LÍLIA ALVES ROCHA

A EXPRESSÃO DO INIBIDOR DE PROTEASE LIBERADA POR LEUCÓCITOS (SLPI) EM GLÂNDULAS SUBMANDIBULARES DE PACIENTES QUE MORRERAM POR AIDS

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Mestre em Estomatopatologia. Área de Patologia

PIRACICABA
2006

LÍLIA ALVES ROCHA

A EXPRESSÃO DO INIBIDOR DE PROTEASE LIBERADA POR LEUCÓCITOS (SLPI) EM GLÂNDULAS SUBMANDIBULARES DE PACIENTES QUE MORRERAM POR AIDS

Dissetação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Mestre em Estomatopatologia. Área de Patologia

Orientadora: Profa. Dra. Thaís Mauad
Co-Orientador: Prof. Dr. Pablo Agustin Vargas

Banca Examinadora:
Prof. Dr. Paulo Hilario Saldiva
Prof. Dr. Ricardo Della Coletta
Profa. Dra. Thaís Mauad

PIRACICABA
2006

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE ODONTOLOGIA DE PIRACICABA
Bibliotecário: Marilene Girello – CRB-8ª. / 6159

R582e

Rocha, Lília Alves.
A expressão do inibidor de protease liberada por leucócitos (SLPI) em glândulas submandibulares de pacientes que morreram por AIDS. / Lília Alves Rocha. -- Piracicaba, SP : [s.n.], 2006.

Orientador: Thaís Mauad
Dissertação (Mestrado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Glândula submandibular. 2. Autópsia. 3. Imunohistoquímica. 4. AIDS (Doença). I. Mauad, Thaís. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.
(mg/fop)

Título em inglês: Expression of secretory leucocyte proteinase inhibitor (SLPI) in submandibular glands of patients that died from AIDS
Palavras-chave em inglês (Keywords): 1. Submandibular gland. 2. Autopsy. 3. Immunohistochemistry. 4. AIDS (Disease)
Área de concentração: Patologia
Titulação: Mestre em Estomatopatologia
Banca examinadora: Paulo Hilario Saldiva, Ricardo Della Coletta, Thaís Mauad
Data da defesa: 24/02/2006

UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

A Comissão Julgadora dos trabalhos de Defesa de Dissertação de MESTRADO, em sessão pública realizada em 24 de Fevereiro de 2006, considerou a candidata LÍLIA ALVES ROCHA aprovada.

PROFa. DRa. THAÍS MAUAD

PROF. DR. PAULO HILÁRIO NASCIMENTO SALDIVA

PROF. DR. RICARDO DELLA COLETTA

DEDICATÓRIA

Aos meus pais, Edna Alves Rocha e Adir de Brito Rocha, por toda dedicação que recebi durante toda a minha vida e por sempre me apoiarem nas minhas escolhas.

Aos meus irmãos Leila Alves Rocha e Luciano Alves Rocha, por toda experiência compartilhada, carinho e paciência

Ao Piero Brandini Bloes, por todos os momentos felizes e por todo apoio e entusiasmo que recebi durante este período.

À minha orientadora Thais Mauad, pela oportunidade dada para realizar este trabalho, por me auxiliar na minha vida profissional e orientação deste trabalho. Muito Obrigada!

Ao meu co-orientador Pablo Agustin Vargas, pelos sábios conselhos dados e pelo incentivo para a realização deste trabalho. Muito Obrigada!

Ao professor Márcio Ajudarte Lopes, pelo apoio e incentivo dado durante a graduação e durante o mestrado.

Ao Professor Oslei Paes de Almeida, responsável pela área de Patologia da faculdade de Odontologia de Piracicaba- UNICAMP, pela oportunidade de realizar o Mestrado.

AGRADECIMENTO ESPECIAL

Á minha orientadora Profa Thaís Mauad por ajudar a me superar e ser cada vez melhor. Pela participação direta na realização deste projeto e pelo exemplo de qualidade e dedicação.

AGRADECIMENTOS

À Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, ao Diretor, Prof. Dr. Thales Rocha de Mattos Filho, ao Prof. Dr. Pedro Luiz Rosalen, Coordenador Geral dos Programas de Pós-graduação e ao Prof. Dr. Jacks Jorge Júnior, atual Coordenador do Curso de Pós-graduação em Estomatopatologia.

À Coordenação do CNPq, pela concessão da bolsa de estudos que permitiu a realização do curso de Mestrado.

À Profa. Dra. Thaís Mauad e ao Prof. Pablo Agustin Vargas, por todas as oportunidades concedidas a mim e por todos ensinamentos. Os senhores têm um papel imprescindível na minha formação acadêmica e científica.

Aos Professores da Área de Patologia e Semiologia da Faculdade de Odontologia de Piracicaba / UNICAMP, Márcio Ajudarte Lopes, Edgard Graner, Ricardo Della Colleta, Osvaldo Di Hipólito Júnior, Jacks Jorge Júnior e Pablo Augustin Vargas, pelos ensinamentos e amizade.

Aos funcionários Ana Cristina, Adriano, João, Rosa Maria, Aparecida Campeão, Rosa, Débora, Katiane, Juliana, Melise, Vanessa, Lucinda, Selma, Aparecida Cassieri, Marco Antonio e Eliseu. pelo apoio, auxílio e amizade.

Aos amigos e colegas Patrícia, Ana Lúcia, Angela Santos, Mônica, Matheus, Ademar, Marco Antônio, Rogério, Lúcia, Bete, Jorge, Guilhermo, Sabrina, Michelle, Michele Kellerman, Lays, Rebeca, Érica, Ito, Ornellas, Chico, Danyel, Dawton, Fabiana, Débora, Andréia, Lucielma e Ana Carolina.

Às amigas e técnicas do laboratório de imunohistoquímica do Departamento de Patologia FMUSP, Angela e Ana, sem as quais este trabalho não seria possível.

Agradeço principalmente aos meus pais, Edna e Adir, aos meus irmãos, Leila e Luciano, e ao meu querido namorado Piero, por todo apoio e carinho.

SUMÁRIO

RESUMO

O Inibidor de Protease Liberada por Leucócitos (SLPI) é um inibidor de protease endógeno, originalmente caracterizado a partir de fluído salivar proveniente da glândula parótida. Além da ação inibitória contra o Vírus da Imunodeficiência Humana (HIV), o SLPI também possui atividades anti-bacterianas e anti-fúngicas. Os tecidos orais são sítios comuns de infecção em pacientes com AIDS. SLPI é expresso em glândulas submandibulares (GSM), mas existem poucos trabalhos sobre a expressão desta proteína em pacientes com infecções associadas ao HIV. O objetivo deste estudo foi analisar a expressão imunohistoquímica do SLPI em GSM de pacientes que morreram de AIDS. Foram analizadas amostras de GSM de 36 pacientes que morreram de AIDS (10 pacientes não apresentavam alterações histológicas em GSM, 10 pacientes apresentavam sialodenite, 08 pacientes apresentavam infecção por micobactérias e 08 pacientes apresentavam infecção por citomegalivírus (CMV)), e 10 pacientes HIV negativos (grupo controle). A expressão do SLPI foi quantificada nos ácinos serosos através de um analisador de imagens, com os resultados expressos em porcentagem de área corada. Foi verificada uma maior expressão de SLPI nas GSM de pacientes com AIDS apresentando infecção por CMV (% SLPI= 37,37±14,45) quando comparado com todos os outros grupos (p= 0,009). Não houve diferença significativa entre o grupo controle (% SLPI= 22,70±9,42) e o grupo de pacientes com AIDS sem alterações histológicas em GSM (%SLPI= 18,10±7,58), pacientes com sialodenite (%SLPI= 17,13±5,36), ou ainda pacientes com infecção por micobactérias (%SLPI= 21,09±4,66). Estes resultados indicam que a infecção por CMV aumentou a expressão de SLPI em GSM de pacientes com AIDS.

ABSTRACT

Secretory Leukocyte Proteinase Inhibitor (SLPI) is an endogen proteinase inhibitor originally characterized from parotid fluids. Besides an inhibitory action on human immunodeficiency virus (HIV), SLPI possess also anti-bacterial and anti-fungical activities. The oral tissues are a common site of infectious conditions in patients with AIDS. SLPI protein is expressed in the submandibular glands, but there are few data on its expression in patients with associated HIV infections. The objective of this study was to analyze the immunohistochemistry expression of SLPI in the submandibular glands (SMG) of patients that died from the AIDS. We analyzed SMG samples of 36 patients that died from AIDS (10 patients with no histological alterations, 10 patients had chronic non specific sialadenitis, 8 had mycobacteriosis and 8 had cytomegalovirus infection (CMV)), and 10 HIV negative (control group). SLPI expression was in the serous acinar cells, quantified with image analysis, results being expressed in percentage of stained areas. There was a statistically significant higher expression of SLPI in AIDS patients with CMV infection (% SLPI = 37.37±14.45) when compared to all other groups ($p = 0.009$). There were no significant differences among control subjects (%SLPI = 22.70±9.42) and AIDS patients without histological alterations (%SLPI = 18.10±7.58), patients with chronic non-specific sialadenitis (% SLPI = 17.13±5.36) or mycobacterial infection (% SLPI = 21.09±4.66). These results indicate that the CMV infection increases SLPI expression in the submandibular glands of AIDS patients.

1. INTRODUÇÃO

A Síndrome da Imunodeficiência Adquirida (AIDS) foi descrita pela primeira vez em 1981, na Califórnia, Estados Unidos. Trata-se de uma doença caracterizada por imunossupressão profunda e está relacionada a infecções oportunistas, neoplasias e manifestações neurológicas. A AIDS é causada pelo vírus da imunodeficiência humana (HIV) tipo 1 ou 2. Estes vírus da família dos lentivírus têm como características o longo período de incubação, tropismo hematopoiético e de sistema nervoso; além da capacidade de causar imunossupressão. A transmissão da doença ocorre através do sangue e alguns fluídos corporais (Cotran et al., 2000).

A molécula CD4 é, de fato, um receptor de grande afinidade para o HIV. Sendo assim os macrófagos, as células dendríticas e os linfócitos T são os alvos da infecção pelo HIV. Contudo, a ligação ao CD4 não é suficiente para a infecção. A proteína gp 120 do HIV também deve ligar-se a outras moléculas da superfície da célula para que a fusão com a membrana celular ocorra. Após a fusão, o cerne viral contendo o genoma do HIV entra no citoplasma celular, onde sofrerá transcrição reversa formando o DNA pro-viral (DNAc). Após a integração do DNAc com o genoma do hospedeiro, o pró-virus pode permanecer no cromossomo por anos ou meses. O DNA pró-viral pode ser transcrito produzindo partículas virais completas. As alterações nas funções celulares e na produção de citocinas possuem um importante efeito nos eventos associados à doença (Cotran et al., 2000).

Atualmente cerca de 40,3 milhões de pessoas foram infectadas pelo HIV, destas 38 milhões são adultos e 2,3 milhões são crianças menores de 15 anos. Noventa por cento (90%) delas vivem em países subdesenvolvidos, segundo estimativas feitas pelo Programa Conjunto das Nações Unidas sobre o HIV/AIDS e a Organização Mundial da Saúde (OMS). Em 2005 ocorreram 4,9 milhões de novas infecções, destas 570.000 acometeram menores de 15 anos. No Brasil, existem cerca de 650.00 indivíduos HIV+ notificados pelo Ministério da Saúde até o ano de 2003, sendo que destes, 277.154 já desenvolveram a AIDS e mais de 11.000 pessoas morreram de doenças relacionadas à AIDS.

O vírus HIV-1 é frequentemente encontrado em secreções salivares. Mesmo assim, considera-se rara ou ausente a transmissão de AIDS pela saliva (Wahl et al., 1997; Fox et al., 1998; Jana et al., 2005). Parece estar bem estabelecido que a saliva humana inibe

a infectividade do vírus HIV em estudos *in* vitro (McNeely et al., 1995; Shugars et al., 1997). Existem vários peptídeos antimicrobianos na saliva que confeririam proteção ao HIV.

Os peptídeos antimicrobianos formam um grupo extremamente diversificado de pequenas proteínas que participam da defesa inata, ou seja, fazem parte da primeira linha de defesa do organismo após uma infecção (Gallo et al., 2002). Dentre elas, acredita-se que o Inibidor de Protease Liberado por Leucócito (SLPI) desempenhe um papel fundamental como inibidor do HIV na saliva. SLPI é um inibidor de protease endógeno associado às superfícies mucosas, inicialmente descrito em secreção da glândula parótida (Ohlson et al., 1984). Originalmente o SLPI foi identificado como um inibidor de elastase, mas estudos subseqüentes revelaram a sua atividade antimicrobiana de amplo espectro (Hiemstra et al., 2002; Chattopadhyay et al., 2004). Na região oral, SLPI é produzido em glândulas salivares maiores e menores e secretado na saliva. (Wahl et al., 1997; Shugars et al., 1997; Lin et al., 2004).

A atividade anti-HIV do SLPI salivar foi descrita pela primeira vez por McNeely et al. (1995), em um estudo que demonstra a atividade anti HIV-1 de um derivado de saliva inibindo a infecção de monócitos, macrófagos e linfócitos T. O trabalho de Wahl et al. (1997) indica que o SLPI atua no vírus depois da sua ligação com a célula alvo, porém antes da ação da transcriptase reversa (ou seja, antes da síntese de partículas virais). O inibidor de protease secretado pelas glândulas salivares é depositado no lúmen do ácino, passa pelo ducto salivar e finalmente chega à cavidade oral. Nas glândulas salivares, o HIV é geralmente encontrado na região periductal. Com bases nessas informações, McNeely et al. (1995) sugeriram que o HIV e SLPI interagem somente na cavidade oral. A depleção do SLPI salivar resulta na diminuição da atividade anti-HIV (McNeely et al., 1995; Wahl et al., 1997) sugerindo assim a importância desta proteína na atividade antimicrobiana da saliva. Lin et al. (2004) verificaram o aumento da concentração de SLPI na saliva de pacientes HIV+ quando comparada com a saliva de pacientes saudáveis.

Além da sabida ação anti-HIV, o SLPI tem demonstrado atividades anti-inflamatórias, anti-fúngicas, anti-bacterianas, além de estar relacionado ao processo de reparo epitelial (Hiemstra et al., 2002; Chattopadhyay et al., 2004). Foi verificado em um estudo *in vitro* utilizando saliva de pacientes HIV+, que o SLPI é capaz de inibir o

crescimento de *Candida albicans* (Chattopadhyay et al., 2004). Pacientes portadores de AIDS geralmente apresentam patologias orais durante o desenvolvimento da doença. Vargas et al. (2003) descreveram alta freqüência (51%) de alterações histopatológicas em glândulas parótidas de pacientes que morreram por AIDS no Brasil. As alterações mais freqüentes foram a tuberculose, a infecção por citomegalovírus e a sialodenite não especifica.

É provável que a expressão de SLPI em tecidos orais de pacientes com AIDS seja diferente da de pacientes não infectados pelo HIV. Existem, porém, poucos estudos em que a expressão tecidual de SLPI tenha sido analisada em pacientes com AIDS apresentando ou não, outras infecções associadas. Desta forma, o presente trabalho teve o objetivo de avaliar a expressão de SLPI em glândulas submandibulares de pacientes que foram a óbito por AIDS. Aumentar o conhecimento sobre os mecanismos de infecção na AIDS é importante para o entendimento da doença e para o descobrimento de novas opções terapêuticas.

2. CAPITULO

O presente artigo foi submetido, conforme carta confirmando o seu recebimento (Anexo 1), ao periódico "Journal of Infectious Diseases".

Expression of Secretory Leukocyte Proteinase Inhibitor (SLPI) is increased in CMV infected submandibular glands of patients with AIDS

[1]Lília A. Rocha, [1]Pablo A. Vargas, [2]Luis F.F. Silva, [1]Jorge E. Leon, [2]Angela B. Santos, [3]Pieter S. Hiemstra, [2]Thais Mauad.

[1]Department of Oral Pathology, Faculty of Odontology of Piracicaba- University of Campinas, Piracicaba-SP, Brazil; [2]Department of Pathology, São Paulo University Medical School- SP, Brazil; [3]Department of Pulmonology, Leiden University Medical Center, Leiden, the Netherlands

Running head: SLPI in submandibular glands of AIDS patients

Abstract word count: 199

Text word count: 2256

(1) None of the authors have a commercial or other association that might pose a conflict of interest with a subject of the manuscript.

(2) Funded by the Conselho Nacional de Pesquisas (CNPq), Brazil.

(3) Corresponding author address:

Thais Mauad
Department of Pathology
São Paulo University Medical School
Av Dr Arnaldo, 455 1st floor
CEP: 01246-903
São Paulo SP, Brazil
Tel 0055 11 30667173
Fax 0055 11 30642744
E-mail: tmauad@usp.br

ABSTRACT

Background: Secretory Leukocyte Proteinase Inhibitor (SLPI) is an endogenous proteinase inhibitor present in mucosal secretions. In addition to its proteinase inhibitory activity, it displays antimicrobial activity including anti-human immunodeficiency virus (HIV) activity. SLPI is expressed in submandibular glands (SMG), but there are few data on its expression in HIV-infected patients. **Objective:** To analyze the expression of SLPI in the SMG of patients that died from AIDS. **Methods:** We analyzed SLPI expression using immunohistochemistry in SMG samples of 36 AIDS patients (10 with no histological alterations, 10 with chronic non-specific sialadenitis, 8 with mycobacteriosis, and 8 with cytomegalovirus (CMV) infection) and 10 HIV negative controls. SLPI staining was quantified using image analysis and expressed as % positively stained area. **Results:** There was a higher expression of SLPI in AIDS patients with CMV infection (% SLPI stained area, mean ± SD: 37.37±14.45) when compared to all other groups ($p = 0.009$). There were no significant differences between control subjects (22.70±9.42%) and AIDS patients without histological alterations (18.10±7.58%), with chronic non-specific sialadenitis (17.13±5.36%) or mycobacterial infection (21.09±4.66%). **Conclusions:** CMV infection increases SLPI expression in the submandibular glands of AIDS patients. Our results suggest that an anti-CMV activity of SLPI should be investigated.

Key-words: Secretory leukocyte proteinase inhibitor, Submandibular gland, AIDS, Immunohistochemistry, Cytomegalovirus, Autopsy.

INTRODUCTION

The oral mucosa is exposed to high concentrations of micro-organisms and relies on an effective innate immune system that provides host defense against infection. In particular, oral tissues are naturally resistant to the human immunodeficiency virus type 1 (HIV-1) infection. A series of secreted antimicrobial factors present in the oral fluids have been implicated in this resistance, and include secretory leukocyte proteinase inhibitor (SLPI), defensins, salivary agglutinin, thrombospondin [1,2].

SLPI is a cationic serine protease inhibitor present in large quantities in mucosal secretions including saliva. Originally it was identified as an inhibitor of neutrophil elastase, but subsequent studies revealed a broad-spectrum antimicrobial activity, anti-inflammatory activities and involvement in wound repair [3-5]. Several studies have shown that SLPI displays marked anti-HIV-1 activity both in vivo and in vitro [6-7]. This activity is thought to result from blocking an early step in infection that occurs prior to virus internalization and reverse transcription [1,2], as demonstrated by binding of SLPI to the HIV-1 co-factor annexin II [8]. Besides its anti-HIV-1 inhibitory action, SLPI has also shown to possess antibacterial and antifungal properties [9].

In oral tissues, expression of SLPI has been demonstrated in keratinocytes, and in the major and minor salivary glands [10,11]. Lin et al. demonstrated that SLPI concentrations in submandibular/sublingual and parotid saliva from HIV-infected individuals are higher than those in healthy subjects [12].

In patients with advanced AIDS, the presence of inflammatory, infectious and neoplastic disease in the oral cavity is frequent. We have previously demonstrated the presence of inflammatory, infectious and neoplastic abnormalities in 51% of the parotid glands of the patients that died from AIDS in Brazil, and showed that mycobacteriosis and cytomegalovirus were the most common infections [13]. Although the role of SLPI in inhibiting HIV infection in the oral cavity has been documented [2], few studies analyzed the association of SLPI with infectious conditions in the oral cavity that are associated with HIV infection [14]. Chattopadhyay *et al.* identified high salivary SLPI levels as one of the risk factors for the development of oralpharyngeal candidiasis in HIV-1 infected subjects [15]. Therefore, the objective of this study was to analyze the expression of SLPI in the

submandibular glands (SMG) of patients that died from AIDS and relate these findings to the presence of infectious and inflammatory processes.

METHODS

The Ethics Committees of the Sao Paulo University Medical School and the Faculty of Odontology of Piracicaba, University of Campinas approved the use of autopsy samples for the present study.

Patient Population

We analyzed SMG samples of 36 patients with AIDS and 10 HIV-1 negative patients who had died in the University Hospital of the São Paulo Medical School between 1996 and 1999 and were autopsied in the Department of Pathology of the Faculty of Medicine of the University of São Paulo.

A complete autopsy was performed in all patients. Clinical records were reviewed in order to obtain age, sex, and CD4 cell count. Final autopsy reports were analyzed for main diseases. We have previously described tongue and parotid histological characteristics of this population [13,16].

Tissue processing and Histological analyses

Submandibular gland samples were fixed in buffered 10% formalin solution for 24h, and routinely processed; two to six sections of each patient were available for analysis. Slides were stained with H&E, Ziehl-Neelsen and Grocott stainings. Four histological categories were analyzed: samples without histological abnormalities, chronic non-specific sialadenitis, mycobacterial infection and cytomegalovirus infection. Mycobacterial infection was determined by the presence of poorly formed granulomas with necrosis and presence of positive bacilli identified by Ziehl-Neelsen staining. CMV infection was defined by the presence of typical enlarged cells with large nuclei with a prominent eosinophilic nucleolus and clear nuclear halo mainly in ductal cells [17]. Chronic non-specific sialadenits was defined by the presence of variable degrees of lymphocytic infiltration in the gland parenchyma without identification of etiologic agents by the techniques cited [13]. No microbiological studies were performed in the samples.

Immunohistochemistry

SLPI expression in tissue was revealed by immunohistochemistry using a monoclonal anti-SLPI antibody (clone 31) essentially as described [18]. In short, the sections were incubated overnight with the primary antibody (1:30,000) at 4°C following antigen retrieval using citrate buffer. As a secondary antibody the horseradish peroxidase conjugated anti-mouse EnVision system (DAKO, Glostrup, Denmark) was used, with Diaminobenzidine (SIGMA) as a chromogen. The sections were counterstained with Mayer's haematoxylin. For negative controls, the primary antibody was omitted from the procedure.

Morphometry

The percentage of SLPI stained area was determined by using image analysis. Measurements were performed with the software Image-Pro® Plus 4.1 for Windows® (Media Cybernetics-Silver Spring, MD, USA) on a personal computer connected to a digital camera (JVC TK-C1380 color video camera; Victor Company of Japan, Yokohama, Japan) camera that was coupled to a light microscope (Leica DMR, Leica Microsystems Wetzlar Gmbh, Germany). We measured the area of positive SLPI staining in at least 10 randomly selected acinar areas of the glands at 200x magnification. Selection of areas to be analyzed was performed by an investigator unaware of the study group. SLPI was expressed as % of stained area ($\mu m^2/ \mu m^2$).

Statistical analysis

Statistical analysis was performed with the SPSS 13.0 software (SPSS, Chicago, Illinois, USA). A multiple comparison using One-Way Analysis of Variance (ANOVA) followed by Tukey's Test was applied for comparison of SLPI content among the different groups. To analyze the correlation between SLPI expression and CD4 counts, the Spearman test was applied. Results were expressed as mean ± standard deviation (SD). The level of significance was set at $p<0.05$.

RESULTS

Patient population

In the control group there were six males and four females. Mean age was 58.4 years, ranging from 32 to 77 years. None of the patients presented salivary gland related complaints, gland enlargement or medical suspicion of submandibular disease prior to death. Autopsy findings of the control group are presented in Table 1.

In the AIDS group there were twenty-five males and eleven females. Ages ranged from 24 to 65 years, with a mean of 37.7 years. The last CD4 levels prior to death were obtained in 21 patients, with a mean of 90.7 ± 124.2 μl^{-1}, with 86% of the patients presenting < 200 cells μl^{-1}. None of the patients presented salivary gland related complaints, gland enlargement or medical suspicion of submandibular gland disease prior to death (Table 2).

Histological characterization

In the control group no histological alterations were found in the submandibular glands (Figure 1A). Among the AIDS group, 10 patients presented no histological alterations, 10 had chronic non-specific sialadenitis (Figure 1B), 8 had mycobacteriosis (Figure 1C) and 8 had CMV infection in the SMG (Figure 1D).

Immunohistochemical findings

SLPI expression was present in the serous acinar cells but not in mucosal acinar cells or in gland stroma, with a granular staining pattern (Figure 2A). SLPI staining could be also observed within the lumen of the gland ducts. There was no SLPI expression in the granulomas or in inflammatory cells (Figure 2B). SLPI expression was observed in the CMV-infected ductal cells (Figure 2C).

Morphometrical analysis

There was no significant difference in SLPI expression among control subjects (% SLPI stained area [mean ± SD]: 22.70±9.42) and AIDS patients without histological alterations in SMG (18.10±7.58 %, p=0.77), with chronic non-specific inflammation

(17.13±5.36 %, p=0.62) or with mycobacterium infection (21.09±4.66 %, p= 0.99). However, there was a statistically significant higher expression of SLPI in patients with CMV infected SMG (% SLPI = 37.37±14.45) when compared to all other groups (p= 0.009) (Figure 3). The inverse correlation between percentage of SLPI and CD4 cells counts did not reach statistical significance (r^2= -0.22, p=0.33).

DISCUSSION

In the present study, we analyzed tissue expression of SLPI in the SMG of patients that died due from advanced AIDS, and demonstrated a higher SLPI expression in CMV infected SMG. No difference was noted in SLPI expression in SMG from AIDS and control subjects. These results suggest that CMV infection increases local expression of SLPI in SMG in AIDS.

There are only few studies that have quantified tissue expression of SLPI in patients with inflammatory and infectious conditions related to AIDS [14] and, to the best of our knowledge; we are the first to detect a higher SLPI expression in patients with CMV infection. SLPI is an endogenous proteinase inhibitor that has two highly homologous domains of about 54 amino acids, each of which contains four disulfide bonds. It was isolated from a variety of mucosal secretions, including human parotid fluids [19]. It was identified based on its ability to inhibit neutrophil elastase, and subsequently shown to inhibit a range of proteinases from inflammatory cells [5]. In addition, SLPI displays anti-inflammatory and antimicrobial activities, and is implicated in epithelial wound repair [5, 20, 21]. Particularly, SLPI plays an important role in defense against HIV-1 infection, especially at the early stages of the infection [22].

Tissue expression of SLPI in the salivary glands has been previously observed. Ohlsson *et al.* described SLPI expression in serous cells of both parotid and submandibular glands [11]. Wahl *et al.* analyzed oral tissue expression of SLPI in HIV positive (including ten patients with a CMV infection) and HIV negative tissue samples, and did not detect qualitative differences in expression between the groups [14]. Our results confirm their descriptive findings. Similarly, we were not able to detect differences in SLPI between control groups and those AIDS patients without an HIV related infectious condition, but detected increased expression in CMV infected glands. The same authors detected the presence of HIV in the mononuclear inflammatory cells in the salivary glands, but not in the epithelial cells, suggesting that the interaction between the virus and SLPI occurs extracellularly in saliva.

More recently, Lin *et al.* studied the salivary SLPI concentration, secretory rate and specific protein concentration in parotid and submandibular/sublingual secretions of

HIV-1 infected and uninfected individuals. The concentration of SLPI was increased in the infected group and most notably, mainly in HIV-1 infected individuals that received highly active antiretroviral therapy (HAART). The authors suggested that this increase in infected patients results from decreased fluid secretion rather than from an alteration in the synthesis or secretion of SLPI *per se* [12]. Gordon *et al.* could not demonstrate differences in SLPI levels in the bronchoalveolar lavage fluid of HIV+ patients and suggested an altered innate pulmonary immunity in these patients [23].

We have demonstrated that the expression of SLPI is increased in the SMG of AIDS patients with a CMV infection, suggesting a role of SLPI in CMV infection. SLPI is synthesized and secreted at mucosal sites in response to microbial exposure, inflammation, injury and repair, and its epithelial expression was found to be increased by e.g. neutrophil defensins, growth factors and pro-inflammatory cytokines, being possibly part of a local inducible defense system. Previous studies have shown that, in addition to its antibacterial and antifungal activities, SLPI also displays antiviral activity. It is not only active against HIV-1, but also against Sendai virus and influenza A virus [24]. The anti-CMV activity of SLPI is not well-documented and therefore the contribution of increased SLPI expression to anti-CMV defenses remains to be established [25], in addition to its antimicrobial activity. SLPI may either provide protection against proteinases derived from inflammatory cells or it may contribute to repair of oral mucosa secondary to CMV-induced tissue injury. The recent observation that mice deficient in SLPI have impaired oral wound healing is in line with this suggestion [26]. The mechanisms by which CMV increases SLPI expression are unknown. The recent observation that CMV causes activation of the epidermal growth factor receptor [27] combined with the observation that the EGFR ligand TGFα increases epithelial SLPI expression [28] suggests that CMV may employ EGFR to increase SLPI expression. This possibility is currently explored.

Although SLPI expression in SMG with mycobacteriosis was higher than in the group without opportunistic infectious, the differences were not statistically significant. It has been previously demonstrated that SLPI expression can be increased in rat macrophages exposed to *Mycobacterium tuberculosis* [29], but further data on humans are not available so far.

Our study has certainly limitations. We did not analyze the presence of HIV-1 protein or RNA in the autopsy samples to check whether the local presence of the virus in the SMG could alter SLPI content expression. In many of the cases, we also did not have access to information on the anti-HIV-1 treatment these patients received. This may be relevant, because it has been recently shown that HAART therapy may enhance SLPI concentration in saliva [12]. However, since our samples date back from 1996 to 1999, it is possible that not all patients were receiving HAART at that time.

Although not statistically significant, there was a negative correlation between CD4 cells counts and SLPI ($r^2 = -0.22$) in our patients. Lin *et al.* also recently described an inverse correlation between SLPI levels in the parotid gland saliva and CD4 counts, and hypothesized that increased SLPI levels in the saliva are indicative of a more advanced stage of the HIV disease [12]. In our samples, most of the patients were profoundly immunosuppressed as indicated by very low CD4 cell counts, which could explain the lack of significance in the correlation analysis.

In summary, we have shown that the presence of CMV infection in SMG of AIDS patients increases local expression of SLPI protein. This increased SLPI expression could serve to provide protection against infection, the action of proteinases derived from inflammatory cells and could promote tissue repair. Further studies are required to delineate the role of SLPI in the pathogenesis of CMV infections and the mechanisms underlying this increased expression.

REFERENCES

1. Shugars DC, Wahl SM. The role of the oral environment in HIV-1 transmission. J Am Dent Assoc **1998**; 129:851-8.
2. Shugars DC, Alexander AL, Fu K, Freel SA. Endogenous salivary inhibitors of human immunodeficiency virus. Arch Oral Biol **1999**; 44:445-53.
3. Sallenave JM. Antimicrobial activity of antiproteinases. Biochem Soc Trans. **2002**; 30:111-5.
4. Hiemstra PS. Novel roles of protease inhibitors in infection and inflammation. Biochem Soc Trans **2002**; 30:116-20.
5. Doumas S, Kolokotronis A, Stefanopoulos P. Anti-inflammatory and antimicrobial roles of secretory leukocyte protease inhibitor. Infect Immun **2005**; 73:1271-4.
6. McNeely TB, Dealy M, Dripps DJ, Orenstein JM, Eisenberg SP, Wahl SM. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. J Clin Invest **1995**; 96:456-64.
7. Shugars DC, Sauls DL, Weinberg JB. Secretory leukocyte protease inhibitor blocks infectivity of primary monocytes and mononuclear cells with both monocytotropic and lymphocytotropic strains of human immunodeficiency virus type I. Oral Dis **1997**; 3:S70-2.
8. Ma G, Greenwell-Wild T, Lei K, et al. Secretory leukocyte protease inhibitor binds to annexin II, a cofactor for macrophage HIV-1 infection. J Exp Med **2004**; 200:1337-46.
9. Hiemstra PS, Fernie-King BA, McMichael J, Lachmann PJ, Sallenave JM. Antimicrobial peptides: mediators of innate immunity as templates for the development of novel anti-infective and immune therapeutics. Curr Pharm Des **2004**; 10:2891-905.
10. Jana NK, Gray LR, Shugars DC. Human immunodeficiency virus Type 1 stimulates the expression and production of secretory leukocyte protease inhibitor (SLPI) in oral epithelial cells: a role for SLPI in innate mucosal immunity. J Virol **2005**; 79:6432-40.

11. Ohlsson M, Fryksmark U, Polling A, Tegner H, Ohlsson K. Localization of antileukoprotease in the parotid and the submandibular salivary glands. Acta Otolaryngol **1984**; 98:147-51.
12. Lin AL, Johnson DA, Stephan KT, Yeh C. Salivary secretory leukocyte protease inhibitor increases in HIV infection. J Oral Pathol Med **2004**; 33:410-6.
13. Vargas PA, Mauad T, Böhm GM, Saldiva PHN, Almeida PA. Parotid gland involvement in advanced AIDS. Oral Dis **2003**; 9:55-61.
14. Wahl SM, Worley P, Jin W, et al. Anatomic dissociation between HIV-1 and its endogenous inhibitor in mucosal tissues. Am J Pathol **1997**; 150:1275-84.
15. Chattopadhyay A, Gray LR, Patton LL et al. Salivary secretory leukocyte protease inhibitor and oral candidiasis in human immunodeficiency virus type 1- infected persons. Infect Immun **2004**; 72:1956-63.
16. de Faria PR, Vargas PA, Saldiva PH, Bohm GM, Mauad T, de Almeida OP. Tongue disease in advanced AIDS. Oral Dis **2005**; 11:72-80.
17. Neville BW, Damm DD, Allen CM, Rouquot JE. Viral Infections. In: Neville BW, Damm DD, Allen CM, Rouquot JE, eds. Oral and Maxillofacial Pathology. 2nd ed. Philadelphia. W B Saunders, **1995**: 226-227.
18. Aarbiou J, van Schadewijk A, Stolk J, et al. Human neutrophil defensins and secretory leukocyte proteinase inhibitor in squamous metaplastic epithelium of bronchial airways. Inflamm Res **2004**; 53:230-8.
19. Thompson RC, Ohlsson K. Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase. Proc Natl Acad Sci USA **1986**; 83:6692-6.
20. Fryksmark U, Jannet M, Ohlsson K, Tegner H, Wihl JA. Secretory leukocyte protease inhibitor in normal allergic and virus induced nasal secretions. Rhinology **1989**; 27:97-103.
21. Vogelmeier C, Hubbard RC, Fells GA, et al. Anti-neutrophil elastase defense of the normal human repiratory epithelial surface provided by the secretory leukoprotease inhibitor. J Clin Invest **1991**; 87:482-8.
22. McNeely TB, Tucker C, Shugars DC, Rosendahl M, Eisenberg SP, Wahl SM. Inhibition of human immunodeficiency virus type 1 infectivity by secretory

leukocyte protease inhibitor occurs prior to reverse transcription. Blood **1997**; 90:1141-9.

23. Gordon SB, Janoff EN, Sloper D, et al. HIV-1 infection is associated with altered innate pulmonary immunity. J Infect Dis **2005**; 192:1412-6.
24. Beppu Y, Imamura Y, Tashiro M, Towatari T, Ariga H, Kido H. Human mucus protease inhibitor in airway fluids is a potential defensive compound against infection with influenza A and Sendai viruses. J Biochem **1997**; 121:309-16.
25. Wahl SM, McNeely TB, Janoff EN, et al. Secretory leukocyte protease inhibitor (SLPI) in mucosal fluids inhibits HIV-I. Oral Dis. **1997**; 3:S64-9.
26. Angelov N, Moutsopoulos N, Jeong MJ, Nares S, Ashcroft G, Wahl SM. Aberrant mucosal wound repair in the absence of secretory leukocyte protease inhibitor. Thromb Haemost **2004**; 92:288-97.
27. Wang X, Huong SM, Chiu ML, Raab-Traub N, Huang ES. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature **2003**; 424:456-61.
28. Sorensen OE, Cowland JB, Theilgaard-Monch K, Liu L, Ganz T, Borregaard N. Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. J Immunol. **2003**; 170:5583-9.
29. Ding A, Yu H, Yang J, Shi S, Ehrt S. Induction of macrophage-derived SLPI by Mycobacterium tuberculosis depends on TLR2 but not MyD88. Immunology **2005**; 116:381-9.

TABLES

Table1. Sex, age and main autopsy findings of HIV negative control group.

Patient	Age (years)	Sex[a]	Autopsy findings
1	63	*f*	Chagas` disease; Atherosclerosis
2	67	*m*	Pneumonia
3	61	*m*	Atherosclerosis; Diabetes Mellitus
4	77	*m*	Cerebral aneurysm
5	32	*f*	Cerebral aneurysm
6	57	*m*	Ischemic cerebral stroke
7	77	*f*	Hemorrhagic cerebral stroke
8	56	*m*	Hemorrhagic cerebral stroke
9	45	*m*	Renal transplantation with gastrointestinal bleeding
10	49	*f*	Myasthenia gravis, Acute pancreatitis

(a); m, male; f, female.

Table 2. Age, sex, CD4 cell counts and main autopsy findings of the AIDS patients.

Patient	Age years	Sex[a]	CD4 count (cells μl^{-1})	Submandibular gland alterations	Autopsy findings
11	45	*f*	26	no	Cachexia; CMV[b] gastritis
12	47	*m*	13	no	Bronchopneumonia
13	41	*m*	41	no	Bronchopneumonia
14	40	*m*	71	no	CMV pneumonia; *Cryptococcus* meningitis
15	32	*m*	na[c]	no	Granulomatous disease; Cachexia
16	33	*m*	05	no	Pneumocystoses; Chronic pancreatitis
17	30	*m*	22	no	Disseminated tuberculosis
18	30	*m*	67	no	Lung mycobacteriosis; Neurotoxoplasmosis
19	31	*m*	na	no	Cachexia; Acute respiratory failure
20	34	*m*	na	no	Disseminated Histoplamosis
21	45	*m*	na	Sialadenitis	Disseminated tuberculosis
22	29	*f*	na	Sialadenitis	Disseminated mycobacteriosis; Septic shock
23	65	*m*	93	Sialadenitis	Pleural tuberculosis
24	50	*f*	na	Sialadenitis	Septic shock
25	40	*m*	375	Sialadenitis	Bronchopneumonia; Meningitis
26	46	*m*	290	Sialadenitis	Neurocryptococosis; Bronchopneumonia
27	36	*f*	31	Sialadenitis	Pneumocystosis
28	38	*m*	434	Sialadenitis	Disseminated Histoplamosis; Septic shock
29	26	*m*	72	Sialadenitis	Intersticial pneumonia; Ganglionic tuberculosis
30	44	*m*	66	Sialadenitis	Disseminated NHL; Esophagic candidiasis

31	46	*m*	50	Mycobacteriosis	Disseminated mycobacteriosis
32	34	*f*	na	Mycobacteriosis	Disseminated mycobacteriosis
33	40	*f*	na	Mycobacteriosis	Disseminated mycobacteriosis; Cachexia
34	24	*m*	na	Mycobacteriosis	Disseminated mycobacteriosis
35	48	*f*	na	Mycobacteriosis	Pneumonia associated with HIV
36	35	*m*	na	Mycobacteriosis	Pneumocystosis
37	43	*m*	na	Mycobacteriosis	Disseminated mycobacteriosis
38	29	*m*	36	Mycobacteriosis	Septic shock ; Disseminated mycobacteriosis
39	30	*f*	05	CMV	Esophagic candidiasis; Disseminated mycobacteriosis; CMV
40	37	*f*	na	CMV	Bacterial bronchopneumonia; Lung and adrenal CMV infection
41	26	*f*	na	CMV	Neurotoxoplasmosis; Lung and adrenal CMV infection
42	32	*m*	47	CMV	Septic shock; CMV; Genital herpes
43	26	*m*	15	CMV	CMV and mycobacteriosis cachexia
44	50	*m*	na	CMV	Bacterial bronchopneumonia; Cachexia; CMV
45	51	*m*	136	CMV	Cachexia; CMV
46	25	*f*	10	CMV	Neurotoxoplasmosis; CMV

(a) m, male; f, female, (b); CMV: cytomegalovirosis, (c) Na: not available.

LEGEND FOR FIGURES

Figure 1. Histological features of submandibular glands in AIDS.

Figure 1A. Submandibular gland without histological alterations in a control patient. Serous acinus (arrow), mucous acinus (asterisk), ductal cells (arrow head). 200X, H&E

Figure 1B. Chronic sialadenitis in an AIDS patient. Observe the chronic inflammation surrounding the acinar structures (arrow). 200X, H&E

Figure 1C. Mycobacterial infection in the submandibular gland of an AIDS patient. Observe the poorly formed granuloma (arrow). 200x, H&E. In the insert, multiple acid fast bacilli. 400x, Ziehl-Neelsen.

Figure 1D. CMV infection in the submandibular gland of an AIDS patient. The ductal cells show the typical large intranuclear inclusions (arrow). 200x, H&E.

Figure 2. Immunostaining for SLPI in submandibular glands.

Figure 2A. SLPI immunohistochemical staining in a submandibular gland without histological alterations. SLPI staining is present in serous acinar cells (arrow) but absent in mucous cells. 200X.

Figure 2B. SLPI immunohistochemical staining in a submandibular gland with mycobacteriosis. Note the absence of staining in the granuloma formation (arrow). 200X.

Figure 2C. SLPI immunohistochemical staining in a submandibular gland with CMV infection. Serous acinar cells (arrow head) and ductal cells (arrow) present strong positive staining.

Figure 3.

Mean values of the % SLPI staining in the submandibular glands (SMG) of the different study groups. (*) There was a significant increase of the CMV infected group when compared to all other study groups, $p = 0.009$. The statistical significance of each comparison was assessed by the One-Way Analysis of Variance (ANOVA) test, followed by the Tukey's post-hoc test.

FIGURE 1

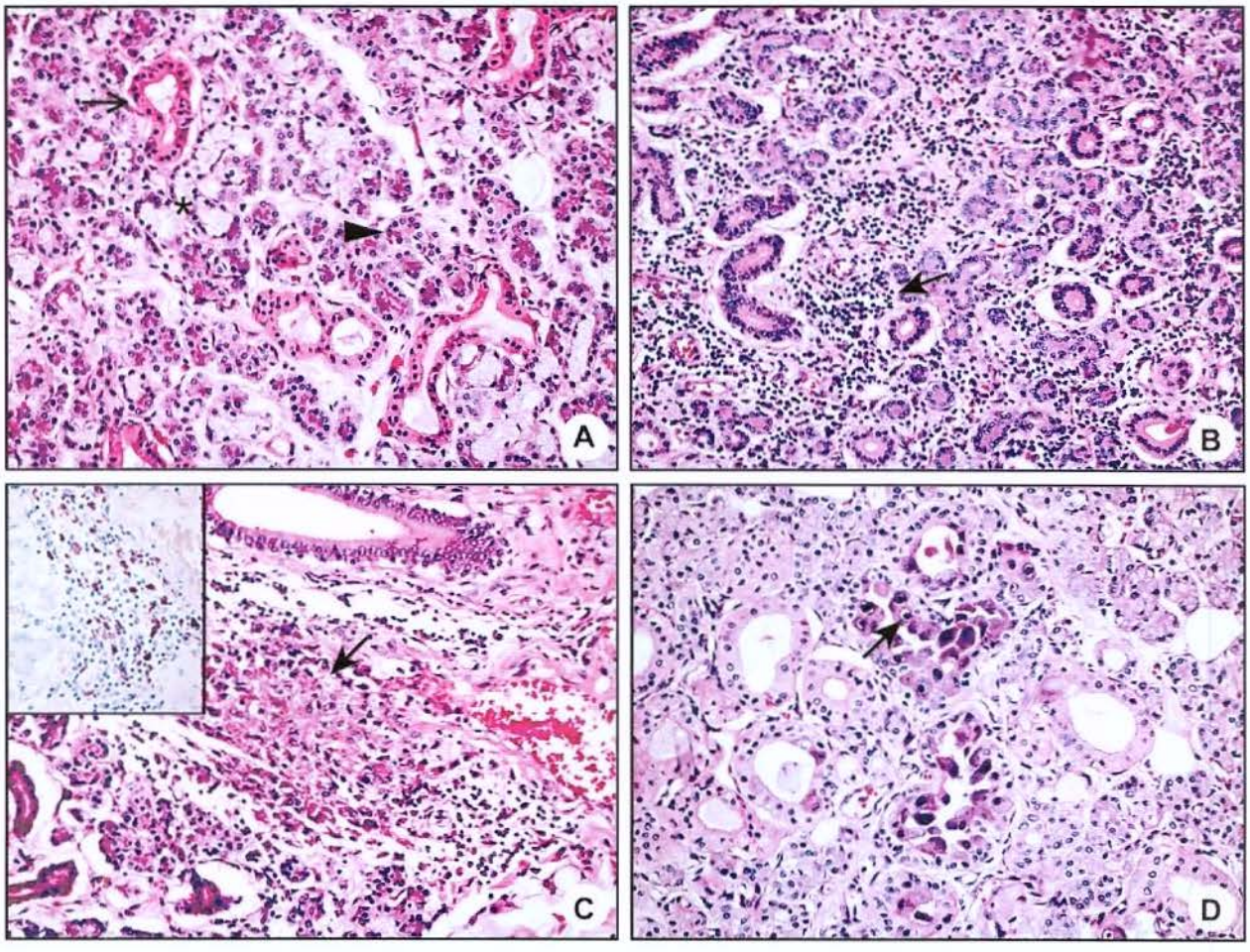


FIGURE 2

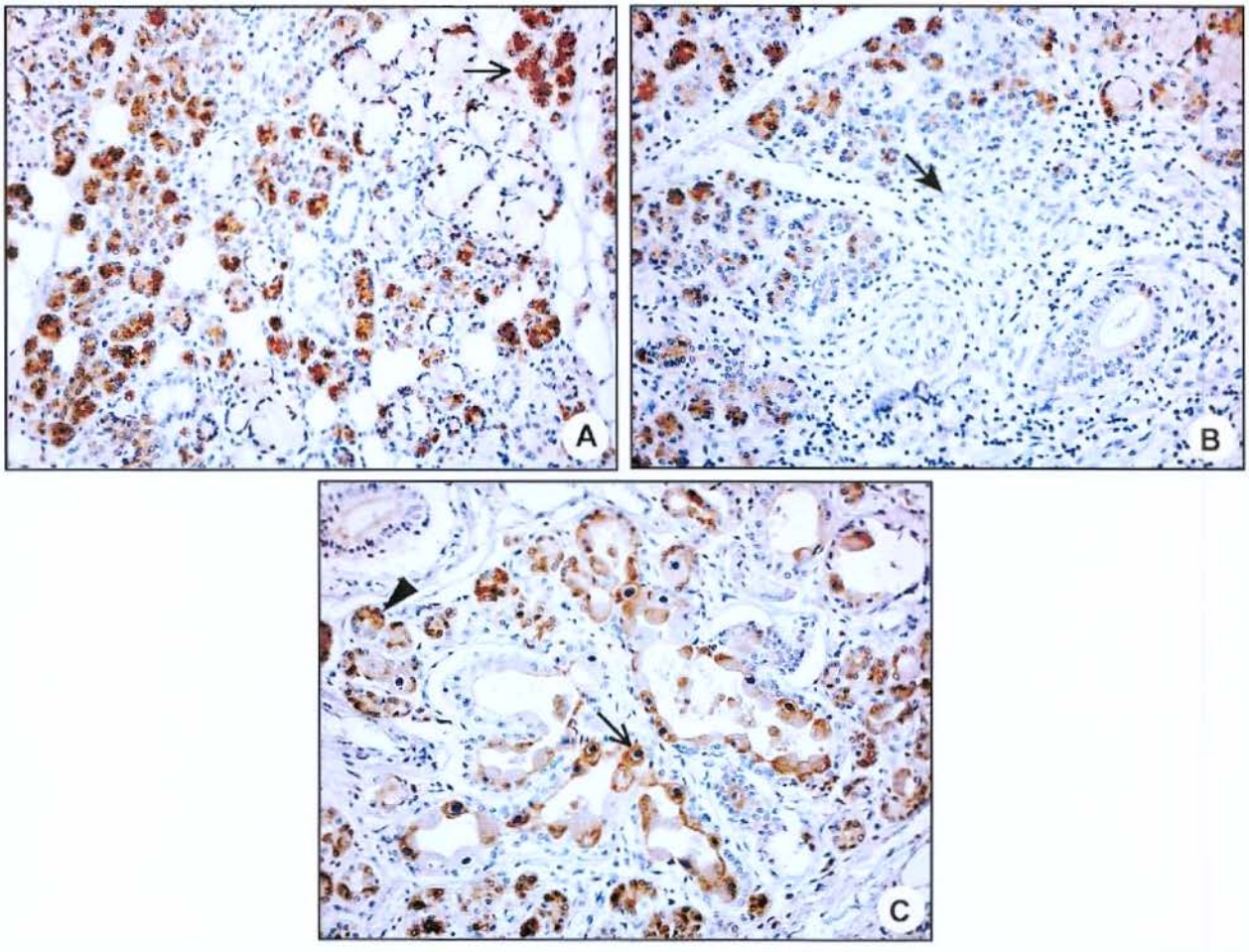
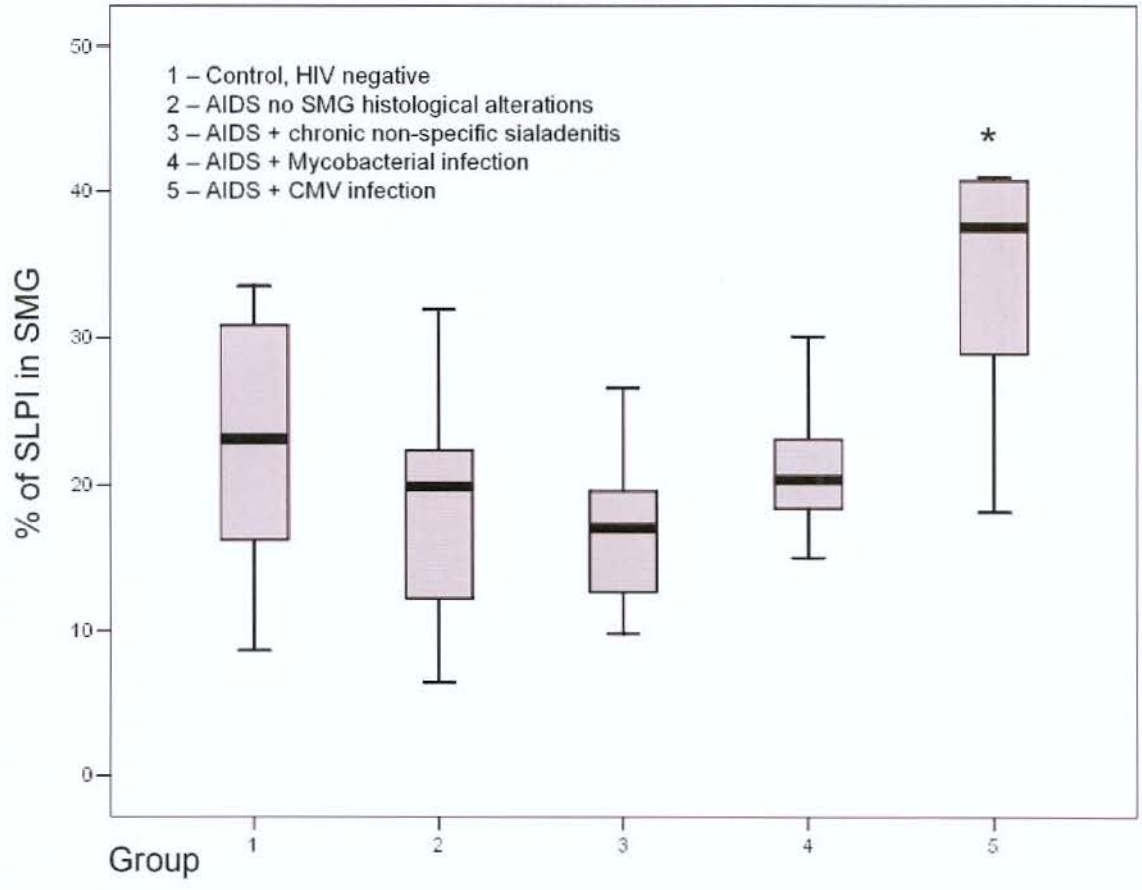


FIGURE 3


1 – Control, HIV negative
2 – AIDS no SMG histological alterations
3 – AIDS + chronic non-specific sialadenitis
4 – AIDS + Mycobacterial infection
5 – AIDS + CMV infection
*
% of SLPI in SMG
50
40
30
20
10
0
Group
1
2
3
4
5

3. CONCLUSÃO

O presente estudo mostra que a infecção por CMV foi capaz de aumentar a expressão do inibidor de protease liberada por leucócitos (SLPI) em glândulas submandibulares de pacientes com AIDS.

REFERÊNCIAS BIBLIOGRÁFICAS

Chattopadhyay A, Gray LR, Patton LL, Caplan DJ, Slade GD, Tien HC, Shugars DC. Salivary secretory leukocyte protease inhibitor and oral candidiasis in human immunodeficiency virus type 1-infected persons. **Infect Immun** 2004;72(4):1956-63.

Cotran RS, Kumar V, Collins T. Doenças da imunidade. Em: Cotran RS, Kumar V, Collins T, editores. **Robbins Patologia Estrutural e Funcional** 6ª.edição. Rio de Janeiro: Editora Guanabara Koogan S.A; 2000. p. 168-232.

Fox PC, Wolff A, Yeh C, Atkinson JC, Baum BJ. Saliva inhibits HIV-1 infectivity. **J Am Dent Assoc** 1988; 116(6):635-7.

Gallo RL, Murakami M, Ohtake T, Zaiou M. Biology and clinical relevance of naturally occurring antimicrobial peptides. **J Allergy Clin Immunol** 2002; 110(6):823-31.

Hiemstra PS. Novel roles of protease inhibitors in infection and inflammation. **Biochem Soc Trans** 2002; 30(2):116-20.

Jana NK, Gray LR, Shugars DC. Human immunodeficiency virus Type 1 stimulates the expression and production of secretory leukocyte protease inhibitor (SLPI) in oral epithelial cells: a role for SLPI in innate mucosal immunity. **J Virol** 2005; 79(10):6432-40.

Lin AL, Johnson DA, Stephan KT, Yeh C. Salivary secretory leukocyte protease inhibitor increases in HIV infection. **J Oral Pathol Med** 2004; 33:410-6.

McNeely TB, Dealy M, Dripps DJ, Orenstein JM, Eisenberg SP, Wahl SM. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. **J Clin Invest** 1995; 96(1):456-64.

Ohlsson M, Fryksmark U, Polling A, Tegner H, Ohlsson K. Localization of antileukoprotease in the parotid and the submandibular salivary glands. **Acta Otolaryngol** 1984; 98(1-3):147-51.

Programa Conjunto das Nações Unidas sobre o HIV/AIDS (ONUSIDA) e Organização Mundial da Saúde (OMS). Situação da epidemia da AIDS. Dezembro, 2005. **http://www.unaids.org/em/resources/epidemiology.asp.**

Shugars DC, Sauls DL, Weinberg JB. Secretory leukocyte protease inhibitor blocks infectivity of primary monocytes and mononuclear cells with both monocytotropic and lymphocytotropic strains of human immunodeficiency virus type I. **Oral Dis** 1997; 3(Suppl 1):S70-2.

Vargas PA, Mauad T, Böhm GM, Saldiva PHN, Almeida PA. Parotid gland involvement in advanced AIDS. **Oral Dis** 2003; 9:55-61.

Wahl SM, McNeely TB, Janoff EN, Shugars D, Worley P, Tucker C, Orenstein JM. Secretory leukocyte protease inhibitor (SLPI) in mucosal fluids inhibits HIV-1. **Oral Dis** 1997; 3(Suppl 1):S64-9

ANEXO 1

The Journal of Infectious Diseases

Logged in: Thais Mauad (tmauad@usp.br)

Finish submission for:

Title	Expression of Secretory Leukocyte Proteinase Inhibitor (SLPI) is increased in CMV infected submandibular glands of patients with AIDS
Authors	Lília A. Rocha (1), Pablo A. Vargas (1), Luis F.F. Silva (2), Jorge E. Leon (1), Angela B. Santos (2), Pieter S. Hiemstra (3), Thais Mauad (2)
MS ID	36385

You chose: **Approve and submit**

Your submission has been received and will be acknowledged in a few business days by the journal office.

Your manuscript number is **36385**. Please use this number in all correspondence with the journal office.

An automatic acknowledgment has been sent to your email address: tmauad@usp.br. Please review your contact information for future communication from the journal office.

Journal office: jid@press.uchicago.edu Tech support: jid-help@mss.uchicago.edu