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ANÁLISE DA DIVERSIDADE DA MICROBIOTA SUPRA E SUBGENGIVAL E PERFIL DE PROTEÍNAS DA SALIVA E PELÍCULA ADQUIRIDA DE INDIVÍDUOS COM PERIODONTITE AGRESSIVA

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ABSTRACT

AMADO, P. P. P. **Diversity analysis of the supra and subgingival microbiota and proteins profile of saliva and acquired enamel pellicle of individuals with periodontitis**. 2019. 132 p. PhD thesis (Microbiology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2019.

Localized aggressive periodontitis (LAgP) is still a poorly understood disease. The subgengival microbiota of LAgP is characterized by the presence of periodontopathogens such as *Aggregatibacter actinomycetemcomitans* (*Aa*), and by the reduction of beneficial bacteria, however, the microbiome associated with LAgP has not yet been described. Since the initial adhesion of bacteria to the dental surfaces is dependent on the composition of the acquired enamel pellicle (PAE) formed by proteins present in the whole saliva (WS), the present study evaluated the PAE and WS proteome of LAgP, compared with healthy patients (HLAgP). In addition, the salivary cytokines/chemokines profile was evaluated in both groups. We also aimed to determine the composition of the oral and gut microbiota in patients with LAgP, and compare to the microbiota of HLAgP. Seven women with LAgP, aged between 19-26 years, Afro-descendants, and 8 healthy controls with the same profile were selected. Samples of supra (SP) and subgingival biofilme of shallow sites (SH) and affected medium/deep sites (MD), WS, AEP and feces were collected. The oral and gut microbiome were analyzed by sequencing of *16S rRNA*, and the presence of the *Aa* JP2 clone was determined in oral biofilme (OB) samples. WS and AEP proteome was analyzed by mass spectrometry, levels of NO in WS were dosed by Griess colorimetric reaction, levels of cytokines/chemokines in WS were quantified in multiplex assay. The proteolytic activity of WS was evaluated through the degradation of histatins 1 and 5 in different time-points. Spearman's correlation was applyed to evaluate correlations between different variables. qPCR analysis revealed that all LAgP patients harbored *Aa*, unlike HLAgP, however, the JP2 clone was detected in only 1 patient. Oral and gut microbiome analysis revealed no differences in the alpha diversity indexes. Beta-diversity analysis revealed that samples of OB and MD of LAgP were different from OB and SH of HLAgP, respectively. In LAgP, there was a reduction of the abundance of benefical bacteria, and increase of putative pathogens such as *Aa*, *Porphyromonas*, *Tannerela* and *Treponema*. The oral dysbiosis was acompained by imbalance in the gut microbiota, with higher abundance of *Desulfovibrio* in LAgP than in HLAgP. The WS of LAgP patients presented lower levels of CCL2 and CCL25, and higher levels of CCL17 and CCL27 than HLAgP, correlated to levels of *Aa*, *Acidovorax ebreus* and *Helicobacter pylori* in the OB. The AEP of LAgP-affected individuals presented undetectable levels of some proteins involved in immune response, antimicrobial activity and anti-inflammatory molecules, differing from the PAE of the HLAgP. AEP proteins such as ALMS1 and Cystatin-S and in WS such as alpha-enolase, profilin-1, dystonin, A2ML1, alpha-actinin-4 and IGHA1 were present differentially between LAgP and HLAgP. The proteolytic activity of WS was more intense in LAgP than in HLAgP. The data revealed new aspects of LAgP and collaborate with the understanding of mechanisms that may be involved in the development and progression of the disease, and indicate that new treatment strategies aiming at reestablishing the balance of the microbiota could be developed.

Keywords: Localized aggressive periodontitis. Oral microbiome. Gut microbiome. Salivary proteome. Saliva. Acquired enamel pellicle. Chemokines.

RESUMO

AMADO, P. P. P. **Análise da diversidade da microbiota supra e subgengival e perfil de proteínas da saliva e película adquirida de indivíduos com periodontite agressiva**. 2019. 132 f. Tese de doutorado (Microbiologia) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2019.

A Periodontite agressiva localizada (PAgL) é ainda uma doença pouco compreendida. A microbiota subgengival da PAgL é caracterizada pela presença de patógenos periodontais como *Aggregatibacter actinomycetemcomitans* (*Aa*), e pela redução de bactérias benéficas, mas o microbioma associado a PAgL ainda não foi descrito. Tendo em vista que a adesão inicial de bactérias às superfícies dentais é dependente da composição da película adquirida do esmalte (PAE) formada por proteínas presentes na saliva total (ST), o presente estudo avaliou o proteoma de PAE e ST de PAgL e comparou-o com o de pacientes saudáveis (S). Além disso, o perfil de citocinas/quimiocinas salivares foi avaliado em ambos os grupos. Também visamos determinar a composição da microbiota oral e intestinal em pacientes com PAgL, e compara-las à microbiota de pacientes S. Foram selecionadas 7 mulheres com PAgL, com idade entre 19-26 anos, afrodescendentes e 8 controles saudáveis com mesmo perfil. Amostras de biofilme de sítios supra (SP) e subgingival de sítios rasos (SR) e médio/profundos (MP), ST, PAE e fezes foram coletadas. O microbioma oral e intestinal foi analisado por sequenciamento de*16S rRNA*, e a presença do genótipo de *Aa* clone JP2 foi determinado em amostras de biofilme oral (BO). O proteoma da ST e PAE foi analisado por espectrometria de massa, níveis de NO na ST foram dosados por reação colorimétrica de Griess, níveis de citocinas/quimiocinas na ST foram quantificados em ensaio multiplex. A atividade proteolítica da ST foi avaliada pela degradação de histatinas 1 e 5 em diferentes intervalos de tempo. Teste de correlação de Spearman foi empregado para avaliar correlações entre diferentes variáveis. A análise de qPCR revelou que todos os pacientes com PAgL albergavam *Aa*, ao contrário de S, mas o clone JP2 foi detectado em apenas 1 paciente. A análise de beta diversidade do microbioma oral e de fezes revelou que amostras de BO e MP de PAgL se diferenciam do BO e SR de S, respectivamente. Na PAgL, houve redução da abundância de bactérias benéficas, e aumento de patógenos putativos como *Aa*, *Porphyromonas*, *Tannerela* e*Treponema*. A disbiose oral foi acompanhada de desequilíbrio no microbioma intestinal, com maior abundância de *Desulfovibrio* em PAgL do que em S. A ST de pacientes LAgP apresentou níveis de CCL2 e CCL25 menores e de CCL17 e CCL27 maiores do que S, e estes foram correlacionados com os níveis de *Aa*, *Acidovorax ebreus* e *Helicobacter pylori* no BO.A PAE de indivíduos com PAgL apresentou níveis indetectáveis de algumas proteínas envolvidas na resposta imune, atividade antimicrobiana e moléculas anti-inflamatórias, diferindo da PAE dos controles S. Proteínas como ALMS1 e Cistatina-S na PAE e como alfa-enolase, profilina-1, distonina, A2ML1, alfa-actinina-4 e IGHA1 na ST estavam presentes de maneira diferencial entre PAgL e S. A atividade proteolítica da ST foi mais intensa em PAgL do que em S. Os dados revelaram novos aspectos da PAgL e colaboram com o entendimento de mecanismos que podem estar envolvidos no desenvolvimento e progressão da doença, e indicam que novas estratégias de tratamento visando o restabelecimento do equilíbrio da microbiota poderiam ser desenvolvidas.

Palavras-chave: Periodontite agressiva localizada. Microbioma oral. Microbioma intestinal. Proteoma salivar. Saliva. Película adquirida do esmalte. Quimiocinas.

1 INTRODUCTION

Periodontitis is multifactorial inflammatory disease affecting the supporting tissues of the teeth, with progressive attachment loss and bone destruction (ARMITAGE, 1999). It is one of the most prevalent diseases in developed and developing countries, affecting about 20-50% of global population. Periodontitis has become a public health concern due to its high prevalence in adolescents, adults, and older individuals (NAZIR, 2017) and due to its associations with other inflammatory conditions such as type 2 diabetes, cardiovascular disease, respiratory tract infection, adverse pregnancy outcomes, neurodegenerative disease and cancer (BUI et al., 2019; KIM; AMAR, 2006; WHITMORE; LAMONT, 2014).

The inflammatory process observed in this disease is induced by a dysbiotic microbiota (HAJISHENGALLIS, 2014) and the continuous local chronic host response may alter the inflammatory profile resulting in deleterious effects in these tissues (BERGLUNDH; DONATI, 2005; GRAVES; COCHRAN, 2003).

Periodontitis was classified, from 1999 to November of 2017, as chronic (ChP) and aggressive periodontitis (AgP), localized or generalized, and necrotizing and as a manifestation of systemic diseases (ARMITAGE, 1999), based on clinical parameters extension and rate of disease progression. Recently, a new classification was proposed, classifying the disease in necrotizing periodontitis (HERRERA et al., 2018), periodontitis as a manifestation of systemic diseases (ALBANDAR; SUSIN; HUGHES, 2018) and “periodontitis”, which is further characterized by severity, progression rate, systemic effects and extension (PAPAPANOU et al., 2018; TONETTI; GREENWELL; KORNMAN, 2018).

The previously defined ChP was characterized by its slow progression, whereas AgP consisted of rapidly progressing disease, and both were further defined by their extension as generalized and localized (ARMITAGE, 1999). However, there are still no biomarkers allowing the differentiation among different clinical features of periodontitis (PAPAPANOU et al., 2018; TONETTI; GREENWELL; KORNMAN, 2018), reinforcing the need of studies on the pathobiology of this disease. The previously described localized AgP (LAgP) exhibits an early onset and affects mostly incisors and molars (ARMITAGE, 1999), and is currently classified as molar incisor pattern periodontitis (LIP) (CATON et al., 2018; FINE; PATIL; LOOS, 2018).

For more than 30 years, *Aggregatibacter actinomycetemcomitans* was considered the etiological agent of AgP, mainly in its localized form (NEWMAN et al., 1976; NEWMAN;

SOCRANSKY, 1977; SLOTS, 1976; SLOTS; REYNOLDS; GENCO, 1980). Six serotypes of *A. actinomycetemcomitans* are known (a, b, c, d and f), and serotype b is more frequently associated with the disease (KAPLAN et al., 2001; ZAMBON; SLOTS; GENCO, 1983). Currently, *A. actinomycetemcomitans* is seen mainly as an opportunistic pathogen of the resident oral microbiota, whereas its clone JP2, belonging to serotype b, may be considered a true exogenous pathogen (HAUBEK, 2010). The endemic presence of the highly leukotoxic JP2 clone was associated with the high prevalence of AgP, whereas a substantial role of non-JP2 clones in the disease was not established (HAUBEK et al., 2001).

Several studies supported the hypotheses that the presence *A. actinomycetemcomitans* would be required to initiate LAgP, which occurs with a higher frequency in African children or Hispanic descendants (HAUBEK et al., 1997; SHADDOX et al., 2010). However, the association between *A. actinomycetemcomitans* and AgP was based on target-directed methods, such as culture in selective media (SLOTS et al., 1982; ZAMBON; CHRISTERSSON; SLOTS, 1983), DNA hybridization (FAVERI et al., 2009; SHADDOX et al., 2012), and quantitative PCR (SARAIVA et al., 2014). The role of *A. actinomycetemcomitans* in LAgP was evidenced by the correlation of its elimination in response to periodontal treatment (MANDELL; EBERSOLE; SOCRANSKY, 1987; VAN WINKELHOFF; TIJHOF; DE GRAAFF, 1992) and by the high antibody response to this microorganism in patients with LAgP (ALBANDAR et al., 2001; ANDO et al., 2010; EBERSOLE; CAPPELLI, 1994; SARAIVA et al., 2014). In addition, its pathogenic role was suggested by the production of the cytolethal distending toxin (CDT), an exotoxin with immunemodulatory activity (ANDO et al., 2010; FERNANDES et al., 2008; SHENKER et al., 1999) and by the production of leukotoxin (LALLY; GOLUB; KIEBA, 1994).

Previous data using cloning and 16SrDNA sequencing, Faveri et al. (2008) could not detect *A. actinomycetemcomitans* in samples from patients with GAgP. However, it should be mentioned that the method could only detect species in proportion higher than 1% (personal information). *A. actinomycetemcomitans* can be detected in healthy individuals or affected by ChP (HÖLTTÄ et al., 1994; YANG et al., 2004), but its serotype b is more associated with AgP both in studies with culture-dependent methods (YANG et al., 2004), and indirectly by measuring serum antibody levels (ANDO et al., 2010).

A. actinomycetemcomitans pathogenic potential may be questioned especially due to the lack of experimental animal models of periodontitis (SCHREINER et al., 2003). Moreover, recognized periodontopathogens such as *Porphyromonasgingivalis*, *Treponema denticola* and

Tannerela forsythia are not only associated to ChP, but also to GAgP and LAgP (CHAHBOUN et al., 2015; FAVERI et al., 2009; SARAIVA et al., 2014; SOCRANSKY; HAFFAJEE, 1994).

It is currently accepted that periodontitis is the result of a microbial community and not exclusively by the presence of a single pathogen (FINE et al., 2013a) and the subgingival dysbiotic microbiota can be evidenced not only by the presence of periodontal pathogens but also by the low proportion of beneficial bacteria (VAN ESSCHE et al., 2013). Among individuals with *A. actinomycetemcomitans*, those with healthy periodontium showed a higher abundance of *Actinomyces* species (FINE et al., 2013b). Other data showed that the oral biofilm of LAgP patients present low levels of *Actinomyces naeslundi*, *A. gerencsiae*, *S. gordonii* and *S. oralis* when compared to healthy control patients (FAVERI et al., 2009). Furthermore, healthy patients harboring *A. actinomycetemcomitans* had a higher proportion of *Streptococcus* and *Actinomyces* species, whereas patients with bone loss had a higher proportion of *Parvimonas micra*, *Filifactor alocis*, *A. actinomycetemcomitans* and *Peptostreptococcus* sp. human oral taxon 113 (HOT-113). In addition, the presence of a bacterial consortium formed by *A. actinomycetemcomitans*, *S. parasanguinis* and *F. alocis* was strongly associated with bone loss in LAgP (FINE et al., 2013b). These data raised the hypothesis that *A. actinomycetemcomitans* is a keystone pathogen, by its ability to alter the subgingival environment by the production of toxins (leukotoxin and CDT), leading to an immunological paralysis and allowing the proliferation of pathobionts. Thus, data on the whole microbial community in LAgP are needed through the application of "open-end" methods, such as new generation sequencing (FINE et al., 2013b).

Due to the absence of evidence of a correlation among the microbial composition of the subgingival sites and the rate and severity of the periodontal tissue destruction, it was postulated that genetically-driven host factors would predispose to dysbiotic changes in the subgingival microbiota and consequently the rapid destruction observed in LAgP (NIBALI, 2015).

AgP seems to be inherited in an autosomal dominant way in Afro-descendants' families (MARAZITA et al., 1994). The incidence of AgP is often very high among certain families, with the percentage of affected siblings and affected lineage members reaching 40-50%, suggesting that genetic factors may be important in susceptibility to AgP (MENG et al., 2011). Few studies on family aggregation in ChP have been conducted so far, but there is evidence that parents with periodontal poor health tend to have children with periodontal disease, suggesting that family history is a valid representation of shared genetic and

environmental factors that contribute to the individual's periodontal condition (SHEARER et al., 2011).

The composition of the gut and oral microbiota differs, but the types of communities observed in one site may be predictive of the communities observed in the other (DING; SCHLOSS, 2014). Both communities are influenced by host factors, and the oral microbiota serves as an inoculum for the intestine. It is interesting to note that oral inoculation with *P. gingivalis* in experimental models leads to alteration of the intestinal microbiota, suggesting that this could be a possible mechanism for the association of other inflammatory diseases with periodontitis, such as cardiovascular diseases (ARIMATSU et al., 2014).

Recently, the gut microbiome of individuals with gingivitis, ChP and periodontal health was evaluated by *16S rRNA* sequencing (LOURENÇO et al., 2018). Abundance of Firmicutes, Proteobacteria, Verrucomicrobia and Euryarchaeota were increased, whereas abundance of Bacteroidetes were decreased in patients with ChP compared to healthy controls. *Mogibacteriaceae*, *Ruminococcaceae* and the genus *Prevotella* were able to discriminate between ChP and healthy individuals. Moreover, significant correlations between OTUs (operational taxonomic unit) representative of periodontopathogens and attachment loss were demonstrated. These findings suggested a relationship between oral and gut dysbiosis in affected individuals (LOURENÇO et al., 2018).

Differences in host factors are likely to play a determinant role on the balance between oral tissues and the microbiota. Populational studies have shown that subgroups of the population show differences in susceptibility to inflammatory diseases, including periodontitis (GORR, 2009). Saliva is a rich reservoir of different proteins and peptides and has been used as a powerful and accessible instrument in the search for host factors that can contribute to the understanding and diagnosis of systemic and oral diseases (ROI et al., 2019).

In the oral cavity, several proteins are released through secretions of different salivary glands and their proportional contribution to saliva varies depending on sympathetic and parasympathetic stimulation, circadian rhythm, health-illness spectrum, and personal habits such as diet and drug intake (LORENZO-POUSO et al., 2018; SIQUEIRA; DAWES, 2011). The total protein concentration in the secretion of each gland varies considerably depending on factors such as flow rate, duration of stimulation, nature of the stimulus and circadian rhythm (SIQUEIRA; DAWES, 2011). Whole saliva (WS) is composed mainly by secretions of three major glands (parotid, submandibular and sublingual) together with the minor salivary glands, but may also contain plasma proteins (such as albumin, transferrin and immunoglobulins) coming from the intimate contact between saliva and gingival crevicular fluid (GCF), which

achieves the oral cavity through the gap between the tooth and the overlying gingiva (LORENZO-POUSO et al., 2018; SIQUEIRA; DAWES, 2011). The contribution of GCF to the WS composition is higher in individuals affected by gingivitis and periodontitis (SIQUEIRA; DAWES, 2011). This complex mixture of fluids is composed by a variety of electrolytes (i.e., Ca^{2+}, Cl^{-}, $H_2PO_4^{-}$, HCO_3^{-}, I^{-}, K^{+}, Mg^{2+}, Na^{+}, and SCN^{-}), proteins, glycoproteins, lipids, desquamated epithelial cells, immune cells and microbial products that can impact the oral homeostasis (HUMPHREY; WILLIAMSON, 2001; SIQUEIRA; DAWES, 2011).

The major families of salivary proteins (structurally related proteins) include acidic and basic proline-rich proteins (PRPs), amylase, high-molecular-weight glycoprotein MUC5B, low-molecular-weight glycoprotein MUC7, aglutinin, cystatin, histatin and statherin (SIQUEIRA; DAWES, 2011). However, through the application of more sensitive analytical techniques (i. g., proteomic analyzes), a large number of other proteins have been detected (LOO et al., 2010), comprising more than 3,652 proteins and 12,562 peptides (LORENZO-POUSO et al., 2018).

The biosynthesis of salivary proteins begins into the salivary glands, followed by the post-translational process within the acinar cells, involving protein glycosylation, phosphorylation, sulfation and proteolysis (HELMERHORST; OPPENHEIM, 2007). When salivary proteins achieve the oral cavity, they can suffer other structural modifications, leading to a formation of a heterogenous protein-protein complex, proteolysis and deglycosylation, mainly by the activity of enzymes produced by oral bacteria (SIQUEIRA; DAWES, 2011). WS contain approximately 700 different species of microorganisms and the total number varies between 10^{6}-10^{9}/ml (CHEN et al., 2010). These microorganisms produce a variety of proteolytic enzymes and other enzymes (HELMERHORST; OPPENHEIM, 2007; ITO; HIROSE; TAKEUCHI, 1959). Many of these enzymes have not yet been characterized and they can quickly break down several proteins in the saliva (SIQUEIRA; DAWES, 2011).

Saliva also contains a large number of epithelial cells desquamated from the oral mucosa. The release of proteolytic enzymes by these cells is still uncertain, but they express a transglutaminase that can catalyze the formation of a g-glutamyl-g-lysine bound between glutamine and lysine residues (BRADWAY et al., 1989). In addition, leukocytes derived from GCF, especially during inflammation in periodontal diseases, when in contact with the hypotonic saliva (which has about one-sixth of the osmotic pressure of the plasma) suffer rupture and release many enzymes, including proteolytic ones (SIQUEIRA; DAWES, 2011).

Proteolysis and deglycosylation processes may alter the profile of salivary proteins by altering the composition of the acquired enamel pellicle (AEP) and, consequently, the receptors for bacterial adhesins. In periodontitis, there is greater neutrophil output from the gingival fluid and, possibly, the proteolysis of the salivary proteins will be greater. In addition, bacteria associated with ChP, such as *P. gingivalis*, *T. denticola* and *T. forsythia* are extremely proteolytic, unlike the bacteria that characterize the biofilm associated with health. On the other hand, some salivary proteins have mechanisms that hinder their degradation. Binding of histamin 1 to hydroxyapatite provides its resistance to proteolytic degradation by salivary enzymes and this binding may serve as a mean of maintaining other proteins in a stable form within the oral cavity (MCDONALD et al., 2011).

Hydroxyapatite has the ability to attract salivary phosphoproteins that bind strongly to the surface of the enamel, forming a protein film called AEP. A study of the total saliva phosphoproteome led to the identification of 65 salivary phosphoproteins (SALIH et al., 2010). Microbial colonization on tooth surfaces can occur by microbial recognition of specific receptors of the adsorbed proteins within the AEP. AEP is a thin acellular film predominantly composed by salivary proteins, but also by non-salivary-derived proteins, carbohydrates, and lipids (SIQUEIRA; CUSTODIO; MCDONALD, 2012). Among 130 proteins forming AEP, the majority is derived from epithelial cells (67.8%) and serum/gingival crevicular fluid (17.8%), and only 14.4% come from salivary glands secretions (SIQUEIRA et al., 2007).

The formation of the AEP is dependent on three major groups of proteins: proteins that bind to calcium ions (18% of the already identified AEP proteins) such as acidic proline-rich proteins (PRPs) and histatins, derived from exocrine salivary secretions; proteins that show a high tendency to bind to phosphate ions (15% of the AEP) such as elongation factor 2 and myosin 9, derived from epithelial cells; and proteins that interact with other proteins (28% of the AEP) (LEE et al., 2013). As an example, MUC5B form complexes with several other salivary proteins, including salivary α-amylase, histatin and statherin (IONTCHEVA et al., 2000; IONTCHEVA; OPPENHEIM; TROXLER, 1997). *In vivo*, the AEP is created by the formation of successive layers of proteins, based initially on binding to the mineral surface of the teeth (calcium and phosphate) and subsequently on protein-protein interactions (LEE et al., 2013).

The oral biofilm is initiated by the adhesion of early colonizers, which present adhesins that interact with saliva, serum, extracellular matrix compounds, host cells and other microorganisms (NOBBS; LAMONT; JENKINSON, 2009). Streptococci are the most successful early colonizers of the tooth surface, by binding to AEP proteins. For instance,

Streptococcus sanguinis, *S. gordonii* and *S. oralis* possess adhesins which recognize statherin, PRPs, amylase and salivary agglutinin (KOLENBRANDER et al., 2010). *S. gordonii* express surface adhesins, such as antigen I/II(AgI/II), SspA and SspB, CshA and CshB which mediate interactions with the salivary agglutinin gp340 (JAKUBOVICS et al., 2005), AbpA which binds to amylase (ROGERS et al., 1998), GspB and Hsa which binds to MUC7 (KESIMER et al., 2009), secretory IgA (S-IgA) (TAKAMATSU et al., 2005), and platelet glycoprotein Ibα (BENSING; LÓPEZ; SULLAM, 2004). The putative pathogens *A. actinomycetemcomitans* have the ability to adhere to AEP (RUDNEY; STAIKOV, 2002) by fimbrial binding to low-molecular-weight salivary mucin (MG2), lactoferrin, and S-IgA (GROENINK et al., 1998). Furthermore, certain salivary proteins can modulate the oral microbiome by their antimicrobial features, affecting bacteria distribution on/in dental biofilms, such as lactoferrin, lysozyme, histatin 1, 3 and 5, statherin, and others (DAWES et al., 2015).

Histatin 1, 3, and 5 are multifunctional proteins involved in the formation of AEP (OPPENHEIM et al., 2007), buffering process and can exhibit antibacterial and antifungal activities (HELMERHORST et al., 2005, 2006; OPPENHEIM et al., 2007; PURI; EDGERTON, 2014). Histatin 1 (his1) inhibits crystal growth of calcium phosphate salts (OPPENHEIM et al., 1988) and is the only phosphorylated histatin in the oral cavity (OPPENHEIM et al., 2007), whereas histatin 5 (his5) is active against pathogenic fungi, such as *Candida albicans* (HELMERHORST et al., 2006; OPPENHEIM et al., 1988; PURI; EDGERTON, 2014). *In vitro* studies revealed that histatins can also present antimicrobial effect against *Streptococcus mutans* (HELMERHORST et al., 1997; PAYNE et al., 1991) and *P. gingivalis* (BORGWARDT et al., 2014; GUSMAN et al., 2001).

Histatins seems to present a modulatory effect, hindering biofilm formation. Clinical trials demonstrated that histatins can reduce gingivitis severity (PAQUETTE et al., 2002; VAN DYKE et al., 2002). Several human oral antimicrobial peptides play important roles including maintenance, repairing of oral tissues (hard or soft) and defense against oral microbes and do not present adverse effects to the host and/or tissues, indicating their great innovative potential for the development of new therapies against oral biofilm associated diseases, such as periodontitis (KHURSHID et al., 2017). The limitation of the use of these peptides/proteins involves the inconsistency between the presence of salivary proteins in glandular secretions and the absence of some of these proteins in WS (JENSEN et al., 1994; HELMERHORST et al., 2006). For example, histatins concentration in glandular secretion varies between 43-120 μg/ml, dropping to 2-8 μg/ml in WS (HELMERHORST, 2007). Thus, several studies aimed to explain these differences and evaluated histatins proteolysis (HELMERHORST, 2007;

HELMERHORST et al., 2006; OPPENHEIM et al., 1988; SIQUEIRA et al., 2012; TAKAMATSU et al., 2005). In the oral cavity, histatins proteolysis occurs extremely fast (HELMERHORST, 2007; HELMERHORST et al., 2006; PAYNE et al., 1991), being triggered by bacterial and host derived enzymes (HELMERHORST, 2007).

The arsenal of salivary proteins and compounds with antimicrobial properties is huge and includes products of immune defense cells such as IgA, defensins, and nitric oxide (NO) (DAWES et al., 2015; FANG; VAZQUEZ-TORRES, 2002). The total concentration and relative proportion of salivary proteins are dependent on several factors (CABRAS et al., 2009; DAWES, 1987; RAYMENT et al., 2001), including age, since hormonal factors were associated with stabilization of the microbiota in adults (CASTAGNOLA et al., 2011). The concentration and activity of antimicrobial components could affect the distribution of bacterial species in the dental biofilm, and levels of these components may vary between individuals, also according to resistance to infection, or even physical exercise (GILLUM et al., 2014).

An early study, evaluating salivary proteins by using two-dimensional gel electrophoresis, revealed that the levels of serum albumin, immunoglobulin (Ig) gamma2 chain C region, Ig alpha2 chain C region, vitamin D-binding protein, salivary alpha-amylase and zinc-alpha2 glycoprotein were increased in whole unstimulated saliva of GAgP subjects when compared to healthy controls, while those of lactotransferrin, elongation factor 2, 14-3-3 sigma, short palate, lung and nasal epithelium carcinoma-associated protein 2 precursor and carbonic anhydrase 6 were decreased (WU et al., 2009). Inflammation influences the WS composition, and gingivitis subjects present increased amounts of blood proteins (serum albumin and hemoglobin), Ig peptides and keratins than healthy controls, assessed by two-dimensional gel electrophoresis and liquid chromatography (GONÇALVES et al., 2011). Although some studies have demonstrated slight differences in cytokines levels such as IL-29 in GCF and serum of GAgP compared to ChP subjects (SHIVAPRASAD; PRADEEP, 2015), there are still conflicting data on differences in cytokines/chemokines profiles between AgP and ChP subjects (DUARTE et al., 2015). For instance, higher GCF levels of IL-8 were reported in GAgP (ERTUGRUL et al., 2013; FINE et al., 2014), whereas others reported reduced IL-8 levels in the GCF of sites with *A. actinomycetemcomitans* infection (SHADDOX et al., 2012). Furthermore, MIP-1α levels in both saliva and GCF were consistently elevated in AgP, and considered as a biomarker for bone loss in adolescents, independently on infection by *A. actinomycetemcomitans* (FINE et al., 2014).

Thus, the identification of saliva and AEP components, by proteomic analysis and immunedetection of chemokines and cytokines, as well as the characterization of the oral and

gut microbiota associated with LAgP, could facilitate the understanding of the pathogenesis of the disease and to clarify the role of salivary proteins in survival and emergence of the dysbiotic microbiota. This knowledge would allow the development of strategies aimed to recover the balance of the microbiota, in homeostasis with the host, useful for the prevention and control of LAgP. Moreover, the analysis of saliva components could help to establish the factors associated with LAgP risk, and healthy associated proteins could be used to modify the AEP or increase saliva antimicrobial properties in order to prevent and control periodontitis, applied as complementary therapy. The analysis of the salivary proteolytic activity of individuals affected by periodontitis against salivary proteins opens avenues for a comprehensive investigation of how these salivary proteins could be used as an additional diagnostic method for the prevention and early treatment of periodontitis.

2 CONCLUSION

Supra and subgingival microbiome analysis of LAgP and HLAgP revealed some aspects not yet described in the literature, and our data contribute to the understanding of the microbiota associated with the disease.

Oral microbiome analysis confirms the microbial dysbiosys in LAgP, with the reduction of abundance of organisms considered beneficial such as species of *Bergeyella*, *Capnocytophaga*, *Gemella*, *Streptococcus*, *Granulicatella*, *Haemophilus* and *Kingella* and increased abundance of pathogenic and putative organisms such as *A. actinomycetemcomitans*, *Porphyromonas*, *Tannerela* and *Treponema*.

The association of organisms such as *Johnsonella, Acidovorax, Desulfobulbus* and other not well known sulfate reducing bacteria with LAgP, as well as the synergistic relation between NO and H_2S producers as an important phenomenon of periodontal destruction should be investigated in future studies.

Dysbiosis of oral microbial community in LAgP may be accompanied by a certain imbalance of the intestinal microbiota, since LAgP patients presented an increased abundance of sulfate reducing bacteria of the genus *Desulfovibrio* in feces, an organism associated with gut inflammatory conditions. Moreover, the association between LAgP in African descendants and enhanced levels of sulfidogenic bacteria in the gut should be also investigated.

Despite the limited number of subjects involved in this study, our data indicated that a combination of chemokines may be useful as biomarkers for AgP, since LAgP showed a profile of decreased salivary levels of MCP-1/CCL2 and TECK/CCL25, and increased salivary levels of TARC/CCL17 and CTAK/CCL27. The altered chemokines profile in LAgP may shape the recruitment of inflammatory monocytes and the traffic and differentiation of regulatory T cells to the periodontal area, thus altering the homeostasis of the gingival tissues to commensal bacteria.

The salivary levels of certain chemokines profile correlated the abundance of oral organisms. Our data suggested that oral colonization may be modultated by host factors such as chemokines, or alternatively, certain organisms such as *A. actinomycetemcomitans* may impair host defenses by altering chemokines production in the oral cavity.

The dysbiosys of the oral microbiota may be influenced by AEP and saliva composition in LAgP subjects, who presented undetectable levels or lacked some proteins involved in immune response processes, antimicrobial activity and anti-inflammatory molecules.

The salivary proteolytic activity in LAgP-affected subjects was higher compared to healthy controls, which may impact the salivary and AEP protein profile. Our findings suggest that the alterations observed in the StS and AEP protein composition in affected individuals may occur not only due to host proteolysis events, but also due to the increased abundance of known proteolytic bacteria in the oral microbiome, such as the periodonthopathogens *P. gingivalis*, *T. denticola*, and *T. forsythia*, and other protease producers such as *A. actinomycetemcomitans*, species of *Prevotella*, *F. nucleatum, E. nodatum*, among others. Moreover, WS proteolytic activities should be tested as diagnostic tool for periodontitis and its progression.

The detection of proteins in AEP and WS samples, in a multianalyte evaluation system should be tested as biomarkers for LAgP, including the detection of Alstrom syndrome protein 1 and absence of Cystatin-S in AEP samples in AEP samples, as well as the detection of alpha-enolase and profilin-1 and absence dystonin, A2ML1, alpha-actinin-4 and IGHA1 in WS samples.

Taken all toghether, our findings enlightened new aspects of AgP and collaborated to the understanding of mechanisms that may be involved in the development and progression of the disease, and open avenues to new innovational treatment strategies, aiming not only the reduction of pathogens, but favoring the colonization of beneficial bacteria, as well as in the use of antimicrobial designed peptides based on salivary proteins in order to enhance its retention time and activity in the oral cavity.

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