodontitis group in comparison to gingivitis and healthy groups (Mann-Whitney test – $p < 0.05$)

† indicates a statistically significantly higher level of arginase in saliva in the gingivitis group in comparison to the healthy group (Mann-Whitney test – $p < 0.05$) and intra-group comparisons of salivary arginase levels (mUI/mg) (C) and total protein levels (mg/ml) (D) between pre- and post-therapy.

* indicates a statistically significant reduction when pre- was compared to post-therapy values (Wilcoxon test – $p < 0.05$).

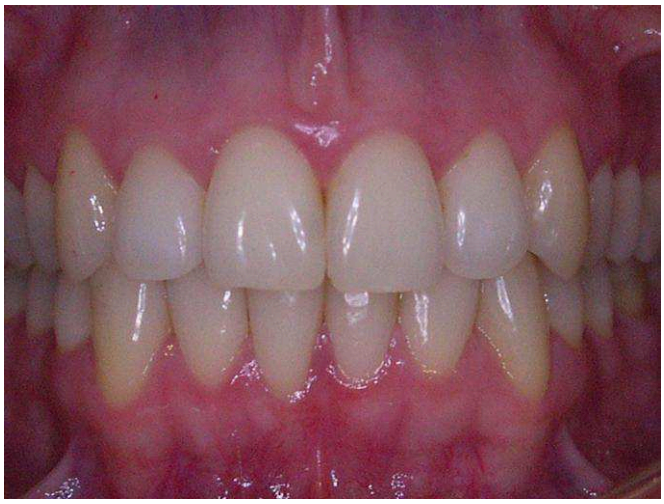

Figure 1

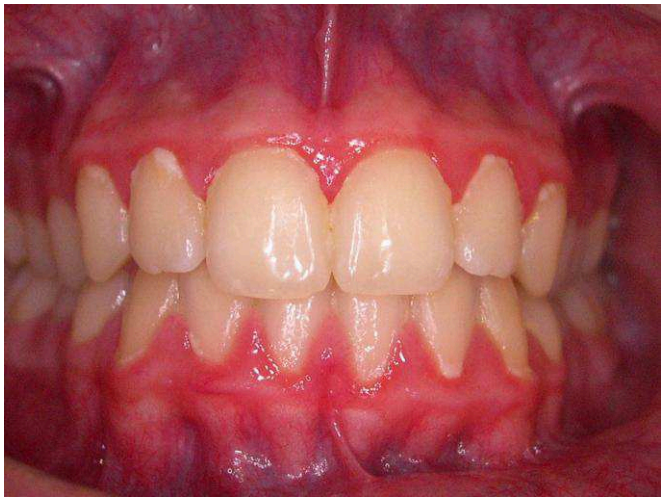

Figure 2

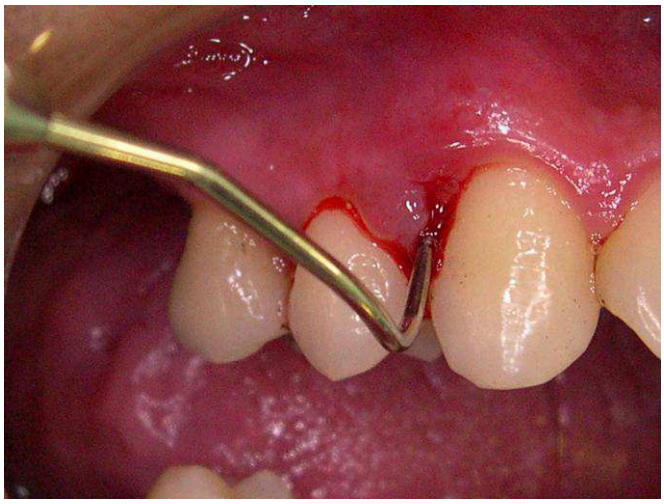

Figure 3

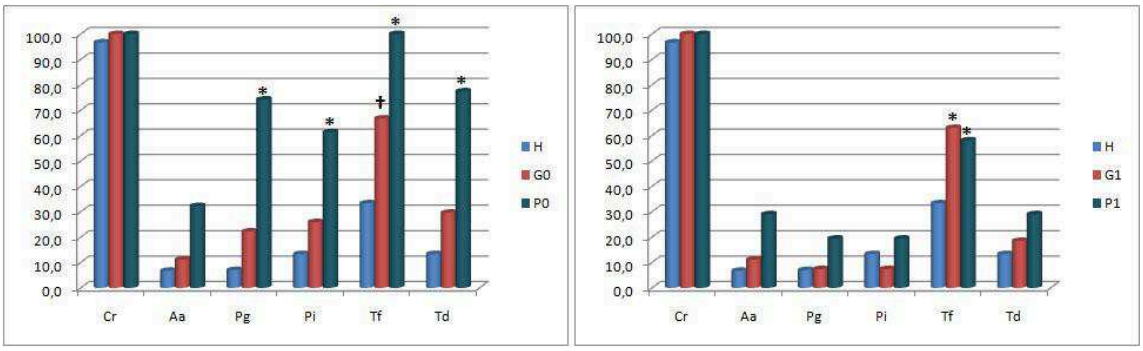

Figure 4

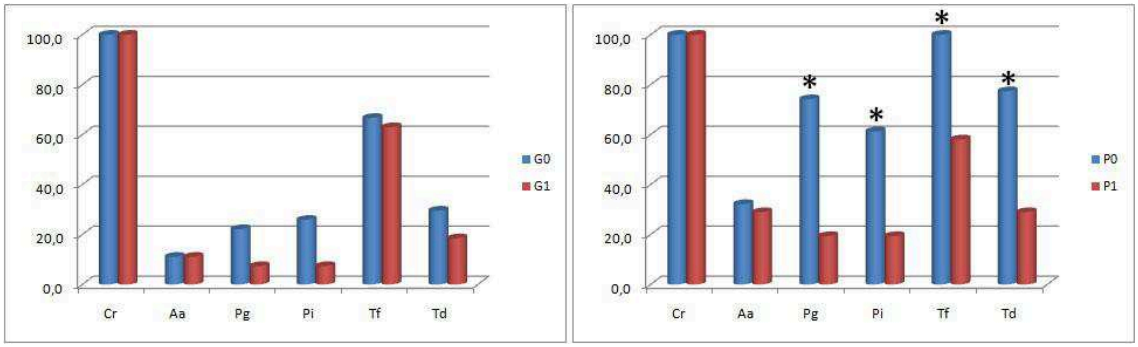

Figure 5

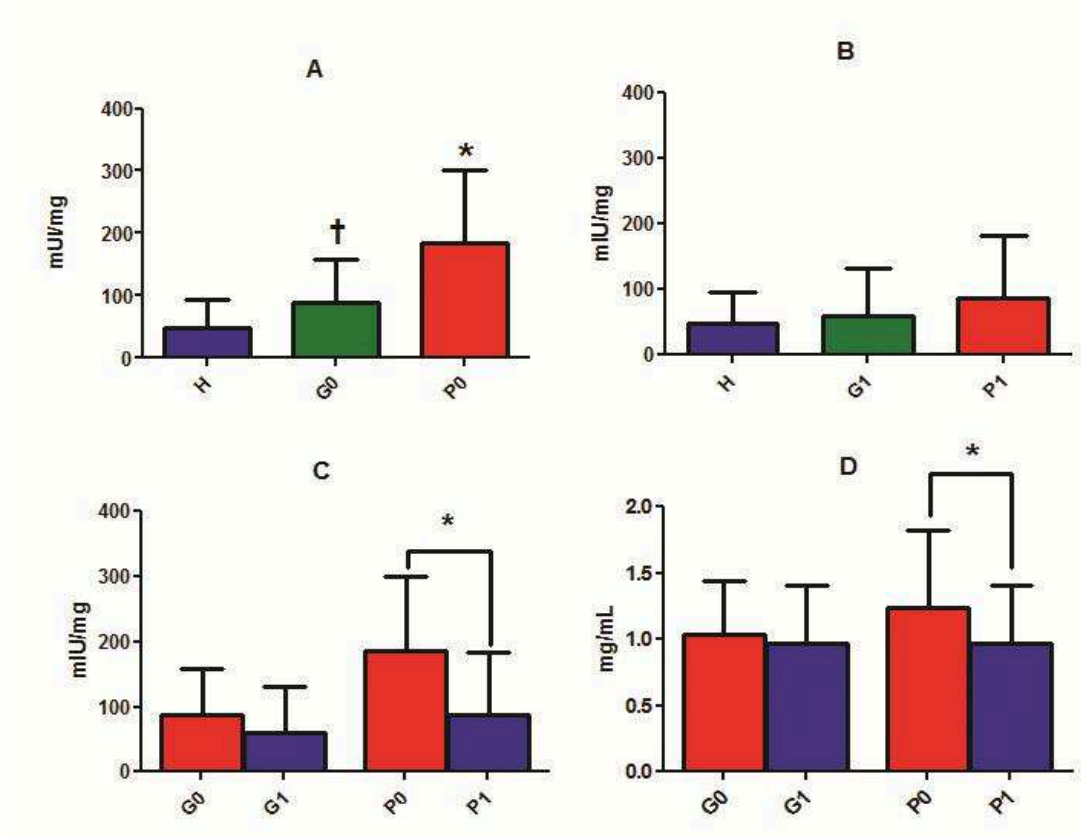

Figure 6

2.2 CAPÍTULO B

Human β-defensin 2 and protease activated receptor-2 expression in patients with chronic periodontitis

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Abbreviations: PAR_2, protease-activated receptor-2, HBD-2, human β defensin-2, GCF, gingival crevicular fluid, PI, Plaque Index;GI, Gingival Index; PD, Probing Depth CAL, Clinical Attachament Level

Short running title: HBD-2 and PAR_2 expression in chronic periodontitis

Pereira AL, Holzhausen M, Franco GCN, Cortelli SC, Cortelli JR. Human β-defensin2 and protease activated receptor-2 expression in patients with chronic periodontitis. Arch Oral Biol 2012; 57(12):1609-14

ABSTRACT

Objective: Some previous studies have shown that gingipains, trypsin-like proteases produced by *Porphyromonas gingivalis*, up-regulate human β defensin-2 (HBD-2) mRNA expression through protease-activated receptor-2 (PAR_2) in gingival epithelial cells. This study aimed at investigating salivary HBD-2 levels and crevicular PAR_2 mRNA expression in human chronic periodontitis and evaluating whether periodontal treatment affected this process. **Methods:** Salivary and gingival crevicular fluid (GCF) samples were collected from periodontally healthy (control) and chronic periodontitis patients at baseline and 50 days after non-surgical periodontal treatment. Salivary HBD-2, and GCF TNF-α levels were analyzed by ELISA, and PAR_2 mRNA at the GCF was evaluated by RT-PCR. **Results:** *P. gingivalis* was significantly ($p < 0.05$) more prevalent in patients with chronic periodontitis when compared to controls. This prevalence decreased after periodontal therapy ($p < 0.0001$). The control group showed statistically significant lower levels of HBD-2, TNF-α, and PAR_2 expression when compared to the chronic periodontitis group. In addition, periodontal treatment significantly reduced PAR_2 expression and HBD-2 levels in chronic periodontitis patients ($p < 0.001$). **Conclusions:** Our results suggest that salivary HBD-2 levels and PAR_2 mRNA expression from GCF are higher in subjects with chronic periodontitis than in healthy subjects, and that periodontal treatment decreases both HBD-2 levels and PAR_2 expression.

Keywords: Chronic periodontitis; Protease activated receptor-2; Human beta defensin 2; Periodontal treatment.

1 INTRODUCTION

Human β-defensins (HBDs) are small cationic peptides produced throughout the body, mainly by epithelial cells, that play an important role in the oral cavity as a first-line defense against gram-negative and gram-positive bacteria, as they are able to create pores into the bacterial membranes, killing the bacteria. Epithelial cells in the oral cavity constitutively express HBDs: HBD-1, HBD-2, and HBD-3[1,2]. However, in the presence of inflammation, a different expression of these peptides might occur[2-5]. Dommisch et al.[2] showed that in healthy gingival tissues there is a similar expression among HBD-1 and -2 mRNA. In contrast, the expression of HBD-2 is statistically higher than human b defensin-1 in both gingivitis and chronic periodontitis subjects.

A recent study by Vardar-Sengul et al.[4] showed that the expression of HBD-1 and -2 mRNA was significantly higher in chronic periodontitis subjects than in the healthy control group. In addition, in a study by Kuula et al.[5], HBD-2 expression was found to be lower in periodontally healthy tissues than in inflamed periodontal and peri-implant tissues. Taken together, these studies suggest a potentially important role for defensins in the host response to infection by periodontal pathogens.

The modulation of the β-defensins expression in the oral cavity can be orchestrated by receptors present in the cell membrane that recognize certain molecular patterns associated to periodontal pathogens, including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*. Previous in vitro studies[6,7] have shown that gingipains, trypsin-like proteases produced by *Porphyromonas gingivalis*, up-regulate HBD-2 mRNA expression

through protease-activated receptor-2 (PAR_2) in gingival epithelial cells. PAR_2 belongs to the family of G-protein-coupled, seven-transmembrane-domain receptors. Its activation occurs through the proteolytic cleavage of the N-terminal domain by serine proteinases such as trypsin, mast cell tryptase, neutrophil proteinase 3, tissue factor/factor VIIa/factor Xa, membrane-tethered serine proteinase-1, and gingipains[8,9]. A recent study by our group[10] compared chronic periodontitis patients to healthy controls and showed that PAR_2 is up-regulated in this first group. We also showed that the presence of *Porphyromonas gingivalis* in the periodontal pocket is associated with this upregulation of PAR_2 gene expression and that a higher pro-inflammatory profile is related to advanced periodontal destruction[11]. In the present study, we hypothesized that HBD-2 levels as well as the expression of PAR_2 are elevated in the saliva of chronic periodontitis subjects. As to assess this hypothesis, the salivary HBD-2 levels and the PAR_2 mRNA expression from GCF were investigated in chronic periodontitis and in healthy subjects. In addition we also evaluated whether periodontal treatment may affect these levels.

2 MATERIALS AND METHODS

2.1 Patients

All subjects were between 20 to 69 years and in good overall health. Patients who reported history of tobacco usage within six months of screening; use of orthodontic appliances; need for premedication with antibiotics for dental treatment; usage of antibiotics, phenytoin, calcium antagonists, cyclosporine, or anti-inflammatory drugs within one month of initial appointment; history of any disease known to compromise immune functions; pregnancy or lactation; immunosuppressive chemotherapy, and/ or periodontal treatment within the last 6 months, were not included in the present study.

The study protocol was approved by the Institutional Committee on Research of the University of Taubate (protocol #385/08) in accordance with the Helsinki Declaration of 1975, as revised in 2000. All patients were instructed in the nature and objectives of the study and signed a consent form agreeing to their participation.

2.2 Clinical examination

Subjects were clinically evaluated with regards to the probing pocket depths, clinical attachment loss and bleeding upon probing recorded at six sites per tooth

(mesiobuccal, buccal, distobuccal, mesiolingual, lingual, distolingual) using a manual periodontal probe (PCPUNC 15 - Hu-Friedy, Chicago, IL, USA). Patients were then divided in two groups: control (i), formed by those showing healthy sites with probing depths of ≤ 3mm, no attachment loss, no bleeding on probing, or no signs of inflammation (10 subjects); and chronic periodontitis (ii), formed by patients with at least four teeth with one or more sites with probing depth ≥ 4mm and clinical attachment loss ≥ 3 mm, and bleeding on probing. For each patient in the chronic periodontitis group the periodontal site showing the deepest probing depth in each oral quadrant was selected for the collection of microbial and GCF samples.

2.3 Periodontal Therapy

After the clinical evaluation, bacterial and saliva samples were taken. Each subject received oral hygiene instructions and a standard kit for mechanical supragingival plaque control. The kit contained fluoride dentifrice, a regular toothbrush, interdental toothbrushes, and dental floss. Subjects in the healthy group were instructed about personal daily oral hygiene care. Periodontitis subjects underwent scaling and root planning under local anesthesia, in a total of four clinical visits.

Clinical data, microbial, crevicular fluid and salivary samples were taken from the same sites at baseline and 50 days after initial therapy.

2.4 Microbial sampling and *Porphyromonas gingivalis* analysis

The periodontal sites selected were isolated and the supragingival plaque was carefully removed. One fine paper point (number 30 – Tanari - Tanariman Industrial Ltda., Manacapuru, Brazil) was inserted into the gingival sulcus/periodontal pocket and left in place for 10 s. Samples collected were stored in 1ml of reduced Ringer's solution (0.9 g sodium chloride, 0.042 g potassium chloride, 0.025 g calcium chloride, 100ml distilled water) at -80°C. Bacterial suspensions were thawed, centrifuged at 12,000g for 1 min. The presence of *Porphyromonas gingivalis* was assessed by polymerase chain reaction (PCR) using specific primers: sense 5'AGGCAGCTTGCCATACTGCGG3', and antisense: 5'-ACTGTTAGCAACTACCGATGT-3' (product size: 404bp) under standard conditions. DNA was extracted using PureLink® Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA). PCR was performed in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) as follows: one cycle 94ºC for 5 min, 35 cycles 94ºC for 30 sec., 57ºC for 30 sec., 72ºC for 1 min, and a final extension of 72ºC for 5 min.

After electrophoresis in 1.5% agarose gel, DNA fragments were stained with SYBR SafeTM (Invitrogen®, Carlsbad, CA, USA) and visualized by UV illumination. PCR amplifications were compared with both positive and negative controls. Molecular weight marker (Ladder 100, Invitrogen) was added in each set.

2.5 Gingival crevicular fluid (GCF) sampling

GCF samples were obtained from the same periodontal sites selected for microbial sampling. One strip of perio-paper (PerioCol Collection Strip, Oraflow, Plainview, NY, USA) was inserted into the gingival crevice/ periodontal pocket and removed after 30 seconds. The volume of the fluid was determined using a moisture meter (Periotron 6000, IDE Interstate, Amityville, NY, USA). After that, all perio-paper strips were placed in tubes containing 500µl of sterile 0.01M sodium phosphate buffer, pH 7.4, and vortex mixed for 30 seconds. Samples were then centrifuged for 10 minutes at 6000g, and supernatant was collected and stored at - 80°. The cell pellet was stored in RNA stabilization solution (Trizol, Invitrogen, Carlsbad, CA, USA) at - 80°.

2.6 TNF-α levels in the GCF

Tumor necrosis factor-α levels were determined by using commercially available enzyme-linked immunosorbant assays (R&D Systems, Minneapolis, Minn, USA). The concentration of the inflammatory mediators was calculated using the Softmax data analysis program (Molecular Devices, Menlo Park, CA, USA).

2.7 PAR_2 gene expression by Reverse-Transcription PCR (RT-PCR)

Total RNA was isolated from the cell pellet of the GCF by the single-step method, using phenol and chloroform/isoamylalcohol. RNA was reverse-transcribed into cDNA by using the ready-to-go RT-PCR beads kit (Amersham Biosciences, Buckinghamshire, UK). Briefly, 2 µg of total RNA was used and the reaction included the random primer p(dN)6. After reverse transcription according to manufacturers' instructions, PCR amplification was performed with the addition of specific primers. For PAR_2, upstream: 5'-TGGGTTTGCCAAGTAACGGC-3', and downstream: 5'-GGGAGATGCCAATGGCAATG-3'. For GAPDH, upstream: 5'-TGGTATCGTGGAAGGACTCATGAC-3', and downstream, ATGCCAGTGAGCTTCCCGTTCAGC-3'. PCR products were loaded in 1.5% agarose gel and, 26µl of PAR_2 or GAPDH PCR products were loaded for each sample. The sizes of the amplified fragments were 324bp and 189bp for PAR_2 and GAPDH, respectively. Amplified samples were visualized under UV light after being stained with SYBR SafeTM (Invitrogen®, Carlsbad, CA, USA). Results are expressed as PAR_2 to GAPDH ratios.

2.8 Saliva sampling

Saliva samples were collected from all individuals. Samples taken from subjects in the control group were collected at baseline, whereas individuals in the

chronic periodontitis group were subjected to two saliva sample collections, one at baseline, previous to periodontal therapy, and the other fifty days after comprehensive periodontal therapy. During the procedure, subjects were instructed to rinse their mouth with water and chew a piece of sterilized rubber tourniquet to stimulate saliva, which was collected to yield a total 1.0ml. Samples were centrifuged for 10 min at 15,000×g at 4℃, and the supernatants were immediately stored at −80°C.

2.9 ELISA for human β-defensin 2 (HBD-2)

The quantification of HBD-2 in saliva was done by an Enzyme Linked Immunosorbent Assay - ELISA (Peprotech, Rocky Hill, NJ, USA) according to manufacturer's instructions. The process was carried as follows: 100µl (0.25µg/ml) of specific antibody (anti-HBD-2) was added to the 96-well polystyrene ELISA plates and incubated overnight (4ºC); after being washed four times with PBST (PBS with 0.05% Tween-20), 300µl of a blocking solution (1% BSA in PBST) was added to the wells and incubated for 1 hour at room temperature. Plates were then washed and 100µl of the samples or standards were added into the respective wells in duplicate and these plates were incubated for 2 hours. After washing, 100µl of detection antibody (0.5µg/ml) was applied to the wells and plates were incubated for 2 hours. After this period, plates were washed and 100µl of streptavidin-conjugated horseradish peroxidase (1:2000 in PBST) was added to the respective wells and incubated for 30 minutes. Colorimetric reactions were developed using

o-phenylenediamine in the presence of 0.02% H_2O_2. Reaction was stopped using H_2SO_4 (2N) and measured by an ELISA reader (OD 490nm).

2.10 Statistical Analysis

One-way analysis of variance was used to compare means among groups. In case of significant differences among groups, posthoc two-group comparisons were assessed with a Tukey-Kramer test. The prevalence of *Porphyromonas gingivalis* among groups was analyzed using the chi-square test. A P value < 0.05 was considered statistically significant. Data are expressed as mean ± SE.

3 RESULTS

3.1 Clinical findings

Mean pocket depth (PD) and mean clinical attachment loss (CAL) were significantly higher ($p < 0.05$) in subjects in the chronic periodontitis group than in those in control. Clinical parameters were significantly ($p < 0.05$) improved by conventional periodontal treatment.

3.2 Laboratory findings

Patients with chronic periodontitis showed a significant increase ($p < 0.001$) in the mean PAR_2 mRNA expression relative to the GAPDH RT-PCR signal. Moreover, conventional periodontal treatment significantly ($p < 0.05$) decreased PAR_2 mRNA expression (Figure 1A).

Although being significantly ($p < 0.05$) more prevalent in patients with chronic periodontitis than in those in the control group, the levels of *Porphyromonas gingivalis* decreased after periodontal therapy ($p < 0.0001$) (Figure 1B).

Levels of TNF-α, that were also higher ($p < 0.01$) in chronic periodontitis patients also decreased after periodontal therapy ($p < 0.001$) (Figure 2A).

The same was observed for the mean HBD-2 salivary levels that were higher in chronic periodontitis subjects than in controls (Figure 2B) and that significantly decreased after therapy ($p < 0.05$).

4 DISCUSSION

While epithelial tissues from gut, trachea and skin only express human beta defensin-2 in the presence of infection or inflammation, the oral epithelium expresses the peptide in normal healthy gingival tissue[12]. HBD-2 expression in normal oral epithelium is due to the constant stimulation of the innate immune response by commensal, non-pathogenic bacteria[13]. In the normal gingival tissue, peptides are detected in the upper spinous, granular, and cornified layers, while mRNA is more strongly expressed in the spinous layer of the tissue. In the presence of pathogenic bacteria, upregulation of HBD-2 expression occurs at the gingival margin, adjacent to the biofilm in the inflamed epithelium[12].

The nature of the epithelial cell receptors which are able to detect microorganisms and induce the production of the antimicrobial peptides is still not well known. Although we already understand that toll-like receptors 2 and 4 can recognize gram positive and gram negative bacteria resulting in the activation of transcriptional factors that mediate several innate and inflammatory responses[14,15], there hasn't been any convincing evidence of their involvement in the regulation of HBD-2 in oral epithelial cells[16].

Protease-activated receptor (PAR) is another family of membrane receptors[17] that probably play a role in the inflammatory and host defense response to pathogenic bacteria, including the modulation of human b defensins[18]. PAR may be activated in the oral cavity through its proteolytic cleavage by *Porphyromonas gingivalis* bacterial proteases[19]. Our results demonstrated that, when compared to periodontally healthy individuals, chronic periodontitis patients show statistically

significant higher levels of HBD-2 and an upregulation of PAR_2. Besides, we also observed that periodontal treatment significantly reduced PAR_2 expression and human b defensin-2 levels in chronic periodontitis patients ($p < 0.001$).

We have previously demonstrated that in subjects with chronic periodontitis a higher expression of PAR_2 in the gingival crevicular fluid was associated with higher levels of pro-inflammatory mediators, total proteolytic activity, *Porphyromonas gingivalis* prevalence and neutrophil-protease 3 mRNA expression[10]. Another study by our group showed that the presence of *Porphyromonas gingivalis* in the periodontal pocket of chronic periodontitis patients is associated with higher proteolytic activity, and a marked increased expression of PAR_2[11]. These evidences suggest that PAR_2 plays an important role in the pathogenesis of periodontal disease in response to proteases secreted by *Porphyromonas gingivalis*.

Chung et al.[7] demonstrated that bacterial proteases such as gingipains from *Porphyromonas gingivalis* induced expression of human b defensins in human gingival epithelial cells by activating PAR_2. Furthermore, Barrera et al.[20] showed that proteolytic antibodies present in the human milk may activate PAR_2, which in turn induces HBD-2 expression. The exact mechanism(s) by which PAR_2 is associated with an increase in HBD-2 levels remains to be established in further studies. A recent study by Lee et al.[21] showed that PAR_2 activation by proteases secreted by *Propionibacterium acnes* leads to both TNF-α and HBD-2 mRNA expression in acne lesions. Accordingly, Shin & Choi[22] showed that *Treponema denticola* suppresses the expression of HBD-2 in gingival epithelial cells by inhibiting TNF-α production. Interestingly, in the present study, increased prevalence of *Porphyromonas gingivalis* and levels of TNF-α were associated with higher salivary levels of HBD-2 in chronic periodontitis. In addition, periodontal treatment resulted in lower levels of both TNF-

alpha and human b-defensin associated with a decreased prevalence of *Porphyromonas gingivalis*. These evidences suggest the hypothesis that PAR_2 activation by gingipains mediates the increased production of TNF-α, therefore leading to increased human β expression in chronic periodontitis.

Several studies have demonstrated that elevated levels of human β defensins are present in saliva and periodontal tissues of patients with gingivitis, periodontitis, and peri-implantitis[1-5]. This is, as far as we know, the first study to show that after periodontal treatment the salivary levels of HBD-2 are decreased and associated with a decreased expression of PAR_2. The exact role of PAR_2 on human periodontal inflammation is still not clearly defined; however, it seems likely that it might play an important role in innate immune defense during periodontal disease by leading to the production of anti-bacterial peptides and pro-inflammatory mediators.

In conclusion, Our results suggest that salivary HBD-2 levels and PAR_2 mRNA expression from GCF are higher in subjects with chronic periodontitis than in healthy subjects, and that periodontal treatment decreases both HBD-2 levels and PAR_2 expression. Thus, anti-bacterial peptides prodution might be an important role played by PAR_2 in innate immune defense during periodontal disease.

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Conflict of interest

The authors have no conflict of interest or competing financial interest with regards to this manuscript.

Ethical approval

Ethical approval given from Institutional Committee on Research Involving Human Subjects of the University of Taubate # 386/08 on August 28, 2008.

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Tables

Table 1: Clinical parameters

	PD (mm)	± SD	CAL(mm)	± SD	PI(%)	± SD	GI(%)	± SD
Control N=10	1.02	0.21	0.95	0.4	38.11	11.9	18.46	9.32
Chronic Periodontitis (CP) N=10	3.22[a]	0.55	2.32[a]	1	78.8[a]	19.31	72.5[a]	18.68
CP post-treatment (CPT) N=10	2.4[ab]	0.36	2.03[ab]	1.26	22.2[ab]	13.13	23.5[ab]	11.19

N: number of subjects; SD: standard deviation; PD: mean probing depth; CAL: mean clinical attachment level; PI: plaque index; GI: Gingival index

Number of sites >4mm on CP-group: 493 of 1326, i.e., about 37% of overall

[a] statistically different compared to Control group, $p<0.05$.

[b] statistically different compared to CP group, $p<0.05$.

Figure legends

Figure 1A: Detection of PAR_2 in human GCF by RT-PCR. Mean ± SEM mRNA ratio PAR_2/GAPDH for control, chronic periodontitis patients at baseline (CP), and after periodontal therapy (CPT) groups; * Significant difference ($p<0.05$) versus control group; N=10 subjects per group;

Figure 1B: Percentage of prevalence of *Porphyromonas gingivalis* (Pg) in control, chronic periodontitis patients at baseline (CP), and after periodontal therapy (CPT) groups; *Significant difference ($p < 0.05$) versus prevalence of *Porphyromonas gingivalis* (Pg) at control group; **Significant difference ($p < 0.05$) versus prevalence of *Porphyromonas gingivalis* (Pg) at baseline; N=10 subjects per group;

Figure 2A: Levels of the pro-inflammatory mediator TNF-α (Mean ± SEM) in the GCF of control, chronic periodontitis at baseline (CP), and after periodontal therapy (CPT) groups; N=10 subjects per group; *Significant difference ($p < 0.05$) versus control group; **Significant difference ($p < 0.05$) versus baseline;

Figure 2B: Levels of HBD-2 (Mean ± SEM) in the saliva of control, chronic periodontitis at baseline (CP), and after periodontal therapy (CPT) groups; N=10 subjects per group; *Significant difference ($p<0.05$) versus control group; **Significant difference ($p < 0.05$) versus baseline;

Figure 1A

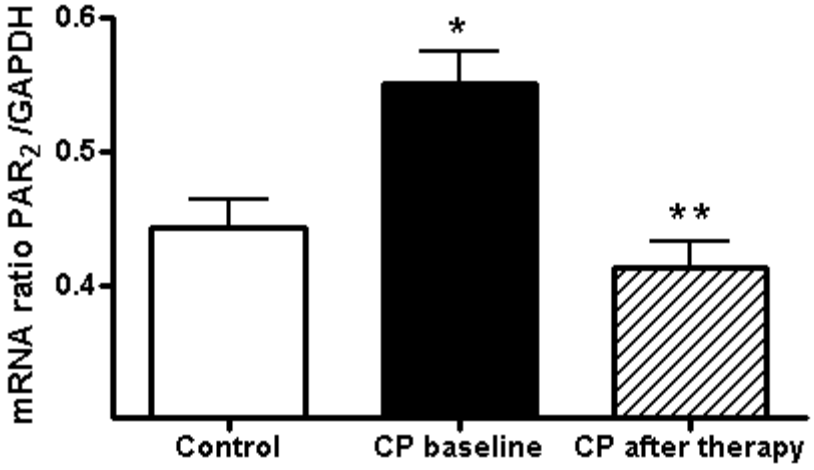


Figure 1B

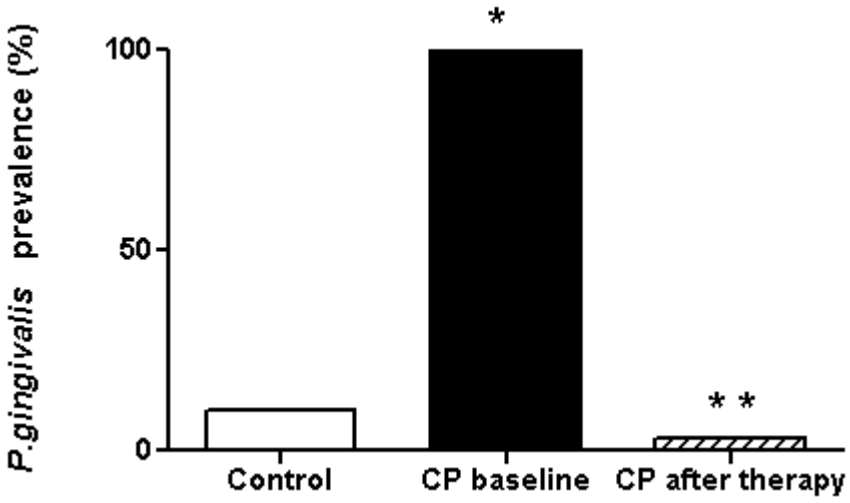


Figure 2A

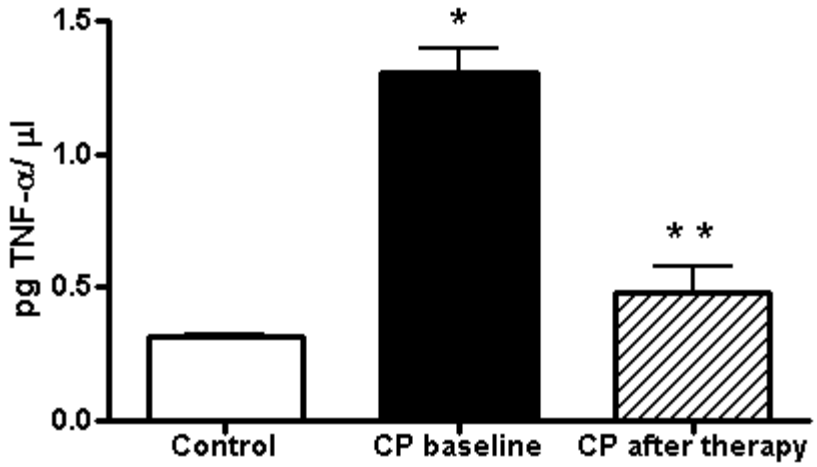
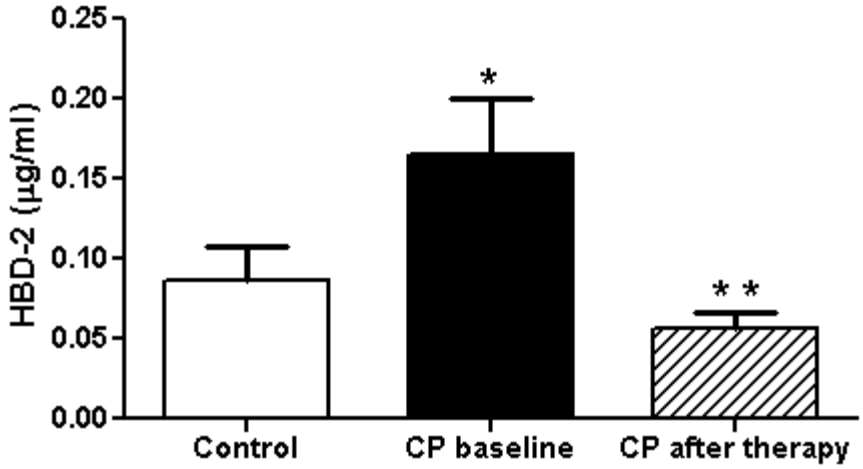


Figure 2B



2.3 CAPÍTULO C

Influence of periodontal status and periodontopathogens on levels of oral human beta-defensin-2 in saliva.

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Short running title: Beta-defensin-2, periodontal status and periodontopathogens

One-sentence summary: Levels of salivary beta-defensins-2 were influenced by periodontal status but were not related with the frequency of periodontopathogens.

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ABSTRACT

Background: Expression patterns of human beta defensing 2 (HBD-2) mRNA or HBD-2 protein concentration and periodontal diseases have been a focus of scientific research. This study compares the salivary levels of HBD-2 protein concentration of healthy, gingivitis and periodontitis patients, and correlates these levels with the presence of periodontopathogens. **Methods:** 89 patients were enrolled in this study: 31 periodontally healthy; 27 gingivitis; and 31 chronic periodontitis. Plaque and gingival indices, probing depth and clinical attachment level were measured. Presence of *Campylobacter rectus, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tanerella forsythia, Treponema denticola*, and *Prevotella intermedia* was evaluated qualitatively by conventional polymerase chain reaction (PCR). HBD-2 quantification in saliva was carried out using an immune enzymatic assay. Frequency of periodontopathogens and HBD-2 protein concentration was assessed. Association between HBD-2 protein concentration ($\geq$ 100pg/ml) and the simultaneous presence of 1-2, 3-4 or 5-6 periodontopathogens was tested. **Results:** While periodontally healthy and gingivitis patients showed similar HBD-2 levels, periodontitis group displayed an increased level of HBD-2. *P. gingivalis, P. intermedia, T. forsythia and T. denticola* were more prevalent in periodontitis; however their mere presence was not related with the increased levels of HBD-2 (Pearson's correlation and multinomial logistic regression model). **Conclusions:** Salivary HBD-2 protein concentration was higher in periodontitis in comparison to healthy or gingivitis patients. These different protein concentrations were not related with the frequency of periodontopathogens. Clinical inflammatory profile had a higher impact on salivary HBD-2 levels than bacteria.

Keywords: Defensin; Bacteria; Periodontitis; Gingivitis; Oral health.

1 INTRODUCTION

Antimicrobial peptides are found in the innate immune system that protects multicellular organisms from a diverse spectrum of microorganisms. In humans, beta-defensins (HBD-human beta-defensins) are small, cationic antimicrobial peptides that can kill a wide variety of gram-positive and gram-negative bacteria by inducing physical holes in their cellular membrane and by stimulating antigen-presenting cells[1-4].

HBD are produced by epithelial cells in many organs, including skin, lung, kidney, pancreas, uterus and eyes, as well as in nasal and oral mucosae[5-8]. HBD in oral cavity play a key role in the maintenance of steady-state levels of normal microbiota[9,10]. Three HBD subtypes have been isolated from human oral tissues. HBD-1 is constitutively expressed, whereas HBD-2 and HBD-3 are considered upregulated in inflamed tissues[11].

In the last years, there has been an interest in the research of the association between oral HBD levels and periodontal disease[11-17]. Antimicrobial activity of HBD against periodontopathogens in the oral cavity is well-documented and the biological mechanisms responsible for the stimulation/inhibition of HBD production have been also investigated[15,18,19]. Some studies have suggested that the severity of periodontal disease could be associated with changes on HBD levels due to the presence of local inflammation[13,14,16]. Vardar-Sengul et al.[14] demonstrated, in a clinical study, that HBD-2 mRNA expression in the gingivitis group was lower than in the control group; however, this difference was statistically significant only in half of the gingivitis patients ($p < 0.001$). HBD-2 mRNA levels were higher in some chronic periodontitis

patients, but lower in the others when compared with the control group ($p < 0.001$). When compared to controls the expression of the HBD-2 gene increased in aggressive periodontitis. These findings are not, however, consensual among researchers. Dommisch et al.[16], for instance, could not find any significant change on HBD oral levels in healthy, gingivitis and periodontitis patients.

Besides the severity of periodontal disease, literature proposes another possible modulator of oral HBD production. Microorganisms such as periodontopathogens can directly affect gingival epithelial cells; however results are also controversial. Taguchi et al.[18] demonstrated that *Porphyromonas gingivalis*, a key periodontopathogen, seems to be able to trigger HBD-2 production on human gingival epithelial cells. However, some studies failed to demonstrate this influence on HBD-2 production[11,15].

This study hypothesized that salivary levels of HBD-2 tend to increase according to periodontal status, i.e, that there is a higher level of the peptide in periodontitis than in gingivitis and periodontal health. In addition, it was hypothesized that this increase in salivary levels of HBD-2 is accompanied by an increase in the frequency of target periodontal bacterial species.

Thus, the aim of this clinical study was, first, to compare the levels of HBD-2 protein concentration in the saliva of healthy, gingivitis and chronic periodontitis patients, and second to correlate these levels with the following key periodontopathogens: *Campylobacter rectus, Porphyromonas gingivalis, Tanerella forsythia, Treponema denticola, Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans*.

2 MATERIAL AND METHODS

2.1 Ethical guidelines

The study protocol was approved by the Institutional Committee on Research of the University of Taubate (protocol #386/08) Taubaté, São Paulo - Brazil in accordance with the Helsinki Declaration of 1975, as revised in 2000. Prior to selection, oral and written explanations about the research protocol were given to eligible participants. All patients provided a written informed consent before their participation in this study which was performed from March 2010 to September 2011.

2.2 Patient population and periodontal evaluation

The population of this study was composed of patients recruited from a Dental Continuing Education Institute named *Escola de Aperfeiçoamento Profissional (EAP)* in Goiânia, Goiás state, Brazil. Prior to the present study, clinical, microbiological and immunological parameters were evaluated in 5 periodontally healthy, 5 gingivitis and 5 chronic periodontitis participants. Results from this pilot study were statistically analyzed (Student t test) to calculate the size of the population (95% of significance and power of 90%). Frequency of *P. gingivalis* in the healthy group required the

highest number of individuals determining groups of at least 20 participants. Data from this pilot study was not considered in the final statistical analysis.

A complete periodontal examination was carried out by a single calibrated examiner (ALP). Measurements of periodontal pocket depth (PD), clinical attachment level, plaque[20] and gingival[21] indices were obtained in six sites per tooth using a manual periodontal probe‡. According to their periodontal diagnoses[22-24], individuals were classified into the following three groups: 1) periodontally healthy[24] (participants showing a mean periodontal attachment level of < 1.5 mm and no sites with > 2.0mm attachment loss); 2) gingivitis[22,23] (no radiographic evidence of periodontal bone resorption and more than 30% of bleeding sites); or 3) chronic periodontitis[22,23] (4 or more teeth with at least one site with attachment loss $\geq$ 3mm and 4 or more teeth with at least one site showing pocket depth $\geq$ 4mm). A panoramic radiograph was taken for each subject.

Data and personal information related to the medical and dental histories were obtained by interview. Only never smokers participated in the present study and the following exclusion criteria were used: (a) less than 14 natural teeth; (b) antibiotics and/or anti-inflammatory drug use in the six months preceding the beginning of the study; (c) systemic diseases or conditions that could influence periodontal status such as uncontrolled diabetes[25,26]; (d) smokers and former smokers[27]; and (e) periodontal treatment performed within six months prior to baseline.

2.3 Samples collection

All samples were collected after clinical examination.

2.4 Subgingival biofilm

Microbial samples were obtained from four periodontal sites, one in each quadrant, based on Cortelli et al.[24]. For periodontally healthy patients mesial sites of first molars were sampled, for Gingivitis patients sites with bleeding on probing and for Periodontitis patients sites showing the highest PD values. Each selected tooth was isolated with sterile cotton rolls and the supragingival plaque was removed with sterile curettes. A sterilized #30 paper point[§] was carefully inserted to the depth of the sulcus or periodontal pocket, and kept in position for 60 seconds. The pooled subgingival samples were stored at -80ºC in microtubes containing 1ml of reduced Ringer's solution.

2.5 Saliva

Saliva samples were collected from all the study population in the morning, from 8:00 to 11:00. Patients were instructed not to eat or drink within 2 hours prior to sampling. Immediately before sampling, patients rinsed their mouth with water. During collection, they remained seated with heads tilted forward (approximately 45°) and chewed a piece of sterilized rubber tourniquet to stimulate saliva production,

which was collected to yield a total of 2.0ml[28]. Samples were centrifuged for 10 min at 15,000×g at 4℃, and the supernatants were immediately stored at −80°C.

2.6 Microbial analysis

As previously published[24], the presence of *C. rectus, P. gingivalis, T. forsythia, P. intermedia, T. denticola* and *A. actinomycetemcomitans* was established qualitatively by conventional polymerase chain reaction (PCR) using specific primers [*P. gingivalis*, sense: 5'- AGGCAGCTTGCCATACTGCGG-3', and antisense: 5'-ACTGTTAGCAACTACCGATGT-3' (product size: 404bp); *T. forsythia*, sense: 5'-GCGTATGTAACCTGCCCGCA-3', and antisense: 5'-TGCTTCAGTGTCAGTTATACCT-3' (product size: 641bp); *C. rectus*, sense: 5'-TTTCGGAGCGTAAACTCCTTTTC-3', and antisense: 5'-TTTCTGCAAGCAGACACTCTT-3' (product size: 598bp); *P. intermedia,* sense: 5'-TTTGTTGGGGAGTAAAGCGGG-3', and antisense: 5'-TCAACATCTCTGTATCCTGCGT-3', (product size: 575bp); *T. denticola,* sense: 5'-TAATACCGAATGTGCTCATTTACAT-3', and antisense 5'-TCAAAGAAGCATTCCCTCTTCTTCTTA-3' (product size 316bp); and *A.actinomycetemcomitans*, sense: 5'-AAACCCATCTCTGAGTTCTTCTTC-3' and antisense: 5'-ATGCCAACTTGACGTTAAAT-3' (product size: 550bp)] under standard conditions. The DNA was extracted‖ and the PCR was performed in a thermocycler¶ as follows: one cycle 94ºC for 5 min, 35 cycles 94ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min, and a final extension of 72ºC for 5 min.

After electrophoresis in 1.5% agarose gel, the DNA fragments were stained with SYBR Safet[#] and visualized by ultraviolet illumination. The PCR products were compared to both positive (DNA isolated from culture collection bacterial strains) and negative (purified PCR-grade water instead of the DNA template) controls. A molecular weight was added in each set.

2.7 HBD-2 quantification

HBD-2 quantification in saliva was carried out using an Enzyme Linked Immunosorbent Assay – ELISA[**] according to manufacturer's instructions. Briefly, 100µl (0.25µg/mL) of specific antibody (anti-HBD-2) was added to the 96-well polystyrene ELISA plates and incubated overnight (4ºC). After being washed four times with PBST (PBS with 0.05% Tween-20), 300µl of a blocking solution (1% BSA in PBST) was added to the wells and incubated for 1 hour at room temperature. Plates were then washed and 100µl of the samples or standards were added into the respective wells in duplicate and they were for 2 hours. After washing, 100µl of detection antibody (0.5µg/mL) was applied to the wells and plates were incubated for additional 2 hours. After incubation, plates were washed and 100µl of streptavidin-conjugated horseradish peroxidase (1:2000 in PBST) was added to the respective wells and incubated for 30 minutes. Colorimetric reactions were developed using o-phenylenediamine in the presence of 0.02% H_2O_2. Color development was stopped using H_2SO_4 (2N) and measured by an ELISA reader (OD 490nm).

2.8 Statistical analysis

The statistical analysis was carried with the support of two softwares[†† ‡‡]. In the initial univariate analysis the frequency of periodontopathogens was assessed through Chi-square (X^2) test, and HBD-2 protein concentration in saliva was evaluated by Mann-Whitney test. Influence of age on salivary HBD-2 within each periodontal diagnoses was evaluated by Mann-Whitney test. After that Pearson's correlation test was used to analyze all parameters. Finally a multinomial model of logistic regression was built up to investigate the association between the levels of HBD-2 in saliva and the simultaneous presence of 1-2, 3-4 or 5-6 periodontopathogens. Since levels of produced salivary HBD-2 of periodontally healthy and gingivitis was up to 99pg/ml and periodontitis was at least 100pg/ml, we considered this cut-off point for this specific statistical analysis.

3 RESULTS

A total of 89 patients were enrolled in this study: 31 periodontally healthy (25.06 ± 5.97 years); 27 gingivitis (33.22 ± 12.09 years); and 31 periodontitis patients (52.16 ± 11.54 years). Clinical periodontal data are shown in Table 1.

3.1 Isolate microbial and salivary HBD-2 results

Although all periodontopathogens were detected in periodontally healthy, gingivitis and periodontitis patients, *P. gingivalis, P. intermedia, T. forsythia* and *T. denticola* were more frequent ($p < 0.05$) in periodontitis group when compared to both periodontally healthy and gingivitis groups (Figure 1). Figure 1 also shows that when compared to healthy and gingivitis, a higher number of periodontitis patients simultaneously harbored the three bacterial species that compose the red complex, i.e *P. gingivalis, T. denticola* and *T. forsythia.*

While periodontally healthy and gingivitis patients showed similar HBD-2 levels in saliva, periodontitis group displayed a statistically significant increased level of HBD-2, indicating an overproduction of this antimicrobial peptide in this disease status (Figure 2).

There was no relation between age and salivary HBD-2 among periodontally healthy ($p = 0.129$), gingivitis ($p = 0.0973$) or chronic periodontitis ($p = 0.229$) groups.

A similar pattern of no influence was observed when age was evaluated in relation to salivary HBD-2 independently of periodontal diagnosis ($p = 0.084$).

3.2 Correlation between periodontal pathogens and salivary HBD-2

The influence of the presence/absence of the searched bacterial species on the mean salivary levels of HBD-2 was evaluated. Although periodontal clinical status determined a pattern of occurrence of certain species (*P. gingivalis, P. intermedia, T. denticola and T. forsythia*) with similar impact on salivary HBD-2 their mere presence did not show a direct relation to the amount of produced HBD-2 protein (Table 2).

Logistic regression revealed no significant difference between HBD-2 and bacterial species on a confidence interval of 95% and $p < 0.05$.

Finally, we looked for a correlation between microbiological and HBD-2 in the different periodontal status (Table 3). Neither the isolate presence nor the combined presence of pathogens was correlated to salivary HBD-2 (Table 3).

4 DISCUSSION

The defensins are a family of antimicrobial peptides that are vital contributors to the host immune response. Being constitutively or inducibly expressed, defensins have been shown to contribute to innate host defense via direct bacteriocidal activity and to adaptive immunity through effector and regulatory functions[29,30].

Taking into consideration that there are many unclear aspects and controversial results regarding the association between HBD-2 and periodontal status, this study was performed aiming at comparing the levels of HBD-2 protein concentration in saliva of healthy, gingivitis and periodontitis patients, and correlating these levels with the presence of six periodontopathogens. To investigate this relation we did not measure mRNA because, as shown by Hosokawa et al.[31], expression patterns of HBD-2 mRNA and HBD-2 protein concentrations do not seem to be correlated in gingival tissues showing different degrees of inflammation. According to these authors[31], concentrations of HBDs, rather than the detection of their mRNA expression levels, would provide the most plausible information as to the expression profile of functional HBD. Furthermore, Dommisch et al.[16], after evaluating biopsies from different clinical stages of health and disease, reported no significant differences in mRNA expression for HBD-2, HBD-1 and HBD-3. However, not all results from mRNA expression are consensual. As an exemplification, Bissel et al.[32] found high levels of HBD-2 mRNA expression in healthy tissues.

Therefore, in accordance to some of previously published researchs[13,31], the main result of the present study was the salivary overproduction of the investigated antimicrobial peptide in periodontitis in comparison to periodontally healthy and

gingivitis patients. These findings highlight the complexity of the periodontal disease process, since only when bone was also compromised levels of HBD-2 were higher; on the other hand these levels did not differ between periodontal health and the pattern of disease limited to soft tissues. Gursoy & Könönen[33] discussed the impact of inflammation on the secretions of inducible defensins such as HBD-2. In an immunohistochemical analysis, Kuula et al.[13] clearly revealed larger amounts of HBD-2 protein in inflamed periodontal tissues. This finding suggests that, in response to the bacterial burden, host defense in periodontitis can be triggered to produce and release HBD-2 due to its antimicrobial effects. Also, this immunohistochemical analysis demonstrated a low expression of salivary HBD-2 in periodontally healthy patients. Similarly, Hosokawa et al.[31] detected HBD-2 protein in slightly higher concentrations in inflamed gingival tissues than in healthy tissues. Although there is a tendency of HBD-2 to be slightly higher in periodontitis patients[13], in some other cases, equal or lower levels were recovered in comparison to periodontally healthy controls.[12,34] In a previous study published by our group[35], HBD-2 as well as two other inflammatory indicators — Protease-Activated Receptor-2 (PAR_2) and Tumor Necrosis Factor-alpha (TNFα) — showed higher levels in a small group of periodontitis patients (n=10) in comparison to healthy controls. In this same study these immune parameters decreased after conventional periodontal therapy accompanying inflammation reduction revealed by clinical improvement.

Besides the inflammatory effect, Gursoy & Könönen[33] also explored the influence of infection on HBD-2 production. Some studies indicate that periodontitis-related bacteria such as *A. actinomycetemcomitans, P. gingivalis*, and *F. nucleatum* induce upregulated expression of HBD by oral keratinocytes[15,16,36,37]. Also, other

studies demonstrated that HBD show antimicrobial activity against periodontal bacteria[38-40].

Although we observed an association between HBD-2 and the most inflamed periodontal tissues, there was no significant relation considering positivity for the searched periodontopathogens and concentration of the protein in saliva. It should be kept in mind that these results derived from a conventional PCR and that the use of other quantitative laboratorial techniques could reveal different patterns of relation. The present findings, as obtained, suggest a possible modulation by other local factors such as cytokines and enzymes. Interestingly, this possible relation was explored by different strategies of statistical analysis. Neither the correlation test nor the multinomial model of logistic regression supported an association between this host factor and isolated or combined frequency of bacteria (confidence interval 95% and $p < 0.05$). Considering that presence and increased proportions of key periodontopathogens according to pattern of periodontal diseases are well documented, our findings can be considered unexpected or intriguing. More intriguing is the lack of association between HBD-2 and presence of the red complex species traditionally related to increased pocket depth and active lesions of chronic periodontitis. However, there are some plausible scientific explanations to support them. Hosokawa et al.[31] reported as an interesting anomaly the fact that only some bacterial species induced HBD-2 production by gingival epithelial cells (GEC) or neutrophils. Similarly, Krisanaprakornkit et al.[11] reported that *F. nucleatum*, but not *P. gingivalis*, was able to induce HBD-2 mRNA expression in primary cultures of GECs. Maybe the inhibition of HBD-2 by *P. gingivalis* is part of the pathogenic activity demonstrated by this bacterium. In the present study we can speculate that this type of inhibition possibly occurred for *P. gingivalis* and also for the other searched

bacterial species. Interestingly, in a previous study, our group[35] found different results by showing that the increased prevalence of *P. gingivalis* and levels of TNF-α were associated with higher salivary levels of HBD-2 in chronic periodontitis. Brissette & Lukehart[41] investigated different mechanisms possibly related to the resistance of *T. denticola* to HBD. They failed to determine a role of proteases, such as dentilisin as responsible for this decreased susceptibility to HBD. However, they demonstrated that *T. denticola* binds significantly less to HBD-2 and -3 than susceptible organisms do. In addition, efflux pump have been shown to be involved in resistance to this peptide. Vankeerberghen et al.[15] reported that both commensal and pathogenic bacteria induced different HBD-2 expression profiles in epithelial cells from human periodontal pocket. Actually, differences in expression profiles were observed for all defensins investigated by these authors. Moreover, they found a correlation between higher bacterial pathogenicity and HBD expression. The authors of the present study are in agreement with Hosokawa et al.[31], who emphasized that these controversies indicate more than complex but also unknown mechanisms underlying the processes of HBD expression in the gingival tissue that may, in turn, regulate the translation of HBD proteins from their mRNA. A post-translational modification activity involving enzymes, cytokines and other environmental factors could be considered. Vankeerberghen et al.[15], considering that the expression profile of defensins is relevant in oral microbial colonization, had previously suggested the use of their specific profiles as biological markers for the pathogenicity of periodontal bacteria. Defensin production is only one of the mechanisms against bacteria in human immune system. If it were possible to simplify biological processes, we could assume that, in the present study, since bacteria did not induce the production of HBD-2, which activates the immune system, they might more easily survive and grow in the

periodontium and, possibly, cause disease. Considering that the levels of HBD-2 secretion seem to correlate to the incubation time with bacteria[33], it is important to keep in mind the cross-sectional design used in the present study. *Per se*, this study design did not allow us to monitor probable fluctuations that HBD could show as a response against bacterial challenge.

The hypothesis of the present study was only partially accepted because we failed to confirm the positive correlation between salivary levels of HBD-2 and frequency of the searched bacterial species. Although an elucidative contribution could arise from this research, our findings reinforce, most importantly, that we have only incipient knowledge regarding HDB-2 effects in periodontal health-diseases processes.

In conclusion, HBD-2 protein concentration in saliva was higher in chronic periodontitis in comparison to healthy or gingivitis patients. These different protein concentrations were not related to the frequency of periodontopathogens. In this population, clinical inflammatory profile had a higher impact on salivary HBD-2 levels than bacteria.

Footnotes

‡ PCPUNC 15 Hu-Friedy Mfg. Co., Inc., Chicago, IL

§ Tanari, Tanariman Industrial Ltda., Manacapuru, Brazil

|| InstaGene Bio-Rad Laboratories Inc., Hercules, CA, USA

¶ Eppendorf, Westbury, NY, USA

Invitrogen, Carlsbad, CA, USA

** Peprotech, Rocky Hill, NJ, USA

†† Bio Estat 5.0, Brazil

‡‡ SPSS 13.0, USA

Conflict of Interest

The authors report no conflicts of interest related to this study.

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Figure Legends

Figure 1 – Frequency of periodontopathogens in the periodontally healthy, gingivitis and periodontitis patients.

***** statistically significant difference in comparison to gingivitis and periodontally healthy patients (Chi-Square test, $p < 0.05$)

† statistically significant difference in comparison to periodontally healthy patients (Chi-Square test, $p < 0.05$)

Figure 2: Salivary HBD-2 levels (pg/ml) in healthy, gingivitis and periodontitis patients.

* Significant statistical difference ($p < 0.05$)

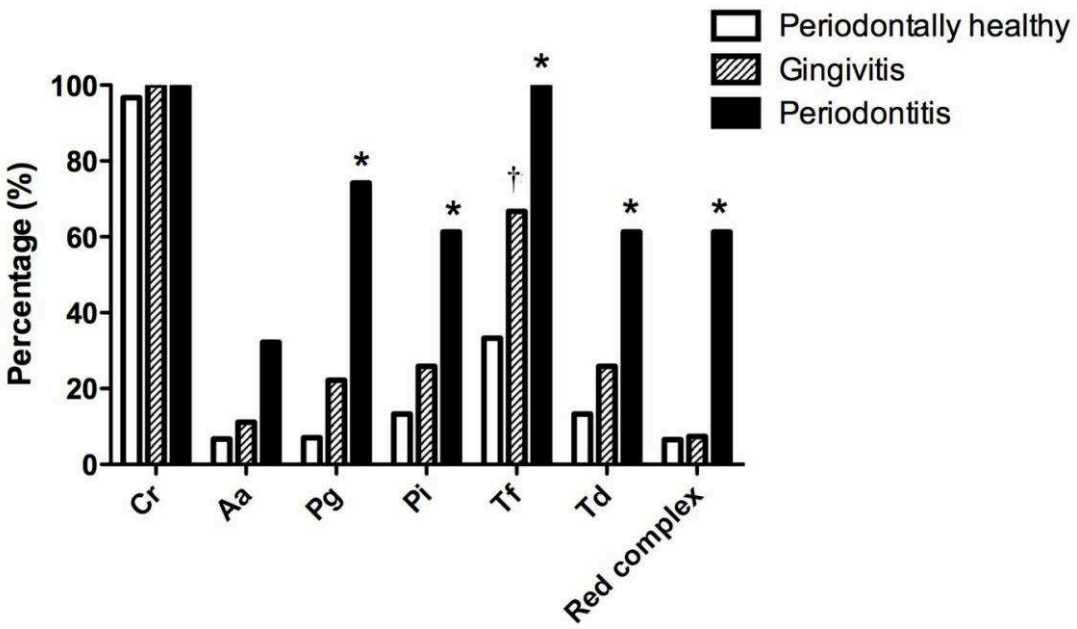

Figure 1

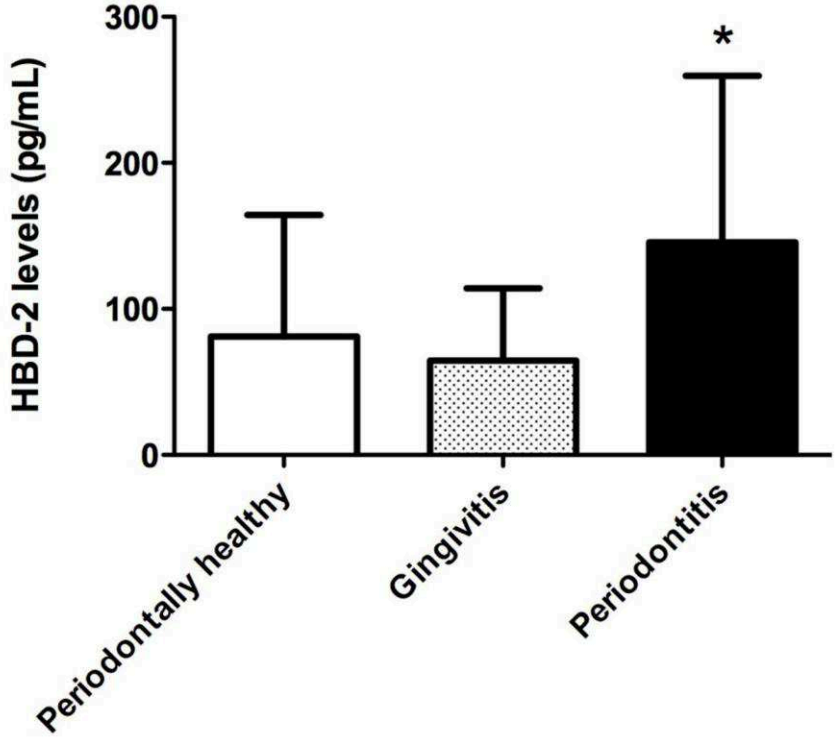

Figure 2

Tables

Table 1 – Periodontal clinical parameters evaluated in all examined population

Group	**PI (%)** *Mean ± SD*	**GI (%)** *Mean ± SD*	**PD (mm)** *Mean ± SD*	**CAL (mm)** *Mean ± SD*
Periodontally Healthy n=31 (12M:19F)	30.70 ± 16.20	5.98 ± 5.73	1.35 ± 0.37	0.67 ± 0.26
Gingivitis n=27 (9M:18F)	70.48 ± 19.73	71.78 ± 22.59	1.78 ± 0.31	0.9 5± 0.28
Periodontitis n=31 (9M:22F)	79.00 ± 17.90	75.03 ± 18.27	3.00 ± 0.45	2.25 ± 0.98

n = number of subjects; M: Male; F: Female; SD: Standard deviation; PI: Plaque index (shown as percentages of teeth having any amount of plaque); GI: Gingival Index (shown as percentages of teeth having any degree of inflammation); PD: Probing Depth; CAL: Clinical Attachment Level; NA: Not applicable.

Table 2: Comparative salivary HBD-2 levels (mean and standard deviations are expressed in pg/ml) among individuals showing negative or positive pooled subgingival samples for each searched bacterial species. Values are shown within each periodontal diagnosis category. Mann-Whitney test did not reveal statistically significant differences between positive and negative bacterial samples.

PERIODONTAL HEALTH

Periodontopathogens	**Negative subgingival samples**		**Positive subgingival samples**	
	n	**HBD-2 levels (pg/mL) Mean (± SD)**	**n**	**HBD-2 levels (pg/mL) Mean (± SD)**
C. rectus	0	-	31	81.01(± 83.44)
A. actinomycetemcomitans	29	77.75 (± 82.21)	2	121.80 (± 123.33)
P. gingivalis	29	82.80 (± 84.56)	2	34.59 (± 37.36)
P. intermedia	27	79.93 (± 83.24)	4	89.69 (± 103.44)
T. forsythia	21	91.47 (± 92.44)	10	60.11 (± 61.07)
T. denticola	27	85.97(± 87.19)	4	41.39 (± 20.96)

GINGIVITIS

Periodontopathogens	**Negative subgingival samples**		**Positive subgingival samples**	
	n	**HBD-2 levels (pg/mL) Mean (± SD)**	**n**	**HBD-2 levels (pg/mL) Mean (± SD)**
C. rectus	0	-	27	64.66 (± 49.39)
A. actinomycetemcomitans	24	65.91 (± 48.23)	3	56.32 (± 67.88)
P. gingivalis	21	64.80 (± 48.21)	6	64.13 (± 59.48)
P. intermedia	20	71.71 (± 52.31)	7	45.55 (± 40.83)
T. forsythia	9	87.10 (± 51.32)	18	56.74 (± 47.70)
T. denticola	19	72.73(± 54.26)	8	49.53 (± 37.11)

PERIODONTITIS

Periodontopathogens	**Negative subgingival samples**		**Positive subgingival samples**	
	n	**HBD-2 levels (pg/mL) Mean (± SD)**	**n**	**HBD-2 levels (pg/mL) Mean (± SD)**
C. rectus	0	-	31	(± 114.07)
A. actinomycetemcomitans	21	145.60 (± 114.07)	10	152.91 (± 117.99)
P. gingivalis	8	145.74 (± 155.20)	23	145.55 (± 99.70)
P. intermedia	12	141.11 (± 128.13)	19	148.20 (± 108.74)
T. forsythia	0	-	31	145.60 (± 114.07)
T. denticola	7	155.20 (± 109.34)	24	142.68 (± 117.71)

n – number of positive or negative subjects for each searched bacterial species
SD – Standard Deviation

Table 3: Person's correlation between isolated or red complex bacterial species and HBD-2 in the different periodontal status

Pearson Correlation Sig. (2-tailed)		Periodontal health (n = 31)			Gingivitis (n = 27)			Periodontitis (n = 31)		
		Red complex	# of bacteria	HBD-2 Levels	Red complex	# of bacteria	HBD-2 Levels	Red complex	# of bacteria	HBD-2 Levels
Periodontal health (n = 31)	Red complex	1								
	# of bacteria	.716 .000	1							
	HBD-2 Levels	-.110 .584	-.094 .642	1						
Gingivitis (n = 27)	Red complex	. .000	.245 .260	-.032 .884	1					
	# of bacteria	. .000	.258 .234	.180 .410	.526 .010	1				
	HBD-2 Levels	. .000	.104 .635	.023 .918	-.222 .309	-.271 .212	1			
Periodontitis (n = 31)	Red complex	.150 .454	.108 .593	.000 1.000	.271 .212	-.040 .855	-.168 .443	1		
	# of bacteria	.104 .605	.065 .784	.138 .493	.399 .059	.071 .748	-.287 .184	.816 .000	1	
	HBD-2 Levels	.162 .421	.079 .697	-.247 .215	.485 .019	.141 .521	-.235 .281	-.105 .581	-.022 .909	1

n – number of participants

3 DISCUSSÃO

O diagnóstico precoce das doenças progressivas é a chave de um tratamento bem sucedido; na periodontite não é diferente. No entanto, uma das grandes dificuldades encontradas nesse caso é que, frequentemente, quando a doença está detectável clinicamente, suas sequelas já estão presentes e são, muitas vezes, irreversíveis.

Assim, é necessário diagnosticá-la na fase mais inicial possível, talvez ainda pré-clínica, para que ela não produza sequelas tão significativas como perda dos tecidos periodontais de suporte e perda dentária.

Algumas substâncias produzidas pelo organismo podem ser mensuradas por meios laboratoriais, e utilizadas como auxiliares dos métodos clínicos para melhor diagnosticar o paciente ou para se prever o momento em que a doença tornar-se-á ativa (Sanz et al., 2007; Nomura et al., 2012). Para essa finalidade, tanto a saliva quanto o fluido gengival podem ser usados (Kaufman & Lamster, 2000; Armitage, 2004; Gheren et al., 2007; Patil & Patil, 2011; Pfaffe et al., 2011; Liu & Duan, 2012; Parwani et al., 2012).

O objetivo deste estudo foi investigar se algumas dessas substâncias poderiam ser úteis para o monitoramento da doença periodontal. Para isso, foram utilizados os parâmetros clínicos e microbianos como referência.

É importante ressaltar que, por se tratar de validação de uma ferramenta diagnóstica, os grupos (saudável, gengivite e periodontite) precisariam estar muito bem definidos; e isso foi comprovado ao avaliarmos os parâmetros clínicos e microbianos (tabela 1 e figuras 4 e 5 — capítulo A).

Nossos resultados foram corroborados por outros autores (Badersten et al., 1981; Greenstein, 1992; Cobb, 1996; Van der Weijden & Timmerman, 2002; Cobb, 2002; Colombo et al., 2005; Ioannou et al., 2009; del Peloso Ribeiro et al., 2008; Swierkot et al., 2009) que demonstraram melhora nos parâmetros clínicos e microbianos após tratamento periodontal não cirúrgico. Isso nos permitiu legitimar a arginase salivar como uma ferramenta útil no auxílio do diagnóstico da doença periodontal.

O ponto mais importante deste primeiro estudo foi que a atividade da arginase salivar estava significativamente ($p < 0,05$) mais elevada quanto mais avançada era a doença periodontal — ou seja, mais elevada na periodontite, seguida pela gengivite, que por sua vez estava mais elevada do que no grupo saudável — e que o tratamento periodontal não cirúrgico tornou o nível de atividade da arginase salivar semelhante nos três grupos ($p > 0,05$).

Nossos resultados foram semelhantes a outro estudo conduzido por nosso grupo com diferentes indivíduos (Gheren et al., 2007). Por outro lado, outros autores (Güllü et al., 2005; Ozer et al., 2011) observaram resultados diferentes dos nossos. Güllü et al. (2005) avaliaram, em tecidos gengivais submetidos a biópsia, a arginase e a óxido-nítrico-sintetase — enzimas que competem pelo mesmo substrato, a arginina — em 13 indivíduos com periodontite crônica, antes e depois do tratamento periodontal cirúrgico e nãocirúrgico. Concluíram que os níveis de arginase eram menores antes do tratamento e maiores após o tratamento. Já os níveis de óxido-nítrico-sintetase seguiram o caminho inverso, estando mais elevados antes do tratamento e mais baixos após o tratamento. Uma possível explicação para esta diferença pode estar no pequeno número de indivíduos participantes do seu estudo. Ozer et al. (2011) também fizeram uma avaliação de arginase e óxido nítrico

em indivíduos saudáveis, com gengivite e com periodontite crônica. Ao contrário do nosso grupo, eles detectaram uma maior atividade de arginase no grupo com gengivite e, assim como Güllü et al. (2005), detectaram um aumento da atividade da arginase após o tratamento periodontal não cirúrgico. Novamente, há que se destacar o menor número de participantes desse estudo em relação ao nosso. Outro ponto intrigante é que, ao observamos a sua tabulação dos resultados clínicos, percebemos uma diferença em relação aos nossos dados clínicos: o seu índice gengival estava quase o dobro no grupo gengivite em relação ao grupo periodontite, ao passo que o nosso índice gengival estava semelhante nos dois grupos; isso talvez possa explicar o fato de que os níveis de arginase salivar de seu estudo estejam maiores no grupo gengivite em relação ao grupo periodontite.

Parwani et al. (2012) utilizaram um delineamento muito semelhante ao nosso para avaliar o óxido nítrico na saliva de indivíduos periodontalmente comprometidos. Como já foi previamente exposto, o óxido nítrico é produzido a partir da arginina pela ação da óxido-nítrico-sintetase que compete com a arginase pelo mesmo substrato. Seus resultados mostram, de maneira interessante, níveis estatisticamente significativos mais elevados de óxido nítrico em indivíduos com periodontite em relação àqueles com gengivite, e destes em relação aos saudáveis. Após o tratamento, houve uma redução dos níveis de óxido nítrico nos indivíduos com gengivite e nos com periodontite. Os autores também encontraram uma correlação positiva entre a profundidade de sondagem e os níveis salivares de óxido nítrico. Menaka et al. (2009) também encontraram níveis estatisticamente mais elevados de óxido nítrico sérico em indivíduos com periodontite em relação a indivíduos saudáveis.

Outro ponto bastante importante a ser ressaltado no nosso estudo é que ele foi — pelo que temos conhecimento — o primeiro a demonstrar que, em indivíduos com periodontite, havia uma maior produção de HBD-2 salivar em relação aos saudáveis, e que o tratamento periodontal não cirúrgico reduziu significativamente seus níveis (figura 2B - Capítulo B).

Isso é respaldado tanto pelos nossos resultados clínicos (tabela 1 - Capítulo B) quanto pela redução da expressão gênica crevicular do PAR_2 (figura 1A - Capítulo B), pela redução da presença de *P. gingivalis* (figura 1B - Capítulo B) e pela redução dos níveis creviculares de TNFα (figura 2A - Capítulo B).

O exato mecanismo pelo qual isso acontece é ainda obscuro. No entanto, alguns estudos anteriores corroboram nossos resultados.

Dommisch et al. (2005) demonstraram que a expressão gênica de HBD-1 e -2 são similares em indivíduos saudáveis e que a HBD-2 está significativamente maior nos indivíduos com gengivite e nos com periodontite crônica. Vardar-Sengul et al. (2007), por sua vez, detectaram maior expressão gênica de HBD-1 e -2 em indivíduos com periodontite crônica em relação aos saudáveis. Kuula et al. (2008) observaram menor expressão gênica de HBD-2 em tecidos periodontais clinicamente saudáveis em relação àqueles com inflamação presente.

Outros estudos (Holzhausen et al., 2010; Fagundes et al., 2011) demonstraram que a expressão gênica do PAR_2 está aumentada em indivíduos com periodontite crônica, e que esta expressão pode ser modulada por proteases (gengipaína) produzidas pela bactéria *P. gingivalis*. Além disso, Chung et al. (2004) observaram que *P. gingivalis* induzem a expressão de HBD por meio do PAR_2.

A influência do TNFα na produção de HBD-2 também foi demonstrada por Lee et al. (2010) e Shin & Choi (2010).

Além disso, nosso estudo também avaliou os níveis de HBD-2 salivares em indivíduos saudáveis, em indivíduos com gengivite e em indivíduos com periodontite, e se havia uma correlação entre eles e periodontopatógenos chave da doença periodontal *Campylobacter rectus, Porphyromonas gingivalis, Tanerella forsythia, Treponema denticola, Prevotella intermedia e Aggregatibacter actinomycetemcomitans.*

Um ponto muito importante do nosso estudo foi a constatação de que os níveis de HBD-2 salivares nos indivíduos com periodontite estavam significativamente maiores do que naqueles saudáveis e nos com gengivite. É interessante notar que nossos resultados estão de acordo com os achados de Hosokawa et al. (2006) e Kuula et al. (2008), que observaram um aumento dos níveis de HBD-2 em tecidos periodontais severamente comprometidos. Isso também corrobora a nossa constatação de que, nos indivíduos com pouco comprometimento periodontal (gengivite), os níveis salivares de HBD-2 não diferiram significativamente dos saudáveis.

Nosso outro estudo (Pereira et al., 2012) demonstrou, similarmente, a maior presença de HBD-2 salivar em um pequeno número de indivíduos com periodontite acompanhada de uma elevação nos níveis de PAR_2 e de TNFα nesses mesmos indivíduos.

Entretanto, outros autores mostraram resultados diferentes dos nossos. Lu et al. (2004) e Brancastisano et al. (2011) demonstraram que os níveis de HBD estavam semelhantes ou menores em tecidos inflamados comparados aos saudáveis.

Quanto a uma possível associação entre HBD-s e periodontopatógenos — inclusive bactérias do complexo vermelho — nosso estudo não conseguiu

demonstrá-la, mesmo usando diferentes estratégias estatísticas como teste de correlação e regressão logística.

Nossos resultados divergem de outros autores. Isso pode ser explicado pelo fato de que algumas bactérias — como a *P. gingivalis* e *T. denticola* — talvez inibam a produção de HBD, mais especificamente HBD-2 (Krisanaprakornkit et al., 2000; Hosokawa et al., 2006; Shin et al., 2010). Entretanto, esta relação ainda é obscura, vez que os resultados da literatura são controversos. Mecanismos complexos e desconhecidos da tradução da proteína HBD-2 a partir de seu mRNA e modificações pós-tradução também podem explicar estas divergências. Há que se ressaltar também o delineamento do nosso estudo — transversal — que nos impossibilitou avaliar possíveis flutuações dos níveis de HBD-2.

Finalmente, podemos chamar a atenção para o fato de que o diagnóstico salivar é hoje uma realidade na pesquisa clínica. Podemos encontrar muitos estudos utilizando a saliva como ferramenta de avaliação. Autores como Streckfus & Dubinsky (2007) e Zimmermann & Wong (2008) tratam da utilização da saliva para fins de diagnóstico de câncer. Outros como Raff & Findling (2010) falam sobre a utilização da saliva como ferramenta para avaliação do hipercortisolismo. Mais especificamente sobre as doenças periodontais, Kaufman & Lamster (2000), Zhang et al. (2009), Patil & Patil (2011), Pfaffe et al. (2011), Liu & Duan (2012) e Parwani et al. (2012) discorrem sobre o valor de alguns biomarcadores salivares como ferramenta de diagnóstico e monitoramento da eficácia do tratamento.

O quanto esta ferramenta estará disponível para a prática clínica diária ou se será útil apenas no âmbito da pesquisa, só o tempo poderá mostrar. O que podemos dizer hoje é que mais estudos são necessários para que ela possa ser validada mais adequadamente.

4 CONCLUSÕES

À luz do que foi exposto nesse estudo, podemos concluir que:

1. O tratamento periodontal não cirúrgico foi eficaz na melhora dos parâmetros clínicos e microbianos dos indivíduos doentes.

2. A arginase salivar está significativamente aumentada em indivíduos com periodontite em relação àqueles saudáveis e aos com gengivite e, por sua vez, nestes em relação aos saudáveis; e que o tratamento periodontal promoveu melhora dos indivíduos doentes; este parâmetro tornou-se estatisticamente semelhante ao dos indivíduos saudáveis. Os achados clínicos e microbiológicos corroboraram os achados salivares.

3. A expressão do RNAm do PAR_2 no fluido gengival e a presença de HBD-2 na saliva de indivíduos com periodontite crônica está significativamente aumentada em indivíduos com periodontite em relação aos indivíduos saudáveis.

4. A presença de HBD-2 salivar está significativamente maior em indivíduos com periodontite em relação àqueles saudáveis e aos com gengivite. Não foi possível demonstrar uma correlação entre ela o os periodontopatógenos investigados.

5. A saliva parece ser uma ferramenta útil para o diagnóstico periodontal e para o monitoramento da eficácia do tratamento periodontal não cirúrgico.

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APÊNDICE

APÊNDICE A – Modelo de Ficha Clínica e Termo de Esclarecimento Livre e Consentido

CD Alexandre Lustosa Pereira	
Periodontista	
FICHA CLÍNICA	
Dados Pessoais do Paciente	
Nome:	
Endereço:	
Fone Res.:	Fone Com.:
Fone Cel.:	Fone Rec.:
Data de Nascimento:	Idade:
Profissão:	
Indicação:	Fone:
Anamnese	
Revisão de Sistemas:	
Cardio-circulatório:	
Respiratório:	
Excretor:	
Endócrino:	
Digestivo:	
Neurológico:	
Outros:	
Gravidez:	
Hospitalizações:	
Cirurgias:	
Medicamentos:	
Reações Alérgicas:	
Hábitos:	
Declaração Declaro, para os fins que se fizerem necessários, que todas as informações acima prestadas são verdadeiras e assumo total responsabilidade sobre as mesmas.	
Data:	Assinatura:

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está sendo convidado (a) a participar, como voluntário (a), em uma pesquisa. Após ser esclarecido (a) sobre as informações a seguir, no caso de aceitar fazer parte do estudo, por favor, assine ao final deste documento, elaborado em duas vias de um só teor. Uma das vias é sua e a outra é do pesquisador responsável. Em caso de recusa em participar do presente estudo, você não será penalizado (a) de forma alguma.

TÍTULO: "Associação dos níveis de atividade de arginase salivar com a severidade da doença periodontal: Avaliação clínica, microbiológica e imunológica, pré e pós terapia não-cirúrgica".

RESPONSÁVEL: C.D. Alexandre Lustosa Pereira.

JUSTIFICATIVA: A doença periodontal (gengival) é uma inflamação que deixa a gengiva inchada, vermelha e, em alguns casos, pode causar o amolecimento e perda do dente. Essa é uma doença muito comum, pois oito em cada dez adultos brasileiros apresentam alguma forma de doença da gengiva. A inflamação da gengiva é causada por bactérias (germes) da boca que vivem ao redor dos dentes e gengiva. Estas bactérias produzem algumas substâncias que, de alguma forma, irritam a gengiva. Em contrapartida, o organismo produz algumas substâncias, chamadas citocinas, que têm o papel de combater estas bactérias. A saliva também apresenta uma substância (arginase) que pode ser usada para se determinar se um paciente apresenta ou não doença da gengiva.

O objetivo deste presente estudo é avaliar se a arginase está presente na saliva dos pacientes com doença na gengiva e se, após o tratamento, a quantidade desta substância diminui.

Neste estudo serão examinados pacientes adultos, entre 20 e 45 anos de idade, para saber se apresentam doença da gengiva. Também será avaliado se estes pacientes possuem na boca algumas bactérias chamadas *Tannerella forzythia, Treponema denticola, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* e *Prevotella intermedia*, e se sua gengiva possui algumas citocinas: interleucina 6, interleucina 1 beta e fator alfa de necrose tumoral. Todos os dentes serão examinados e será coletado um pouco de líquido da gengiva com um cone de papel. Também será coletada um pouco de saliva em um pote plástico. O exame leva aproximadamente 20 minutos, não dói e não causa qualquer problema para a pessoa. Se o paciente não quiser participar deste estudo será feito apenas um exame mais rápido para ver que tipo de tratamento dentário a pessoa precisa. Se o senhor (a) quiser participar, mas durante o exame não gostar de alguma coisa é só falar com o dentista que ele irá parar na mesma hora. O tratamento dentário será realizado mesmo que o (a) senhor (a) não queira participar do estudo.

CUSTO E PAGAMENTO: Não haverá nenhum custo extra para participar deste trabalho. Todo o material necessário será fornecido pelo pesquisador.

SIGILO: Caso você aceite participar deste estudo será feito um cadastro numa ficha que pertence ao responsável pela pesquisa. Será mantido segredo do seu nome e não será divulgado o seu nome em trabalhos apresentados na faculdade, congressos etc.

INDENIZAÇÃO E DANOS: Você deve saber que a coleta do líquido da bochecha e língua com um cotonete de algodão ou da gengiva com uma cone de papel não vai causar nenhum dano a você. Assim, não haverá qualquer tipo de pagamento pelo exame.

CONSENTIMENTO VOLUNTÁRIO: Você deve ter entendido tudo o que leu. Uma cópia deste Termo será entregue para você e outra ficará arquivada com o pesquisador responsável. A assinatura abaixo significa que você concorda em participar deste estudo.

DECLARAÇÃO

Eu, ... nacionalidade ....., estado civil ..... profissão....., nascido (a) em / /

na Cidade de....., Estado de ..... portador da cédula de identidade número ..... CPF....., residente e domiciliado a .....

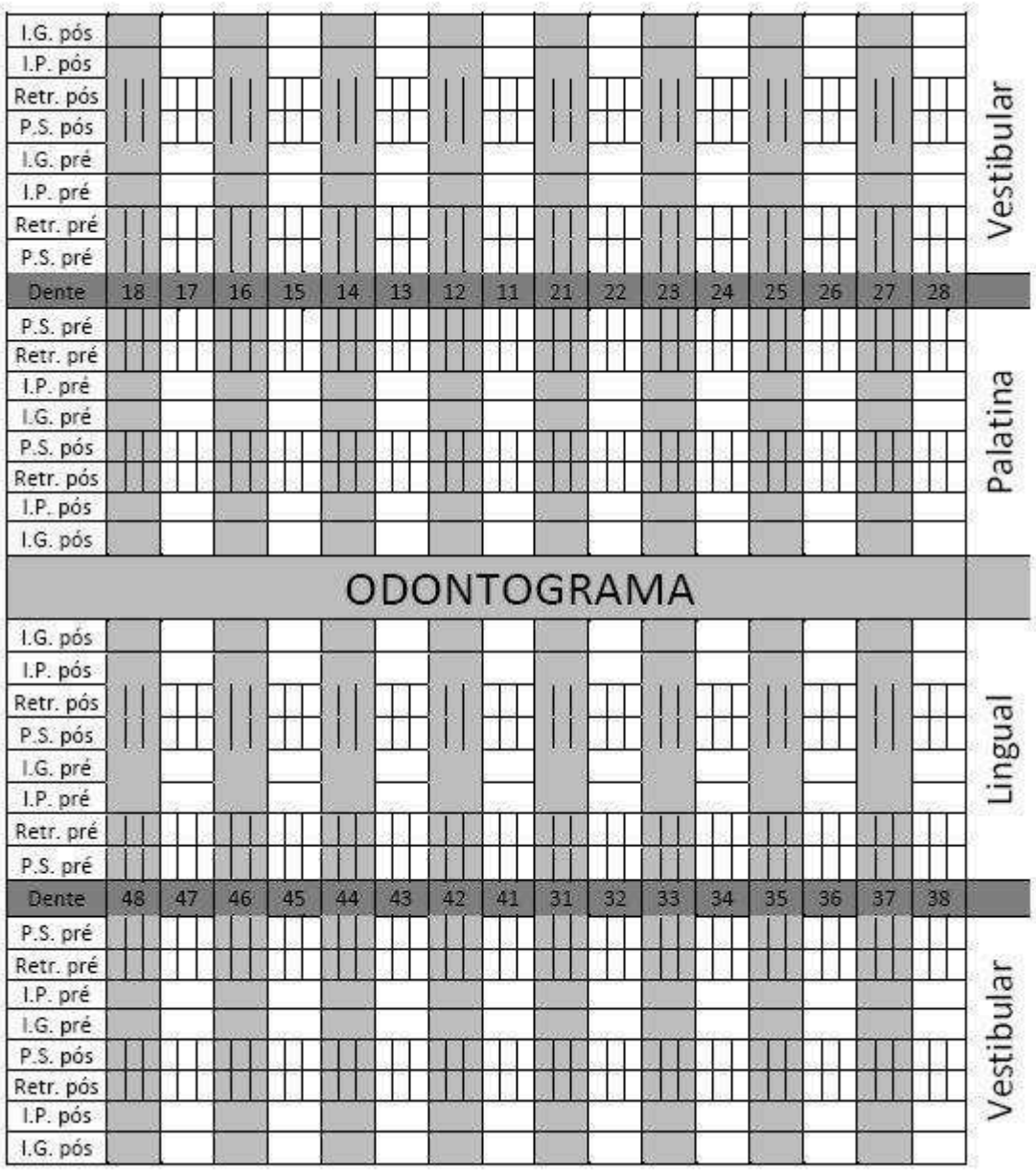
.....

, declaro ter sido inteiramente esclarecido (a) sobre o estudo e ter lido e entendido o termo que estou assinando abaixo.

_____ _____

Assinatura do paciente Assinatura do responsável

Goiânia, _____de _____ 200__.


I.G. pós
I.P. pós
Retr. pós
P.S. pós
I.G. pré
I.P. pré
Retr. pré
P.S. pré
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Vestibular
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Retr. pré
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I.G. pré
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Palatina
ODONTOGRAMA
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Retr. pós
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Dente 48 47 46 45 44 43 42 41 31 32 33 34 35 36 37 38
Lingual
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Retr. pós
I.P. pós
I.G. pós
Vestibular

Data	Procedimentos Realizados	Assinatura do Paciente

ANEXOS

ANEXO A – Declaração do Comitê de Ética em Pesquisa

UNITAU

PRPPG-Pró-reitoria de Pesquisa e Pós-graduação
Comitê de ética em Pesquisa
Rua Visconde do Rio Branco, 210 Centro Taubaté-SP 12020-040
Tel.: (12) 3625.4143 – 3635.1233 Fax: (12) 3632.2947
cep@unitau.br

DECLARAÇÃO Nº 385/08

Protocolo CEP/UNITAU nº 386/08 (Esse número de registro deverá ser citado pelo pesquisador nas correspondências referentes a este projeto)

Projeto de Pesquisa: *Associação dos níveis de atividade de Arginase Salivar com a severidade da doença Periodontal: Avaliação clínica, microbiológica e imunológica, pré e pós terapia não-cirúrgica*

Pesquisador(a) Responsável: Alexandre Lustosa Pereira

O Comitê de Ética em Pesquisa, em reunião de **08/08/2008,** e no uso das competências definidas na Resolução CNS/MS 196/96, considerou o Projeto acima **aprovado,** após atendimento às pendências.

Taubaté, 28 de agosto de 2008

Prof. Robison Baroni
Coordenador do Comitê de Ética em Pesquisa da Universidade de Taubaté

ANEXO B – Questionamentos e respectivas respostas aos revisores da revista Quintessence International

Decision and reviewer reports

Dear reviewers,
Below are changes made as requests.
Thank you.

Comments to authors:
This paper was aimed to determine the SAA before and after nonsurgical periodontal therapy. Overall, the study could be of interest to the Journal readers. There are, however, several issues to be address in order to improve this paper presentation.
1. Scientific English Editing might improve the presentation of this paper.

2. The introduction should be written in a more concise and to-the-point manner.
It was removed second paragraph.

3. Why did the authors chose not to use a common and acceptable classification for periodontal disease such as the AAP classification??
AAP classification was used to categorize health, gingivitis and periodontitis subjects. However, we refined this touchstone with Lopez parameters.

4. What is the reason for the discrepancy in measurements time in the different groups (30 vs. 50 days)?
Gum disease reflects an inflammatory process confined to the gingiva and its clinical resolution occurs by reduction of this process and by sulcular epithelium regeneration. Periodontitis, in turn, presents bone support loss for the formation of periodontal pocket and its clinical resolution is achieved by bone loss stabilization and by periodontal pockets reduction, usually through long junctional epithelium. Thus, for the second group, there is need for a longer clinical reassessment [Kon et al. (1969), O'Bannon (1964), Ramfjord; Kiester (1954), Waerhaug (1955)].

5. The Discussion should be shortened and re-written in a less cumbersome and more summarizing way.
It was done according your suggestions.

6. The fact that this issue requires further studies with larger cohort and better definitions of the periodontal disease should be referred to in the Discussion section.
It was done according your suggestions.

7. The conclusions should be tuned down – please remember this is only a short term, small cohort, study.

It was done according your suggestions.

Review:
Date of review:2011-10-22
Comments to authors:
Please emphasize the relevance to the general practitioner (Quintessence International readership is based on general practitioners) **(done – last paragraph in discussion)** and add clinical pictures of the discussed pathology. **(done – figures 1, 2 and 3)**
2 of 2

Decision and reviewer reports

Dear reviewers,
Below are changes made as requests.
Thank you.

Review:
Date of review:2011-11-19
Comments to authors:
The manuscript is accepted with a condition that the conclusion will be modified in the abstract from:

"we concluded that SAA represents a reliable indicator to the therapeutic response of chronic periodontitis subjects treated by non-surgical periodontal therapy."
To
"SAA has a potential to serve as a reliable indicator to the therapeutic response of chronic periodontitis subjects treated by non-surgical periodontal therapy."
And in a similar manner in the discussion.
Done in abstract and discussion.

One study on 58 patients with various periodontal diseases cannot have such a definitive conclusion

ANEXO C – Questionamentos e respectivas respostas aos revisores da revista Archives of Oral Biology

Reviewer comments:

Reviewer #1: This study comparatively evaluates salivary human beta defensin-2, GCF TNF-alpha and PAR2 mRNA in chronic periodontitis patients as well as periodontally healthy control subjects. The study is interesting and provides interesting data however, there are some points to be addressed by the authors before the manuscript can be accepted for publication in Archives of Oral Biology. We appreciate your suggestions and provide explanations and/or answers below each comment and through the correction of the manuscript.

1. The language usage needs to be improved throughout the manuscript. There are quite many errors.
Text was revised for grammar and language use.

2. Please do not repeat the findings in the conclusion of the abstract only make a comment.
Text was rewritten according your request.

3. The first sentence of page 5 needs to be rewritten for clarity.
Sentence was rewritten.

4. Do not number the sections.
Corrections made according to your suggestion.

5. Write "Saliva samples were collected from all individuals." The first sentence under the subheading of Saliva sampling.
Sentence was included.

6. Write "Reaction was stopped using …" The first line of page 9.
Sentence was rewritten.

7. I suggest presenting the data under two subheading; Clinical findings and Laboratory findings instead of 5 sections.
Topic 'Results' is now presented in Clinical Findings and Laboratory Findings.

8. Please rewrite the conclusion at the end of discussion.
Conclusion was rewritten according your request.

9. Please provide a power analysis, the number of individuals in each study group is rather low.
A sample size calculation done previously to the study concluded that, by using a power of 0.85, having a statistical significance level of 0.05 and by applying the Student *t* test per group, 10 was an accurate number of individuals to be included in each experimental group.

10. In Table 1, it would be good to see the number of sites with a PD>4 mm to better document the disease severity and localisation of chronic periodontitis.
Before treatment, periodontal sites showing PD>4mm were 493 out of 1326, i.e., 37.17% of them.

11. Please discuss the reason for recollecting the biological samples 50 days after completion of non-surgical periodontal treatment.
In periodontitis, bone support loss leads to the formation of periodontal pocket. Its clinical treatment is done by the stabilization of bone loss, reduction of periodontal pockets and gain in attachment level, usually through the long junctional epithelium. Traditional papers have demonstrated that the healing process is slow and takes about 50 days [Kon et al. (1969), O'Bannon (1964), Ramfjord; Kiester (1954), Waerhaug (1955)]. As this research was a preliminary investigation about the subject, the time for recollection was determined based on clinical parameters.

Reviewer #2: Human <beta>-defensin-2 and protease activated receptor-2 expression in patients with chronic periodontitis.
This clear and well-presented manuscript describes a study to investigate salivary human <beta> defensin-2 levels and associated protease activated-2 mRNA expression in chronic periodontitis and to evaluate if treatment affects their expression.
Before this manuscript is acceptable for publication attention should be paid to the following:
We appreciate your nice comments. We provide explanations and/or answers below each comment and through the correction of the manuscript.

* Attention to grammar is needed in places.
Text was revised for grammar and language use.

* Introduction - line 4 - the constitutive expression of defensins - does this relate to an in vivo situation? Other in vitro studies with oral cell lines have shown human <beta> defensin-2 to be inducible.
You are right about your first question. The first two references mentioned in the manuscript are from *in vivo* studies related to the constitutive expression of defensins. In addition, there are *in vitro* studies which indicted this same constitutive pattern [Harder et al (1997)]. However, there are studies showing that defensins could be upregulated by some stimuli [Vankeerberghen et al. (2005), Beckloff; Diamond (2008), Diamond; Ryan (2011)]. Our focus on line 4 was to highlight these differences.

* The saliva samples from the periodontitis subjects were taken 50 days after therapy - please provide justification.
In periodontitis, bone support loss leads to the formation of periodontal pocket. Its clinical treatment is done by the stabilization of bone loss, reduction of periodontal pockets and gain in attachment level, usually through the long junctional epithelium. Traditional papers have demonstrated that the healing process is slow and takes about 50 days [Kon et al. (1969), O'Bannon (1964), Ramfjord; Kiester (1954), Waerhaug (1955)]. As this research was a preliminary

investigation about the subject, the time for recollection was determined based on clinical parameters.

* Is it possible to speculate as to the importance of actual levels of *Porphyromonas gingivalis* as the PCR detection method only determined presence vs. absence.
We agree that determining the presence/absence of bacteria has a limited predictive value as an isolated diagnostic tool. However, in the present study we aimed at analyzing the possible association between HBD-2 levels and PAR_2 expression. Although we are able to classify patients as healthy or periodontally diseased through clinical parameters, in the preset study we decided to combine a microbiological indicator due to our previous research that demonstrated the association between *P. gingivalis* and an upregulation of PAR_2 gene expression.

Editor: Extensive revision is needed especially in the English. Please revise and resubmit.
Text was revised for grammar and language use.

ANEXO D – Questionamentos e respectivas respostas aos revisores da revista Journal of Periodontology

Reference: Manuscript JOP-12-0321
Title: Influence of periodontal status and periodontopathogens on levels of oral human beta-defensin-2 in saliva

29-Jun-2012

Dear Editor,

We are grateful for the opportunity of reviewing our manuscript. Please, find below all answers and explanations. In the revised manuscript all changes are highlighted in yellow.
Regards,
Jose Roberto Cortelli

Dear Reviewers,
We appreciate your comments and efforts to help us improve our paper. Bellow you will find answers to your questions and specific explanations. Please check manuscript for final changes highlighted in yellow.

Reviewers: 1
Reviewer Comments to Author:
The aim of this study was to assess the influence of periodontal status and periodontopathogens on levels of oral human beta-defensin-2 (HBD-2) in saliva. The study concluded that salivary levels of HBD-2 are higher in patients with periodontitis as compared to those with gingivitis and periodontally healthy controls.
In general this study is interesting; however, there are several technical issues that should be clarified before this study is considered further for publication.

General comments:
1. The authors did not adhere to the "Authors instructions" of the Journal of Periodontology. Please correct.
2. Throughout the manuscript the authors used two referencing styles in conjunction.
3. There are many undefined abbreviations throughout the manuscript.
4. The reference list should have be revised. It does not precisely follow the style recommended by Journal of Periodontology.
Answers (questions 1 to 4): Changes were made accordingly.

5. Figure 3 is unclear and redundant.
Answer: Figure 3 was updated. Information was given just once to avoid redundancy.

6. The text throughout the manuscript should clarify that "salivary" HBD-2 levels were investigated.
Answer: We added the terms “salivary” or “in saliva” in key-points to help readers keep this information in mind.

7. English vocabulary and expression should be revised by a professional scientific writer.
Answer: It was done. Please, see the certificate attached.

Introduction:
1. Page 3, lines 43-50 ("antimicrobial activity----also investigated"): This statement should be supported by an adequate number of references.
Answer: References ***(****Vankeerberghen A, Nuytten H, Dierickx K, Quirynen M, Cassiman JJ, Cuppens H. Differential induction of human beta-defensin expression by periodontal commensals and pathogens in periodontal pocket epithelial cells. J Periodontol 2005;76:1293-1303 / Taguchi Y, Imai H. Epression of beta-defensin-2 in human epithelial cells in response to challenge with Porphyromonas gingivalis* in vitro*. J Periodontal Res 2006;41:334-339 / Lu Q, Darveau RP, Samaranayake LP, Wang CY, Jin L. Differential modulation of human {beta}-defensins expression in human gingival epithelia by Porphyromonas gingivalis lipopolysaccharide with tetra- and penta-acylated lipid A structures. Innate Immun 2009;15:325-335)* **were added.**

2. Page , line 50 ("some studies have suggested…"): Please cite these studies that you have referred.
Answer: References ***(****Dommisch H, Açil Y, Dunsche A, Winter J, Jepsen S. Differential gene expression of human beta-defensins (hBD-1, -2, -3) in inflammatory gingival diseases. Oral Microbiol Immunol 2005;20:186-190 / Vardar-Sengul S, Demirci T, Sen BH, Erkizan V, Kurulgan E, Baylas H. Human beta defensin-1 and -2 expression in the gingiva of patients with specific periodontal diseases. J Periodontal Res 2007; 42: 429-37 / Kuula H, Salo T, Pirilä E, Hagström J, Luomanen M, Gutierrez-Fernandez A, et al. Human beta-defensin-1 and -2 and matrix metalloproteinase-25 and -26 expression in chronic and aggressive periodontitis and in peri-implantitis. Arch Oral Biol 2008; 53: 175-86)* **were added.**

3. Where is the hypothesis?
Answer: The hypothesis of the present study was that salivary levels of HBD-2 tend to increase according to periodontal status, i.e, that there is a higher level of the peptide in periodontitis than in gingivitis and periodontal health. In addition, it was hypothesized that this increase in the salivary levels of HBD-2 is accompanied by an increase in the frequency of target periodontal bacterial species.
This information is cited in the revised manuscript before the aims of the study. Also, we added under Discussion that this hypothesis was only partially confirmed.

Materials and Methods:

1. The complete name and location of the ethical committee and its associated

institution should be given. In this same section you could address the consent form issue. Also, a subheading such as "Ethical guidelines" is required.
Answer: This subheading as well as the other required information were included in the revised manuscript.

2. How did the authors come up with this sample size?
Answer: Patients were recruited from a Dental Continuing Education Institute named Escola de Aperfeiçoamento Profissional (EAP) in the central area of the Country. Information about sample size calculation was also provided as follows:
Prior to the present study, clinical, microbiological and immunological parameters were evaluated in 5 periodontally healthy, 5 gingivitis and 5 chronic periodontitis participants. Results from this pilot study were statistically analyzed (Student t test) to calculate the size of the population (95% of significance and power of 90%). Frequency of *P. gingivalis* in the healthy group required the highest number of individuals determining groups of at least 20 participants. Data from this pilot study was not considered in the final statistical analysis.

3. How were periodontally healthy, patients with gingivitis and patients with periodontitis defined? What was the criteria? Definitions of disease conditions in each group should also be supported with references.
Answer: Besides two references we added a brief description of periodontal status definition as follows: 1) periodontally healthy (participants showing a mean periodontal attachment level < 1.5 mm and no sites with > 2.0mm attachment loss); 2) gingivitis (no radiographic evidence of periodontal bone resorption and more than 30% of bleeding sites); or 3) periodontitis (4 or more teeth with at least one site with attachment loss > 3mm and 4 or more teeth with at least one site showing pocket depth > 4mm)

4. Did you take intraoral or panoramic radiographs?
Answer: Panoramic radiographs were taken. This data is cited in the revised manuscript.

5. How did the authors classify gingivitis in their study population? Mild, moderate, severe?
Again this categorization should be supported by references. Severity of gingivitis could have had an influence on your overall results.
Answer: The gingivitis classification used by the authors is the definition given by Lopez et al. which does not distinguish severity of gingivitis. We can, however, by analyzing our results (GI 71.78 ± 22.59), classify patients as showing moderate gingivitis.

6. There is no methodology criteria and references stated for clinical measurements.
Answer: Periodontal clinical examination was described as follows: A complete periodontal examination was carried out by a single calibrated examiner (ALP). Measurements of periodontal pocket depth, clinical attachment level, plaque (Silness & Löe.1964) and gingival indices (Löe & Silness, 1963) were obtained in six sites per tooth using a manual periodontal probe.

7. How did the authors know about the systemic health and tobacco habits of the participants? Was it self-reported? Did you use a questionnaire? If yes, then this should have been mentioned in the methods that demographic data and information about tobacco habits and systemic conditions was collected using a questionnaire. Also references should be entered showing the influence of tobacco smoking and systemic conditions (such as poorly-controlled diabetes, prediabetes etc.) on periodontal health.
Answer: Data and personal information related to the medical and dental histories were obtained by interview. Only never smokers participated in the present study. References were added. Please, check the revised manuscript.

8. Page 5, line 43: What is "60s? Is it 60 seconds?
Answer: Yes, it is. The word "seconds" is now spelled out.

9. "Patients were instructed not to eat or drink prior to sampling…" For how long were they instructed to refrain from eating and drinking?
Answer: For 2 hours *[**Cortelli SC, Cortelli JR, Holzhausen M, Franco GC, Rebelo RZ, Sonagere AS, Queiroz Cda S, Costa FO. Essential oils in one-stage full-mouth disinfection: double-blind, randomized clinical trial of long-term clinical, microbial and salivary effects. J Clin Periodontol. 2009 Apr;36(4):333-42****]*****. This information is mentioned in the revised manuscript.**

10. Why were the patients instructed to rinse their mouths with water before saliva collection? Would this somehow influence the salivary HBD-2 concentrations?
Answer: To remove any possible residual food and debris. Different research and even previous studies published by our group applied this same protocol *(**Özmeriç N, Elgün S, Uraz A. Salivary arginase in patients with adult periodontitis Clin Oral Invest (2000) 4:21–24; Gheren LW, CORTELLI JR, Rodrigues E, Holzhausen M, Saad WA. Periodontal therapy reduces arginase activity in saliva of patients with chronic periodontitis. Clin Oral Investig. 2008 Mar;12(1):67-72.; Rocha AD, Zenóbio EG, Van Dyke T, Silva KS, COSTA FO, SOARES RV. Differential expression of salivary glycoproteins in aggressive and chronic periodontitis. J Appl Oral Sci. 2012;20(2):180-5.****)***

11. From the manuscript flow, it seems that clinical measurements were made after saliva collection. If this is true, then there is a possibility that rinsing the mouth with water could have influenced the clinical measurements, especially the plaque index. Please respond.
Answer: In the revised manuscript periodontal measurements come first. Therefore the authors believe that now it is clearer that sampling was performed after clinical examinations. To emphasize this order under Samples collection we added "All samples were collected after clinical examinations".

12. Why was stimulated saliva collected specifically? Why not unstimulated whole saliva? This means that the saliva collected was more serous (because of the chewing stimulus). Couldn't this have influenced your overall all results?
Answer: For different purposes, our group has been working with unstimulated whole saliva and/or stimulated saliva *(**1- Cortelli SC, Feres M, Rodrigues AA, Aquino DR, Shibli JA, Cortelli JR. Detection of Actinobacillus actinomycetemcomitans***

in unstimulated saliva of patients with chronic periodontitis. J Periodontol. 2005 Feb;76(2):204-9; 2- Gheren LW, CORTELLI JR, Rodrigues E, Holzhausen M, Saad WA. Periodontal therapy reduces arginase activity in saliva of patients with chronic periodontitis. Clin Oral Investig. 2008 Mar;12(1):67-72.; 3- Cortelli SC, Cortelli JR, Holzhausen M, Franco GC, Rebelo RZ, Sonagere AS, Queiroz Cda S, Costa FO. Essential oils in one-stage full-mouth disinfection: double-blind, randomized clinical trial of long-term clinical, microbial and salivary effects. J Clin Periodontol. 2009 Apr;36(4):333-42; 4 - Queiroz DA, Cortelli JR, Holzhausen M, Rodrigues E, Aquino DR, Saad WA. Smoking increases salivary arginase activity in patients with dental implants. Clin Oral Investig. 2009 Sep;13(3):263-7; 5 - Cota LO, Aquino DR, Franco GC, Cortelli JR, Cortelli SC, Costa FO. Gingival overgrowth in subjects under immunosuppressive regimens based on cyclosporine, tacrolimus, or sirolimus. J Clin Periodontol. 2010 Oct;37(10):894-902; 6- Rocha AD, Zenóbio EG, Van Dyke T, Silva KS, COSTA FO, SOARES RV. Differential expression of salivary glycoproteins in aggressive and chronic periodontitis. J Appl Oral Sci. 2012;20(2):180.). **Actually, in our first research on defensins (***Forte LF, Cortelli SC, Cortelli JR, Aquino DR, de Campos MV, Cogo K, Costa FO, Franco GC. Psychological stress has no association with salivary levels of β-defensin 2 and β-defensin 3. J Oral Pathol Med. 2010 Nov;39(10):765-9. doi: 10.1111/j.1600-0714.2010.00933.x.***) we worked with unstimulated saliva collected from periodontally healthy individuals. Before doing additional studies with patients showing different diagnosis – including the present one – we decided to carry a pilot study with individuals not considered for the final statistical analysis. In the pilot study we found high unstimulated salivary levels of HBD-2 in all groups without significant differences among them. Also, although showing lower HBD-2 levels, stimulated saliva provided significant differences among groups (periodontitis > gingivitis and periodontal health). Based on this pilot study we decided to work with stimulated saliva. This type of saliva also revealed differences in the present population. The use of stimulated saliva to detect HBD-2 is described in our paper published on Arch Oral Biol. (***Pereira AL, Holzhausen M, Franco GC, Cortelli SC, Cortelli JR. Human β-defensin 2 and protease activated receptor-2 expression in patients with chronic periodontitis. Arch Oral Biol. 2012. http://dx.doi.org/10.1016/j.archoralbio.2012.04.018***). In a recent publication stimulated salivary concentration of HBD-2 also differed between HIV infected subjects with or without antiretroviral therapy (***Nittayananta W, Kemapunmanus M, Amornthatree K, Talungchit S, Sriplung H. Oral human β-defensin 2 in HIV-infected subjects with long-term use of antiretroviral therapy. J Oral Pathol Med. 2012 Jun 9. doi: 10.1111/j.1600-0714.2012.01183.x.***)

13. There is no reference for microbial analysis.
Answer: A reference was added.

14. What were the positive and negative controls?
Answer: DNA isolated from culture collection bacterial strains was used as positive control while purified PCR-grade water served as the negative control. This information was added to the revised manuscript.

Results:
1. The results are haphazardly arranged.

2. The authors could have used subheadings to make thir results more reader friendly.
Answer: We apologize for this inconvenience. We used subheadings to clarify this chapter.

Discussion:
1. The discussion mainly reports work done by others instead of discussing the main findings.
Answer: We explored our own findings more deeply. However, we kept in mind that this was a cross-sectional study.

2. The English vocabulary and reference style is questionable.
Answer: As mentioned before English and style were revised.

3. Please note that phrases such as "in a very nicely written paper", page 10, line 41) are not scientific. There are other ways to applaud others' work.
Answer: Non-scientific "adjectives" were deleted.

4. The Discussion overall looks haphazardly arranged. This reviewer read it many times but everytime was remained confused. Please address.
Answer: We reorganized some parts and ideas to clarify the text. The same order used under Results was maintained. Only new sentences are highlighted.

Reviewer: 2 (Format)
Reviewer Comments to Author:
AFFILIATIONS: please put degrees & positions with author list & only use * for affiliation.
Answer: Done as requested.

TERMINOLOGY
Please:
Avoid use of "subject" – individual, participant, patient are all acceptable.
Answer: The term "subject" was substituted throughout the text.

Spell out all terms at first mention in the abstract & again in the main text.
Answer: Done as requested.

KEY WORDS: please confirm all are from the MeSH documentation.
Answer: Confirmed as requested.

REFERENCES IN TEXT: please remove all author/publication year information you have in parentheses after citing the reference numbers.
Answer: Done as requested.

MATERIALS & METHODS: please include study dates in this section.
Answer: Dates were included as requested.

PAGE 5, line 7: please identify the examiner by initials if author; by name & affiliation if not.

Answer: Examiner was identified by initials.

PRODUCT FOOTNOTES:
Please:
Follow symbol sequence begun with affiliations.
Answer: Done as requested.
Remove all brand names from the text & replace with generic terms [add brand names to footnotes]
Answer: Done as requested.
Add location for software programs.
Answer: Done as requested.

CONFLICT OF INTEREST STATEMENT: please add one before the reference list.
Answer: The statement was added as requested.

REFERENCES
Please:
Use a period, not a dash, after each reference number.
Put a period at the end of each citation.
Put bacteria names in italics.
Use MEDLINE journal abbreviations throughout.
Answer: All changes were properly done.

21: What does “University of Southern California School of Dentistry” mean?
Answer: We apologize for this mistake. The wrongly typed information was erased.

31: Provide print publication data when available.
Answer: DOI was changed to print publication.

FIGURE NOTENOTES: please follow symbol sequence beginning with *.
Answer: Done as requested.

TABLES 3 & 4: these are not called out in the text.
Answer: Callings were included.

Figure 3: suggest you identify this as a table.
Answer: Figure 3 now is identified as a table 4.

Reviewer 3: Reviewer Comments to Author:
Comments to Author:
The study entitle “Influence of periodontal status and periodontopathogens on level of oral human beta-defensin-2 in saliva” by Alexandre L. Pereira *et al.* is very interesting. Authors concluded from this study that levels of beta-2 defensins-2 were influenced by periodontal status but were not related with the frequency of periodontal pathogens. The manuscript is confirmatory what already reported in literature.
Answer: The authors are grateful for your nice appreciation of our research.

This study was planned to investigate from a total of 89 subjects:31, periodontally healthy,27
gingivitis and 31 chronic periodontitis. In the abstract under subheading background authors have mentioned expression pattern of human beta defensin 2(HBD-2) mRNA or HBD 2 protein concentration and periodontal disease have been a focus of scientific research. Authors have not checked expression level of defensin 2 at transcription level anywhere in this study, so this is very misleading. Three HBD subtypes have been isolated from human oral tissue. Its better if author discuss the reason for measuring HBD-2 level. HBD-1 is constitutively expressed, whereas HBD-2 and HBD-3 are considered upreglated in inflamed tissue.
Answer: You are right. We did not check transcription. Our intention under Background was to mention that two types of investigation had been conducted. However, based on your point of view, authors revised the manuscript and now abstract starts by showing the types of human beta defensins.

This manuscript contain four tables out of which Table 1 is clinical periodontal measurements of patients with chronic periodontis and healthy subject which is not essential as these are well established parameters in literature.
Answer: This table was deleted.

The manuscript needs the minor revision in light of following published literature.

1-Authors have following manuscript recently accepted in Arch of Oral Biology. So it's essential to discuss and compare the work in accepted manuscript and this one. Accepted manuscript should be included in this manuscript.
"Human beta-defensin 2 and protease activated receptor-2 expression in pateins with chronic
periodontitis." Pereira *et al*. Arch Oral Biol 2012 May 28.
Answer: In fact Arch Oral Biol sent us this acceptance letter just after the present manuscript had been submitted to JOP. In the revised manuscript the Arch Oral Biol paper is mentioned.
Our group has been working with human beta-defensins (*Forte LF, Cortelli SC, Cortelli JR, Aquino DR, de Campos MV, Cogo K, Costa FO, Franco GC. Psychological stress has no association with salivary levels of β-defensin 2 and β-defensin 3. J Oral Pathol Med. 2010 Nov;39(10):765-9.***). Therefore, now we have results from a larger population. In the AOB paper only *P. gingivalis* positive patients were investigated. Therefore, a small final sample composed of 10 chronic periodontitis individuals (pre vs. post-treatment) and 10 periodontally healthy controls were analyzed. For the AOB study former-smokers were considered; only patients who reported tobacco usage within 6 months of screening were excluded. In the present study only never smokers were able to participate.**

In the present study, in addition to a higher number of periodontitis and healthy participants, we investigated a third group of gingivitis patients. Also, the study published in AOB detected only the presence of *P. gingivalis* in relation to TNF-α, PAR-2, and HDB-2 while the present research investigated six bacterial species in relation to HBD-2 only.

2-Mechanism of decreased susceptibility to beta-defensins by *Treponema denticola*. Brissette and Lukehart. Infect Immun 2007, 75(50)307-3015.
Answer: Thanks for your suggestion. This paper helped us to discuss our results and now it is mentioned under discussion.

Reference: Manuscript JOP-12-0321.R1
Title: Influence of periodontal status and periodontopathogens on levels of oral human beta-defensin-2 in saliva

13-september-2012

How were the bacteria determined? Qualitatively or quantitatively? This is a major aspect in the study because this might influence and question the results of the present study. An only qualitatively assessment does not allow to regard the amount of present bacteria, which might influence the salivary level of HBD-2.
Answer: Bacteria were determined qualitatively. We know the presence of bacteria itself is not enough to diagnose disease, and that is why we associated this data with clinical measurements. This association is well documented and is commonly used in clinical studies. Furthermore, in spite of its limitations, literature supports the use of qualitative methods for bacterial detection associated with HBD (Brancatinsano et al, 2011 and Pereira et al, 2012). The manuscript you reviewed did not contain information on clinical data because it had been removed according to suggestions given by another reviewer. Also, we know that in further studies the use of quantitative techniques will improved the observed data.

- Were chronic and/or aggressive periodontitis patients included? is anything known in the literature about differences between chronic and aggressive periodontitis regarding HBD-2 levels? what about the aspect of differences in bacterial colonization between these 2 disease entities?
Answer: Only chronic periodontitis patients were included. Manuscript was slightly revised to make this clearer. So far, information on literature is not enough to sustain a division between chronic and aggressive periodontitis regarding HBD. This is one of the reasons to include only one type of periodontitis.

- Page 5, last paragraph: I do not see a connection between the references #22,23 and the periodontal diagnosis (which is actually not reported!).
Answer: These references are our main support – they were considered along with a panoramic radiograph - to determine periodontal diagnosis and will be helpful to answer this and your next questions.
Reference # 22 (*López NJ, Smith PC, Gutierrez J. Higher risk of preterm birth and low birth weight in women with periodontal disease. J Dent Res 2002;81:58-63.***) shows a reliable clinical criteria to define both periodontitis and gingivitis. However, it is not enough to eliminate mild gingivitis from the periodontally healthy group. For this reason, we combined another criterion, previously used by our research group, for a better selection of the periodontally healthy participants. Reference #23 refers to this last criterion (***Cortelli JR, Aquino DR,*

Cortelli SC et al. Etiological analysis of initial colonization of periodontal pathogens in oral cavity. J Clin Microbiol 2008; 46: 1322 - 1329.)
Please see below the applied parameters:

	PERIODONTALLY HEALTHY	GINGIVITIS	PERIODONTITIS
Periodontal parameters	**a mean periodontal attachment level of < 1.5 mm and no sites with > 2.0mm attachment loss**	**no radiographic evidence of periodontal bone resorption and more than 30% of bleeding sites**	**4 or more teeth with at least one site with attachment loss ≥ 3mm and 4 or more teeth with at least one site showing pocket depth ≥ 4mm**

- how was gingivitis excluded in the control group? Which gingivitis or bleeding parameter was applied? Further, you write for the gingivitis group, "bleeding at more than 30% of sites" - did you assess bleeding on probing or another bleeding parameter? Further, what about hygiene and plaque parameters in all 3 groups?
Answer: Gingivitis was excluded in the control group according to Lopez et al (2002)[22]; this criterion is based on bleeding on probing. Based on your questions, we have readded the information (see table below). This table also shows plaque index values.

Table 1 – Periodontal clinical parameters evaluated in all examined population

Group	PI (%) *Mean ± SD*	GI (%) *Mean ± SD*	PD (%) *Mean ± SD*	CAL (%) *Mean ± SD*
Periodontally Healthy n=31 (12M:19F)	**NA**	**NA**	**NA**	**NA**
Gingivitis n=27 (9M:18F)	**70.48±19.73**	**71.78±22.59**	**NA**	**NA**
Periodontitis n=31 (9M:22F)	**79.00±17.90**	**75.03±18.27**	**3.00±0.45**	**2.25±0.98**

N = number of subjects; M: Male; F: Female; SD: Standard deviation; PI: Plaque index; GI: Gingival Index; PD: Probing Depth; CAL: Clinical Attachment Level; NA: Not applicable.

- The clinical and general parameters should be presented in a table (the periodontitis group seemed to include also rather slight forms of periodontitis according to the "inclusion criteria") and I am missing a periodontal diagnosis.
Answer: The table containing from slight to moderate forms of chronic periodontitis (as shown by a mean whole-mouth PD of 3.00 ± 0.45 and a mean whole-mouth CAL of 2.25 ± 0.98) was included according to your suggestion. In the present study we chose to use the criteria proposed by Lopez et al. (2002) after having demonstrated satisfactory sensitivity and positive predictive values in comparison to other criteria (Costa FO et al. Impact of different periodontitis case definitions on periodontal research. J Oral Sci. 2009 Jun;51(2):199-206).

- What about salivary flow rate? Was this parameter assessed? Does this affect HBD-2 levels?

Answer: The assessment of salivary flow rate was not part of the present study although we could reach some indicative values considering the pre-established collected volume (2ml) and the time needed to produce this amount. During screening we did not select individuals taking medicines or having systemic problems which could alter saliva. Furthermore, in the literature, there is no established relation between salivary flow rate and HBD. In 2011, a group in Japan (*Usui T et al. Changes in salivary antimicrobial peptides, immunoglobulin A and cortisol after prolonged strenuous exercise Eur J Appl Physiol. 2011 Sep; 111: 2005 - 14***) performed a research which evaluated if some host-produced antimicrobial products, i.e HBD-2, cathelicidin (LL-37), and immunoglobulin A (IgA), might be affected by prolonged strenuous physical exercise. As expected, salivary flow rate decreased during exercise. These authors reported that both salivary concentrations and secretion rates of HBD-2 and LL-37 increased during and after the exercise.**

- There are huge differences concerning age distribution between the groups. Were these differences significant? Does this affect HBD-2 levels? Is there a correlation between age and HBD-2 levels?

Answer: The wide range of age among participants is compatible with periodontal diagnosis. Age increase is accompanied by an increase in periodontal breakdown, especially revealed by higher values of clinical attachment loss (this topic was reviewed by *Albandar JM. Global risk factors and risk indicator for periodontal diseases. Periodontology 2000 2002; 29: 177-206***). In the gingival epithelium Matsuzaka et al. (2006) observed that expression of HBD-2 was higher in the superficial layer among young subjects; elderly were also positive in the spinous cell layer. In addition, the elderly group showed the strongest HBD-2 immunoreaction (***Matsuzaka K et al. Age-related differences in localization of beta-defensin-2 in human gingival epithelia. Bull Tokyo Dent Coll. 2006 Nov;47(4):167-70.***)**

In the present study there was no relation between age and salivary HBD-2 among periodontally healthy (p = 0.129), gingivitis (p = 0.0973) or chronic periodontitis (p = 0,229) groups. A similar pattern of no influence was observed when age was evaluated in relation to salivary HBD-2 independently of periodontal diagnosis (p = 0.084). These evaluations were done by a Mann-Whitney test. Based on your question this information was reported in the revised manuscript.

- Page 9, 4th paragraph: Were the increased levels of HBD-2 significantly?

Answer: Yes, they were statistically significantly increased. This information was clarified in the revised manuscript.

- Results, correlation part: This part is hard to read and parts of it should be written in the materials part but not in the results part (describing the applied statistical methods)

Answer: We moved some information from results to statistical methods.

- according to your summary the available literature is already controversy, therefore your study should aim to solve at least one aspect - maybe the quality of the study

would improve if salivary levels of HBD-3 are also assessed, maybe there are differences in expression between HBD-2 and -3? maybe HBD-3 is influenced to a higher degree by the bacteria? maybe you have the possibility to remeasure the salivary samples for HBD-3?
Answer: Because we understand that aiming at only one objective is more effective we worked only with HBD-2. In a previous study (*Forte LF et al. Psychological stress has no association with salivary levels of β-defensin 2 and β-defensin 3. J Oral Pathol Med. 2010 Nov;39(10):765-9***) we worked with both HBD-2 and HBD-3 but only one type of periodontal status was included. For now, as we are working with three categories of periodontal status we decided to focus on HBD-2. After reaching a better understand on HBD-2 and periodontal status in future studies we will be confident to work with HBD-3.**

- The discussion is not easy to read and to follow your thoughts, e.g. in the introduction you write P.g. stimulates HBD-2 expression (ref 18), but in the discussion you write P.g. inhibits HBD-2 expression which might explain the results of the present study...? the discussion needs to be rewritten to present it more fluently.
Answer: We checked the discussion section. Controversial results were used under "Introduction" to show that the literature has not come to a consensus on the matter. As you can see, the influence of periodontopathogens on epithelial cells is not clear. While Taguchi et al[18] demonstrated that *P.g* is able to trigger HBD-2 production, other authors[11,15] failed to sustain this influence. The controversy of the subject is one of the aspects that lead us to its study. The first one motivated our group to develop this study while the others partially justify our findings.

- Table 1-3: Those tables take you some time to get an idea what you want to tell with them, further the footnote "ns = non-significant" is not appearing in the tables
Answer: These tables were combined and its title was clearly written. "NS" should having been erased during the first revision but it was not. Therefore, we apologize for this mistake that was properly corrected.

- Table 4: Why are the number of participants different in the three groups (e.g., only 23 patients with gingivitis)? Again the abbreviations explained in the footnote are not appearing in the table; and the presentation of the data is not well chosen in my opinion
Answer: After reaching the minimum number of individuals per group, previously defined as 20 by the sample size calculation procedures, we included pre-scheduled individuals who met inclusion/exclusion criteria according to their availability. The number 27 could seem to be low but in fact it was higher than the required 20.
Not mentioned abbreviations were excluded. We worked on improving the presentation of data in this table.

Minor points:
- Professional english language editing might improve the quality of the manuscript
Answer: Actually it has been previously done as you can check in the attached certificate emitted by American Journal Experts.

- The abstract is rather long, I would prefer not to include the statistical methods
Answer: Statistical methods were erased from abstract.

- The keyword "health" is not appropriate in my opinion
Answer: "Health" was changed to "oral health".

- Page 11, first sentence of second paragraph: "previously published research" - references are missing
Answer: References 13 and 30 were added.

- Page 13, at the beginning of the yellow highlighted part "in a previous study..." - reference is missing!
Answer: Reference 34 is cited in the revised manuscript.

Reviewer: 1

Reviewer Comments to Author:

The manuscript has improved, but this reviewer still has some suggested points that need to be addressed before publication in the Journal of Periodontology.

Dear Reviewer,
Thanks again for your efforts in improving our paper. Please see below our specific comments. Last changes in the revised manuscript can be found highlighted in yellow.

- The fact, that the bacteria were assessed qualitatively should be stated clearly in the abstract, the M&Ms section and definitely in the discussion.
Answer: This fact is mentioned in these three sections according to your suggestion.

- In my opinion the criterion, "chronic" periodontitis should be related to the current AAP classification from 1999 - The manuscript is missing this reference.
Answer: You are right because this reference states "chronic" periodontitis. This aspect was revised.

- The authors should be more specific with the applied indices. The original indices from Silness and Löe (the GI as well as the PI index) are graded indices, but you are presenting percentages.
Answer: Although plaque and gingivitis were collected in grades we showed these data in percentages of teeth having any amount of plaque and any degree of inflammation. This information was detailed in table 1' footnote.

- In Table 1 many clinical parameters of the healthy and gingivitis group are missing, which is a clear limitation of your present study.

Answer: This data was collected. As you can check, the second paragraph of *Patient population and periodontal evaluation* section starts informing that: "A complete periodontal examination was carried out by a single calibrated examiner (ALP). Measurements of periodontal pocket depth (PD), clinical attachment level, plaque and gingival indices were obtained in six sites per tooth using a manual periodontal probe". As the first reviewer asked us not to show clinical data we just selected the most important clinical parameters according to periodontal diagnosis category. However, taking into consideration your opinion in the revised manuscript you can see a full table.

- You state, that you excluded a correlation between age and HBD-2 levels by Mann-Whitney test. As far as I am aware, this is not the appropriate test to prove or exclude a correlation - Spearman or Pearsons's correlation should be applied.
Answer: In the previous answer that we provided it was written "in the present study there was no relation between age and salivary HBD-2 among periodontally healthy (p = 0.129), gingivitis (p = 0.0973) or chronic periodontitis (p = 0,229) groups. A similar pattern of no influence was observed when age was evaluated in relation to salivary HBD-2 independently of periodontal diagnosis (p = 0.084). These evaluations were done by a Mann-Whitney test. ." **You are right as a hypothesis statistical test Mann-Whitney does not offer information about correlation. Maybe in the original format our sentence lead you to misinterpretation. Actually, the absence of statistical significance in this preliminary univariate analysis did not allow the use of any correlation test.**

- Especially in Table 3 and sometimes in the text, you alternatively use point and comma among your presented data. Please be uniform and use the English format.
Answer: We apologize for this mistake. Text was checked.

Alexandre Lustosa Pereira

Taubaté, dezembro de 2012.