



ISADORA LUANA FLORES

**“Decreased expression of angiotensinogen and dipeptidyl peptidase 1  
may be associated with the development of Proliferative Verrucous  
Leukoplakia”**

**“Diminuição da expressão de angiotensinogênio e dipeptidil  
peptidase 1 pode estar associada ao desenvolvimento de Leucoplasia  
Verrucosa Proliferativa”**

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**UNIVERSIDADE ESTADUAL DE CAMPINAS**  
**FACULDADE DE ODONTOLOGIA DE PIRACICABA**  
**ISADORA LUANA FLORES**

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may be associated with the development of Proliferative Verrucous  
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peptidase 1 pode estar associada ao desenvolvimento de Leucoplasia  
Verrucosa Proliferativa”**

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**Orientador: PROF. DR. MARCIO AJUDARTE LOPES**

Este exemplar corresponde a versão final da tese  
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## ABSTRACT

**OBJECTIVE:** Proliferative verrucous leukoplakia (PVL) is a rare variant and still poorly understood of oral leukoplakia with a behavior of persistent progression to malignancy showing a rate of malignancy between 40-100%. Moreover, the early detection of PVL is sometimes challenging for clinicians, but plays a crucial role to establish a continuous and rigorous follow-up. Underlying molecular aspects are relevant and no previous study investigated the saliva of PVL patients. The increased interest in the salivary proteome study is because proteins are considered the most important molecules in the salivary fluid with potential to act as biomarker for diagnosis of various systemic and local diseases. Based on these aspects, the present study aimed to draw the salivary proteome profile of patients with PVL in order to identify potential biomarkers to better understanding of this entity targeting the possible clinical use.

**MATERIALS AND METHODS:** Unstimulated whole-mouth saliva was collected of 30 voluntaries (15 PVL patients and 15 controls). Proteomic approach based to liquid chromatography coupled to tandem mass spectrometry was performed to 20 µg of proteins of the samples. Chi-Square, analysis of variance and logistic regression test were used in the statistical analysis.

**RESULTS:** A total of two hundred eighty-three proteins were identified. Among of them, 31 proteins showed statistical significance difference in relation to abundance, being 25 proteins with higher abundance in control group and 6 proteins with higher abundance in PVL group. The combination of angiotensinogen and dipeptidyl peptidase 1 created a model for group differentiation with a concordance index of 94.2% revealing both proteins as potential biomarkers for diagnosis of PVL.

**CONCLUSIONS:** Although this study is the first to evaluate the salivary proteome in PVL patients, the results showed that saliva screening may be helpful test to diagnosis of individuals with risk to PVL development.

**Keywords:** Saliva, angiotensinogen, cathepsin C, biomarkers, oral leukoplakia.





## RESUMO

**OBJETIVO:** A leucoplasia verrucosa proliferativa (LVP) é uma variante rara e ainda pouco compreendida de leucoplasia oral com um comportamento de progressão persistente para malignidade apresentando uma taxa de malignização entre 40-100%. Além disso, a detecção precoce da LVP às vezes é um desafio para os clínicos, porém desempenha um papel crucial para estabelecer um contínuo e rigoroso acompanhamento. Aspectos moleculares subjacentes são relevantes e nenhum estudo anterior investigou a saliva de pacientes com LVP. O aumento do interesse no estudo do proteoma salivar ocorre porque as proteínas são consideradas as moléculas mais importantes do fluido salivar com potencial para atuar como biomarcador para o diagnóstico de várias doenças sistêmicas e locais. Com base nestes aspectos, o presente estudo teve como objetivo traçar o perfil do proteoma salivar de pacientes com LVP, a fim de identificar potenciais biomarcadores para a melhor compreensão desta entidade visando o possível uso clínico.

**MATERIAIS E MÉTODOS:** A saliva total não estimulada foi coletada de 30 voluntários (15 pacientes com LVP e 15 controles). Uma abordagem proteômica baseada na associação de cromatografia líquida acoplada à espectrometria de massa foi realizada para análise de 20 µg de proteínas das amostras. Os testes de qui-quadrado, análise de variância e regressão logística foram utilizados na análise estatística.

**RESULTADOS:** Um total de duzentas e oitenta e três proteínas foram identificadas. Entre estas, 31 proteínas apresentaram diferença estatisticamente significativa em relação à abundância, sendo 25 proteínas com maior abundância no grupo controle e 6 proteínas com maior abundância no grupo LVP. A combinação das proteínas angiotensinogênio e dipeptidil peptidase 1 criaram um modelo de diferenciação de grupo com um índice de concordância de 94,2% revelando ambas as proteínas como potenciais biomarcadores para o diagnóstico de LPV.

**CONCLUSÕES:** Apesar deste estudo ser o primeiro a avaliar o proteoma salivar em pacientes com LVP, os resultados mostraram que a triagem da saliva pode ser um teste útil no diagnóstico de indivíduos com risco para o desenvolvimento de LPV.

**Palavras-chave:** Saliva, angiotensinogênio, catepsina C, biomarcadores, leucoplasia oral.



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“A nossa maior glória não reside no fato de nunca cairmos, mas sim em levantarmo-nos sempre depois de cada queda.”

*Confúcio*



# INTRODUÇÃO

## **Leucoplasia verrucosa proliferativa**

O termo leucoplasia verrucosa proliferativa (LVP) foi definido pela primeira vez por Hansen et al. em 1985 como uma doença agressiva e de etiologia desconhecida e que apesar da apresentação clínica inicial de uma lesão leucoplásica que tende a se tornar multifocal ao longo do tempo (Hansen et al., 1985; Gouvêa et al., 2010a). A LVP é considerada uma lesão potencialmente maligna incomum da cavidade oral que afeta principalmente mulheres idosas a partir da sexta década de vida em uma proporção homem/mulher de 1:4 (Gouvêa et al., 2010a; Gouvêa et al., 2013; Carrard et al., 2013). Etiologicamente, as pacientes geralmente não apresentam histórico de exposição aos fatores de risco convencionais para a leucoplasia, tais como o consumo de tabaco e álcool e nenhuma associação com infecções pelo papiloma vírus humano (HPV) (Pentenero et al., 2014) ou pelo vírus do Epstein-Barr (EBV) foram encontradas (Gillenwater et al., 2013a). As lesões de LVP têm tendência a várias recidivas e resistência a todos os tipos de tratamento (Gouvêa et al., 2010a; Gouvêa et al., 2013; Carrard et al., 2013).

Segundo a Organização Mundial de Saúde (OMS), a leucoplasia oral é classificada como uma lesão potencialmente maligna e caracteriza-se por "uma placa branca de risco questionável definida após a exclusão de outras doenças ou distúrbios conhecidos que mimetizam este aspecto, mas que não carregam nenhum risco conhecido para o câncer" (van der Wall, 2009; van der Wall, 2010; Gillenwater et al., 2013a). Logo, leucoplasia representa um termo clínico e a biópsia é fundamental para observação de possíveis alterações microscópicas (van der Waal, 2009; van der Waal, 2010). Além disso, a maioria das leucoplasias convencionais permanece com alterações clínicas e histológicas ao longo do tempo caracterizando-se por acantose ou hiperqueratose com um pequeno grupo de lesões progredindo para displasias de alto grau ou carcinoma francamente invasivo (van der Waal, 2009; van der Waal, 2010). Interessantemente, a LVP é uma variante rara e

menos conhecida entre as leucoplasias com um comportamento único de persistente progressão para malignidade. Entretanto, como compartilha similaridades com a leucoplasia convencional, o seu diagnóstico pode ser adiado, pois depende da avaliação temporal das lesões (van der Waal e Reichart, 2008; Gouvêa et al., 2010b; Gouvêa et al., 2013; Carrard et al., 2013; Gillenwater et al., 2013).

As lesões de LVP podem acometer qualquer sítio da mucosa oral sendo muito frequentes na mucosa jugal e na língua (Hansen et al., 1985; Gouvêa et al., 2010a), porém, as áreas preferenciais para o desenvolvimento de carcinoma espinocelular (CEC) são as lesões de gengiva, palato e de rebordo alveolar (Bagán et al., 2004; Cerero-Lapiedra et al., 2010; Gillenwater et al., 2013a, Gillenwater et al., 2013b). Classicamente, apresentam-se como placas brancas hiperqueratóticas que progridem para um aspecto verrucoso e exofítico (Hansen et al., 1985; Gouvêa et al., 2010a) com o reconhecimento nos últimos anos de que algumas lesões podem apresentar-se como áreas atróficas ou eritematosas e até mesmo estar associadas a uma franca ulceração (Hansen et al., 1985; Müller, 2011; Gouvêa et al., 2013; Gillenwater et al., 2013a). Histologicamente, hiperplasia epitelial e hiperqueratose localizada sem displasia epitelial, expansão multifocal com ou sem graus variados de displasia, hiperplasia verrucosa e carcinoma verrucoso ou um CEC francamente invasivo são os quatro aspectos histológicos geralmente encontrados durante o curso da doença (Gouvêa et al., 2010b; Gillenwater et al., 2013a; Gillenwater et al., 2013b).

Esta apresentação clínica e histopatológica distinta associada ao comportamento biológico agressivo permite que a LVP seja classificada como uma entidade distinta da leucoplasia oral convencional sendo descrita pela OMS como a lesão potencialmente maligna com mais alto risco de progressão para o CEC oral (Hansen et al., 1985; Ge et al., 2011). Tal taxa de transformação maligna ocorre em mais de 40% dos casos, com uma tendência para a evolução de mais de uma área para CEC oral sendo muito importante a avaliação temporal das lesões (Hansen et al., 1985; Zakrzewska et al., 1996; Silverman and Gorsky, 1997; Fettig et al., 2000; Bagán et al., 2003; Morton et al., 2007; Bagan et al., 2003; Gouvêa et al., 2010a; Gouvêa et al., 2013). Atualmente, quatro grandes fases poder ser encontradas durante o desenvolvimento da LVP: (1) envolvimento focal inicial, (2) expansão multifocal ao longo do tempo, (3) desenvolvimento de uma aparência verrucosa,

e, finalmente, (4) o desenvolvimento de um câncer (Batsakis et al., 1999; Gillenwater et al., 2013b).

Inúmeras condições devem ser consideradas como diagnósticos diferenciais para LVP, especialmente em estágios iniciais, desde uma leucoplasia convencional localizada ou multifocal, hiperplasia verrucosa, carcinoma verrucoso, líquen plano, reações liquenóides e leucoplasia pilosa oral (LPO) (Gillenwater et al., 2013a; Gillenwater et al., 2013b). As leucoplasias convencionais diferentemente da LVP acometem mais frequentemente homens idosos com histórico de exposição crônica aos fatores de risco de fumo e álcool. Pode acometer um ou mais sítios, principalmente nas bordas laterais e ventre de língua e o assoalho bucal. Em cerca de 5% a 15% dos casos pode evoluir com alterações displásicas ou CEC (Lee et al., 2000; Gillenwater et al., 2013a). Uma hiperplasia ou carcinoma verrucoso esporádico podem mimetizar a LVP devido ao aspecto clínico e acometimento principalmente da mucosa alveolar e gengiva de mulheres idosas. Entretanto, tais lesões não apresentam a evolução natural característica da LVP (Murray e Batsakis, 1994).

A LVP em estágio inicial pode mimetizar clínica e histologicamente o líquen plano e as reações liquenóides, principalmente por acometerem mais mulheres, serem multifocais e apresentarem infiltrado linfocítico denso sub-epitelial em banda. Por outro lado, quando há displasia epitelial líquen plano e reações liquenóides devem ser desconsideradas (Batsakis et al., 1999; Müller, 2011; Gillenwater et al., 2013a). Portanto, lesões com aspecto que varia de placas hiperqueratóticas a áreas eritematosas atróficas ou de franca ulceração associadas à displasia em mulheres com o mesmo perfil demográfico da LVP precisam ser acompanhadas, pois podem representar um quadro inicial de LVP no qual a observação temporal é fundamental para o diagnóstico definitivo (Gillenwater et al., 2013a; Gillenwater et al., 2013b).

A LPO está associada a um quadro de imunodepressão, principalmente da síndrome da imunodeficiência adquirida, e apresenta-se como placa branca espessa e de superfície verrucosa que acomete as bordas laterais de língua (Greenspan et al., 1992; Simi et al., 2013). Apesar das lesões de LVP poderem ser clinicamente semelhantes às lesões de LPO, os achados histológicos diferenciam plenamente as duas entidades (Gillenwater et al.,

2013b; Simi et al., 2013). Uma vez confirmado o diagnóstico de LVP, inúmeras modalidades terapêuticas isoladas ou combinadas têm sido realizadas, tais como cirurgia, crioterapia, laserterapia e terapia fotodinâmica (Ge et al., 2011). No entanto, a resposta ao tratamento é limitada persistindo a natureza progressiva e o envolvimento multifocal característico da LVP mesmo após a remoção cirúrgica completa das lesões (Ge et al., 2011). Logo, buscar o controle da doença é a melhor abordagem de forma a manter os pacientes sob avaliação permanente das lesões a fim de facilitar a detecção precoce de um câncer e melhorando o tratamento e prognóstico dos mesmos.

### **Marcadores biológicos e a leucoplasia verrucosa proliferativa**

Apesar das características clínicas e histopatológicas serem bem aceitas, os aspectos moleculares subjacentes que podem estar envolvidos com a etiopatogenia e progressão da LVP ainda são desconhecidos e precisam ser melhor investigados. Um aspecto único e desafiador desta entidade é o fato de acometer principalmente mulheres idosas sem histórico de exposição aos fatores de risco conhecidos para leucoplasia sugerindo a possível existência de eventos moleculares atuantes (Gillenwater et al., 2013). Os estudos de biomarcadores em LVP são escassos na literatura inglesa e nenhum marcador biológico é rotineiramente utilizado na prática clínica até o presente momento. Entretanto, alguns eventos e candidatos já foram propostos, tais como aberrações na regulação do ciclo celular e nos genes *p16INK4a* e *p14ARF*, deleção homozigótica, perda de heterozigosidade, expressão variável do gene supressor de tumor p53 e uma deleção homozigótica rara do éxon 1 $\beta$  do gene p14 (Klanrit et al., 2007; Kresty et al., 2008). A ploidia do DNA também tem sido sugerida para prever a transformação maligna das lesões de LVP em CEC oral (Klanrit et al., 2007; Gouvêa et al., 2013). Kannan et al. (1996) através de análises por imunistoquímica mostraram maior positividade para o fator de crescimento transformador alfa (TGF alfa) em amostras teciduais de LVP e CEC oral em comparação ao tecido normal.



As proteínas p53, Ki-67, Mcm-2 e Mcm-5 já foram identificadas por imunohistoquímica com expressão aumentada em lesões de CEC oral que progrediram de LVP. A alta expressão de Mcm-2 e Mcm-5 nas lesões de LVP com displasia leve ou moderada, especialmente nos pacientes em que as lesões evoluíram para CEC oral indicam que tais moléculas podem ser úteis como marcadores de transformação maligna da doença, com destaque para Mcm-2 (Gouvêa et al., 2010, Gouvêa et al., 2013). Achados semelhantes detectados em outras lesões potencialmente malignas displásicas convencionais e no CEC oral confirmam a natureza agressiva dessa entidade, porém ainda não esclarecem o verdadeiro significado dessas moléculas com relação à etiopatogenia ou progressão da LVP (Kresty et al., 2008; Gillenwater et al., 2013). Além disso, nenhum biomarcador é ainda utilizado rotineiramente como auxiliar na prática clínica.

### **A proteômica e a busca de biomarcadores**

Um biomarcador pode ser uma única molécula ou uma combinação de várias moléculas e, idealmente, deve permitir análises fáceis e confiáveis e demonstrar alta sensibilidade analítica e especificidade (Kulasingam e Diamandis, 2008). Atualmente, a alta sensibilidade na detecção do perfil de expressão de proteínas diferenciais, modificações proteicas e interações proteína-proteína com a possibilidade de utilização efetiva faz da proteômica um campo emergente (Liang et al., 2009).

Entre as abordagens proteômicas mais modernas, a espectrometria de massas (MS) é uma ferramenta de análise que tem por objetivo medir um grande número de proteínas desconhecidas em uma amostra através da separação química e física dos íons e pela determinação da razão massa/carga ( $m/z$ ) dos mesmos (Sparkman, 2000). Tal metodologia tem sido amplamente utilizada para descoberta de proteínas diferencialmente expressas em diferentes doenças a partir de diversos tipos de amostras, desde linhagens celulares, tecido, saliva e sangue, a fim de identificar potenciais candidatos a biomarcadores (Schaaij-Visser et al., 2010a; Schaij-Visser et al., 2010b). Além disso, a MS tem demonstrado ser uma tecnologia em desenvolvimento, tanto para análises

qualitativas e quantitativas de proteínas, sendo que recentes avanços nesta área vem gerando enorme impacto sobre a abrangência da proteômica nos estudos sobre câncer de cabeça e pescoço, inclusive em CEC oral (Matta et al., 2008).

O desconhecimento biológico prévio das proteínas presentes em uma amostra possibilitando a descobertas de marcadores até então desconhecidos é outra vantagem da MS em relação às demais abordagens proteômicas (Bertucci e Gonçalves, 2008). Para isso, as premissas básicas da maioria dos estudos é usar a alta capacidade de coleta de dados da MS para comparar amostras biológicas a fim de identificar proteínas que são diferencialmente abundantes entre as amostras (Veenstra, 2007). Inúmeros potenciais marcadores relacionados ao diagnóstico e ao prognóstico em CEC oral já foram descobertos por MS. Entretanto, estudos em que a MS tenha sido utilizada como metodologia para a análise de leucoplasias convencionais são escassos (Schaij-Visser et al., 2010) e ainda não existem estudos prévios que demonstrem a utilização dessa ferramenta para a análise de amostras de pacientes com LVP.

Neste contexto, o emprego da *mass spectrometry* (MS) pode contribuir de forma decisiva no traçado do perfil proteico da saliva de pacientes com LVP possibilitando a detecção de candidatos a marcadores moleculares importantes para o melhor manejo dos pacientes considerando que tanto o diagnóstico precoce quanto o tratamento eficaz são os aspectos mais desafiadores e cruciais nesta doença.

### **A saliva humana e seus potenciais marcadores biológicos**

A saliva humana é um fluido oral com múltiplos constituintes secretada principalmente pelas três glândulas salivares maiores: parótida, submandibular e sublingual e pelas glândulas menores, as quais produzem cerca de 1-1,5 L de saliva por dia (Fabián et al., 2008; de Almeida Pdel et al., 2008; Liu et al., 2012). A saliva é constituída por cerca de 98% de água e 2% de sais minerais e eletrólitos, ácidos nucléicos, mucos, proteínas, substâncias antissépticas (peróxido de hidrogênio e imunoglobulina A), enzimas ( $\alpha$ -

amilase, lisozimas e lipases) (de Almeida Pdel et al., 2008; Pink et al., 2009; Pfaffe et al., 2011) e hormônios oriundos dos capilares e do plasma sanguíneo (Chiappin et al., 2007).

A saliva é considerada um dos fluidos corporais mais complexos e importantes com uma ampla gama de funções fisiológicas de proteção e manutenção da integridade da membrana mucosa da porção superior do trato digestório (de Almeida Pdel et al., 2008; Pfaffe et al., 2011). A possibilidade da realização de uma coleta não invasiva sem a necessidade de um profissional especializado associada ao fácil armazenamento e custos reduzidos são vantagens da saliva em relação à coleta de amostras de sangue. Alguns estudos já tem proposto a substituição da avaliação dos componentes do plasma pela análise da saliva (Pfaffe et al., 2011; Zhang et al., 2013).

As inúmeras proteínas de defesa presentes na saliva tais como as imunoglobulinas, lisozimas, BPI (*bactericidal/permeability increasing protein*), proteínas semelhantes à BPI, PLUNC (*palate, lung and nasal epithelium clone*), amilase salivar, cistatinas, mucinas, peroxidases, proteínas ricas em prolina e peptídeos catiônicos, todas moléculas relacionadas com a imunidade inata e adquirida, têm sido alvo de estudos proteômicos recentes (Fabian et al., 2008). Além disso, o interesse pela investigação de biomarcadores salivares para inúmeras doenças, inclusive em CEC oral, também tem aumentado nos últimos anos através do uso da recente tecnologia da proteômica baseada em MS (Jou et al., 2010; Wang et al., 2014). Isto se deve especialmente porque, do ponto de vista bioquímico, as proteínas têm sido consideradas as moléculas mais importantes da saliva podendo trazer informações importantes sobre a patogênese de muitas doenças bucais e apresentando-se com um crescente potencial para revolucionar o campo do diagnóstico (Pfaffe et al., 2011; Zhang et al., 2013). Logo, uma análise abrangente com a identificação do perfil proteico da saliva de pacientes com LVP pode ser o primeiro passo para a descoberta e caracterização de biomarcadores que facilitem o melhor entendimento dessa rara entidade.

## **CAPÍTULO 1**

Artigo submetido para publicação no periódico (Oral Diseases)

### **Decreased expression of angiotensinogen and dipeptidyl peptidase 1 may be associated with the development of Proliferative Verrucous Leukoplakia**

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## **ABSTRACT**

**OBJECTIVE:** Proliferative verrucous leukoplakia is a persistent oral pre-cancerous condition still poorly understood and challenging for clinicians particularly because the difficulty to establish the diagnosis and reach a successful treatment. As the articles that investigated molecular aspects are relevant, this study aimed to draw the salivary proteome profile in order to identify potential biomarkers with possible clinical use.

**MATERIALS AND METHODS:** Unstimulated whole-mouth saliva was collected of 30 voluntaries (15 PVL patients and 15 controls). Proteomic approach based to liquid chromatography coupled to tandem mass spectrometry was performed to 20 µg of proteins of the samples. Chi-Square, analysis of variance and logistic regression test were used in the statistical analysis.

**RESULTS:** A total of two hundred eighty-three proteins were identified. Among of them, 31 proteins showed statistical significance difference in relation to the abundance, being 25 proteins with higher abundance in control group and 6 proteins with higher abundance in PVL group. The combination of angiotensinogen and dipeptidyl peptidase 1 created a model for group differentiation with a concordance index of 94.2% revealing both proteins as potential biomarkers for diagnosis of PVL.

**CONCLUSIONS:** Although this study was the first to evaluate the salivary proteome in PVL patients, the results showed that saliva screening can be a useful test to diagnosis of individuals with risk to PVL development.

**KEYWORDS:** Saliva, angiotensinogen, dipeptidyl peptidase 1, biomarkers, proliferative verrucous leukoplakia, LC-MS/MS.

## INTRODUCTION

Proliferative verrucous leukoplakia (PVL) is a rare variant and less known among the leukoplakias with a unique behavior of persistent progression to malignancy (Hansen et al., 1985, van der Wall and Reichart, 2008, Gouvêa et al., 2010a, Carrard et al., 2013, Gouvêa et al., 2013, Gillenwater et al., 2013a, Gillenwater et al., 2013b). The rate of malignant transformation of PVL ranges from 40% to 100% (Hansen et al., 1985, Zakrzewska et al., 1996, Silverman and Gorsky, 1997; Fettig et al., 2000, Bagán et al., 2003, Morton et al., 2007, Gouvêa et al., 2010a, Gouvêa et al., 2013). This variability mostly depends on the time of follow-up of patients and the criteria for diagnosis of LVP (Cabay et al., 2007, Bagan et al., 2010). Due to lack of specific baseline characteristics, the diagnosis of PVL is made retrospectively based on the observation of progressive clinical and histopathological characteristics of the lesions (Gouvêa et al., 2013). Thus, the evaluation of biopsy specimens of representative areas is quite relevant to establish the existence of epithelial dysplasia or carcinoma along with the clinical evolution (Navarro et al., 2004).

Recent reviews about PVL have considered in addition to clinical aspect of multiple hiperkeratotic white plates with verrucous and expansive nature, lesions with lichenoid aspects that made progress to classical PVL (Müller, 2011, Gillenwater et al., 2013a, Gillenwater et al., 2013b). Nevertheless, the histological aspects of located hyperplasia and hyperkeratosis with no epithelial dysplasia; multifocal expansion with or without varied degrees of dysplasia; verrucous hyperplasia and verrucous carcinoma or frankly invasive oral squamous cell carcinoma (OSCC) remain as the four histological features encountered during the course of the disease (Gouvêa et al., 2013, Gillenwater et al., 2013a, Gillenwater et al., 2013b). Currently, four main stages may be encountered during the development of PVL: initial focal involvement, geographic expansion (multifocal) over time, developing a warty appearance and, finally, developing of a cancer (Batsakis et al., 1999, Gillenwater et al., 2013a).

Despite the clinical and histopathological features are well accepted, the underlying molecular aspects involved in the pathogenesis and progression of PVL are still

unknown and need to be further investigated. An intriguing characteristic of this entity is the preference for older women with no history of exposure to known risk factors for leukoplakia suggesting the existence of active molecular events (Batsakis et al., 1999). Studies of biomarkers in PVL are scarce in the English-language literature and no biological marker is routinely clinical practice has been used. However, some events and candidates have been proposed, such as aberrations in cell cycle regulation, p16INK4a and p14ARF genes homozygous deletion, loss of heterozygosity, variable expression of the tumor suppressor gene p53 and a rare homozygous deletion of exon 1 $\beta$  gene p14 (Klanrit et al., 2007, Kresty et al., 2008). The DNA ploidy has also been suggested to predict the malignant transformation of PVL in OSCC (Klanrit et al., 2007, Gouvêa et al., 2013a).

Additionally, p53, Ki-67, Mcm-2 and Mcm-5 proteins have been identified by immunohistochemistry with increased expression in the OSCC lesions that have progressed from PVL (Gouvêa et al., 2010a, Gouvêa et al., 2013). A high expression of Mcm-2 and Mcm-5 lesions in PVL with mild or moderate dysplasia was also found in lesions that progressed to OSCC being an indication that such molecules may be useful as markers of malignant transformation of the disease, especially Mcm-2 (Gouvêa et al., 2010a, Gouvêa et al., 2013). Similar findings detected in other conventional dysplastic premalignant lesions and oral SCC reinforced the aggressive nature of this entity, but without clarifying the true meaning of these molecules with respect to the pathogenesis or progression of PVL (Kresty et al., 2008, Gillenwater et al., 2013b).

Furthermore, the interest in salivary biomarkers for numerous diseases, including OSCC, has increased in recent years through the use of the technology of the proteomic based to mass spectrometry (MS) (Jou et al., 2010, Wang et al., 2014). This interest in the salivary proteome study is because the biochemical aspects of the proteins are considered as the most important molecules in the salivary fluid (Pfaffe et al., 2011, Zhang et al., 2013, Wang et al., 2014). This proposition is based on the salivary proteins activity, which provides important information about the pathogenesis of many oral diseases. In addition, salivary proteins have presented a growing potential to revolutionize the field of diagnosis (Pfaffe et al., 2011, Zhang et al., 2013).

Therefore, the aim of this study was to draw the salivary proteome profile of patients with PVL through high efficiency MS technology in order to identify proteins that may contribute to the better understanding this intriguing entity.

## **MATERIALS AND METHODS**

### **Human subjects**

This study followed the guidelines of the Declaration of Helsinki and Tokyo for research in humans and was approved by the local ethics committee of University of Campinas (protocol number: 105/2013). All patients received verbal explanations concerning the study before signed a written informed consent. In this study, 15 patients with diagnosis of PVL according to World Health Organization (WHO) (Barnes et al., 2005; Gouvêa et al., 2010a; Gouvêa et al., 2013) and 15 control patients with no history of cancer or any oral lesion and with the same demographic aspects were chosen from patients assisted by the Orocentro (Oral Diagnosis Clinic) of Piracicaba Dental School. Clinicopathological data were collected retrospectively from the patients' charts.

### **Saliva collection and preparation**

After a mouthwash with 5 mL of drinking water for removal of oral residues, whole unstimulated saliva produced during 5 minutes was collected in sterile plastic tubes of 50 mL. After collection, the saliva was immediately placed on dry ice and subsequently stored at -80°C in sterile plastic tubes of 2mL. Before preparation for MS analysis, 10µl of PMSF and 2µl of EDTA to each 1 mL of saliva, both at a final concentration of 1 mM were added followed by centrifugation per 12.000g for 10 minutes in refrigerated centrifuge at 4°C. The supernatant was collected and protein concentrations were determined with a Bradford assay (Bio-Rad, Hercules, CA, USA) previously the protein digestion.



## **Sample preparation to MS and LC-MS/MS analysis**

Proteins (20 µg) were treated with final concentration of 5 mM dithiothreitol, 25 min at 56°C to reduction followed by alkylation with 14 mM iodoacetamide, 30 min at room temperature in the dark and digestion with trypsin (1:50, w/w). The reaction was stopped with 1% formic acid and the samples were dried in a vacuum concentrator and reconstituted in 0.1% formic acid.

An aliquot containing 1 µg of proteins was analyzed on an ETD enabled LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific) connected to nanoflow liquid chromatography (LC-MS/MS) by an EASY-nLC system (Proxeon Biosystem) through a Proxeon nanoelectrospray ion source. Peptides were separated by a 2-90% acetonitrile gradient in 0.1% formic acid using a pre-column EASY-Column (2cm x id 100 µm, 5 µm particle size) and an analytical column PicoFrit Column (20 cm x ID75 µm, 5 µm particle size, New Objective), at a flow of 300 nl/min over 65 min. The nanoelectrospray voltage was set to 2.2 kV and the source temperature was 275°C.

All instrument methods for the LTQ Orbitrap Velos were set up in the data dependent acquisition mode. The full scan MS spectra (m/z 300-1600) were acquired in the Orbitrap analyzer after accumulation to a target value of  $1e^6$ . Resolution in the Orbitrap was set to  $r= 60.000$  and the 20 most intense peptide ions with charge states  $\geq 2$  were sequentially isolated to a target value of 5,000 and fragmented in the linear ion trap by low-energy CID (normalized collision energy of 35%). The signal threshold for triggering an MS/MS event was set to 500 counts. Dynamic exclusion was enabled with an exclusion size list of 500, exclusion duration of 60 s, and repeat count of 1. An activation  $q= 0.25$  and activation time of 10 ms were used.

## **Raw data analysis**

Peak lists (msf) were generated from the raw data files using Proteome Discoverer version 1.4 (Thermo Fisher Scientific) with Sequest search engine and searched against Human Uniprot (actualized in June, 2013) (88.771 sequences; 35.204.890 residues)

with carbamidomethylation as fixed modification, oxidation of methionine as variable modification, one trypsin missed cleavage and a tolerance of 10 ppm for precursor and 1 Da for fragment ions. All datasets were processed using the workflow feature in Proteome Discoverer software and the resulting search data were further analyzed in ScaffoldQ+v.3.3.1. The scoring parameters in Scaffold were set to obtain a false discovery rate of less than 1%. Using the number of total spectra output from ScaffoldQ+, it was identified the differentially expressed proteins using spectral counting.

### **Statistical analysis**

File (.csv) containing the identified proteins and their normalized spectral counts were created to 15 healthy patients (control) and to 15 PVL patients (PVL). All statistical analyses were performed using SAS<sup>®</sup> software (version 9.3; SAS Institute Inc, Cary, NC, USA, 2010). A *Chi-Square* test ( $\chi^2$ ) was realized to evaluate the expression of proteins in relation to groups. *Analysis of Variance* test (*ANOVA*) were used to compare the abundance of each protein in relation to groups and fold change (FC) calculation for proteins with difference in relation to abundance was performed according to the equation:

$$\text{Mean of abundance values (MV) in PVL group} / \text{Mean of abundance values (MV) in control group} = \text{Fold change } (\geq +1.00)$$

Moreover, for the fold change less than 1 was used the below equation:

$$1 / \text{Fold change } (< +1.00) = \text{Fold change } (-1.00)$$

Finally, to develop a method to predict the probability of pertinence to one specified group, an analysis based on the regression logistic model was conducted in conjunction with the stepwise method to variable selection as from of proteins identified. A p-value less than 0.05 was considered statistically significant to all statistical tests.

## **RESULTS**

### **Clinicopathological findings**

All thirty patients (15 PVL and 15 control) were females with mean age of 68.13 years (SD 9.82 years) for PVL patients and mean age of 65.20 years (SD 8.36 years) for control patients and with no history of alcohol consumption and smoking. Figures 1 and 2 show clinical features of PVL patients and figures 3 and 4 display some histopathological findings. Demographic and clinicopathological data of the population are showed in Tables I and II.

### **Salivary proteome of proliferative verrucous leukoplakia identified by LC-MS/MS**

Using ScaffoldQ+v.3.3.1, a list of 283 proteins with less than 1% false discovery rate was generated after the high efficient analysis of LC-MS/MS. The complete list of proteins and their respective normalized spectral counts to each patient is showed in the Supplemental Table 1.

### **Potential biomarkers of proliferative verrucous leukoplakia**

Thirty-nine proteins showed association statistically significant among expression and group (control and PVL) through  $\chi^2$  test. A list of proteins is presented in the Table III. Moreover, 31 proteins showed difference statistically significant in relation to abundance between the control and PVL groups according to ANOVA. Among these 31 proteins, 25 had higher abundance in control group and 6 proteins were higer in PVL group. Table IV shows the complete list to these proteins with their respective fold changes.

## **Angiotensinogen and dipeptidyl peptidase 1 are found as potential biomarkers to group predictors**

Screening tests for logistic regression were used as a form of group differentiation and 2 potential biomarkers met the final prediction: dipeptidyl peptidase 1 and angiotensinogen. When likelihood analysis was applied to the 2 variables selected stepwise within the logistic regression, the following logistic function was obtained:

$$\text{Logit} = 2.5647 - 12.4678 \times P192 - 4.3839 \times P206$$

A negative logit indicated a control group landmarks and a positive logit indicated a PVL landmarks. The logit value can also be used to calculate the probability of PVL group ( $P_{PVL}$ ) using the function, being  $e$  the Neperian constant equal to 2.71828.

$$P_{PVL} = e^{\text{logit}(p)} / (1 + e^{\text{logit}(p)})$$

The probability of relevance of an individual patient be of PVL group ( $P_{PVL}$ ), which indicates the degree of confidence in the analysis, can be found by inserting the logit value in the above equation. Combining the 2 selected variables in this study, a concordance index of 94.2% was found. The parameters selected by Stepwise method are presented in Table V and the reliability values to the model built by the logistic regression test are showed in Table VI.

## **DISCUSSION**

PVL is a form of oral leukoplakia with particular features, specially related to etiology, high rate of recurrence and its persistent evolution to malignancy. The epidemiological, clinical and histopathological aspects found in the present study are well known and accepted by various studies (Hansen et al., 1985; Barnes et al., 2005; Bagan et al., 2010). However, some points remain obscure such as early diagnosis criteria and treatment. The search in the PubMed Medline revealed around 81 articles about PVL with only 10 studies describing some molecular aspects involved in this entity (Migliorati et al., 1992, Kannan et al., 1996, Gopalakrishnan et al., 1997, Fettig et al., 2000, Campisi et al.,

2004, Bagan et al., 2007, Bagan et al., 2008, Kresty et al., 2008, Gouvêa et al., 2010, Gouvêa et al., 2013). Nevertheless, no previous study investigated the presence of potential biomarkers of PVL through complete proteome salivary analysis using LC-MS/MS.

A major challenging to use saliva as a diagnostic fluid is in relation to lower amounts of the informative analytes present in saliva when compare with the levels in the serum (Miller, 1994; Nagler et al., 2006). However, through highly efficient techniques of detecting small quantities, such as LC-MS/MS (Bigler et al., 2009), any salivary components including proteins that can be found in blood can be also measured in saliva (Krishna Prasad et al., 2013). Thus, saliva can be seen as the blood stream of oral cavity and the proteins with levels of modified abundance detected for any pathologies through this sophisticated approach (Krishna Prasad et al., 2013, Bigler et al., 2009) can be considered as potential biomarkers with a significant disease specificity as was found to PVL in the present study.

Among the 283 proteins identified in the saliva of sample studied by LC-MS/MS, 31 proteins showed statistical significance difference in relation to abundance, being 25 proteins with higher abundance in control group and 6 proteins with higher abundance in PVL group. Interestingly, no previous study showed association these molecules with PVL; however, salivary proteomic approaches have already revealed the molecular potential of some proteins in other oral pathologies. Leukocyte elastase inhibitor (LEI) (anova  $p=0.002$ ,  $FC=-2.12$ ) is an oral fluid proteolysis highly important due to inhibitor activity in the saliva being that its unbalance was related with a variety of pathological conditions including oral cancer (Sun et al., 2009). Actin-related protein 3 (ARP3) (anova  $p=0.0268$ ,  $FC=-7.69$ ), an actin ATP binding active in the movement of immune cells was observed with decreased expression in saliva of patients with Type-2 diabetes mellitus and periodontitis (Chan et al., 2012). Moreover, moesin (MSN) (anova  $p=0.0107$ ,  $FC=-4.16$ ), a linking protein of the submembraneous cytoskeleton with role in the control of cell morphology, adhesion, and motility was showed through immunohistochemical analysis as adjunct to screening of premalignant and OSCC lesions with decreased expression in oral cancer patients with higher locoregional metastatic potential (Kobayashi., 2003, Kobayashi et al., 2004). The proteins LEI, ARP3 and MSN

also presented downexpression in PVL patients. The same expression pattern was observed by other authors in some oral diseases, including OSCC in which become possible to suggest the involvement of these molecules in PVL progression until malignancy. However, additional investigations need to be performed to confirm their possible role in relation to this illness.

On the other hand, the analysis of tissue samples by MS-based proteomics revealed adenylyl cyclase-associated protein 1 (CAP1) (anova  $p=0.005$ ,  $FC=-3.84$ ), a cytoskeletal protein associated with transport, differentiation and cell cycle progression as overexpressed in OSCC patients (Thiel et al., 2011). Calreticulin (CALR) (anova  $p=0.0172$ ,  $FC=-4.76$ ) an endoplasmic reticulum protein which plays a pivotal role since intracellular calcium homeostasis regulation until cell adhesion and clearance of apoptotic cells presented overexpression in tissue samples of OSCC at the same study (Thiel et al., 2011). Cofilin-1 (CFL1) (anova  $p=0.0423$ ,  $FC=-3.12$ ), a structural protein that plays a role in the regulation of cell morphology, cytoskeletal organization and cell migration was found with increased expression in saliva of patients with head and neck squamous cell carcinoma (Dowling et al., 2008). Finally, peroxiredoxin 6 (PRDX6) (anova  $p=0.0255$ ,  $FC=-12.5$ ), a thiol-specific antioxidant protein responsible to eliminates  $H_2O_2$  in cells has been already reported overexpressed in various tumors including in OSCC samples (Huang et al., 2011). Interestingly, CAP1, CALR, CFL1, PRDX6 were found downexpressed in saliva of the PVL patients showing a different expression pattern than previously revealed by other studies. Thus, our results suggested that additional studies about the profile of the salivary proteome may contribute to improve understanding this pattern of expression for these molecules found by the first time for PVL entity.

Although above proteins presenting a potential cited also by previous studies, angiotensinogen (AGT) (anova  $p=0.0006$ ,  $FC=12.5$ ) and dipeptidyl peptidase 1 (DPP1) (anova  $p=0.0150$ ,  $FC=$ not calculated) were found with statistical significance in expression, abundance and revealed as better biological parameters for model created to screening of PVL patients according to regression logist test. These proteins were revealed among the 25 proteins with higher abundance in control group as from 31 proteins with difference in

abundance. Based in these findings, both proteins can be considering as the first salivary proteins with biomarker potential to diagnosis of PVL.

AGT (53 kDa) is a circulating protein quite known as precursor of the renin–angiotensin aldosterone system being synthesized and secreted mostly by hepatocytes, adipocytes and artrocytes (Gaillard-Sanchez et al., 1990, Vairaktaris et al., 2008). Physiologically, AGT is a unique precursor of the angiotensin peptides and the only natural rennin substrate (Gaillard-Sanchez et al., 1990). The hydrolysis of AGT into angiotensin I by rennin results in the vasoactive molecules of angiotensin II and III by angiotensin-converting enzyme (Gaillard-Sanchez et al., 1990, Vairaktaris et al., 2008). AGT, also called of Serpin A8, is derived from serine protease inhibitor with a role in blood pressure control as a potent vasoconstrictor of arteries and veins and prothrombotic action (Vairaktaris et al., 2008, Gourin et al., 2009).

Interestingly, recent studies have also related the protein AGT with in vitro inhibition of human endothelial cell proliferation, cell migration and angiogenesis (Vairaktaris et al., 2008, Bouquet et al., 2006, Gourin et al., 2009). Antitumoral effect of AGT in relation to blocking of primary tumor growth, suppression of intratumoral vascularization, and decreased number of metastases was showed through in vivo models (Vairaktaris et al., 2008, Bouquet et al., 2006). Moreover, AGT protein was found downexpressed in tissue samples of esophageal squamous cell carcinoma and in serum of the patients with recurrent head and neck cancer, demonstrating a possible action in facilitate tumorigenesis when in reduced levels (Zhou et al., 2005, Gourin et al., 2009). A previous study also demonstrated that women with low levels of AGT presented an increased risk of breast cancer (González-Zuloeta et al., 2007, Gourin et al., 2009).

In the present study, low AGT abundance was observed in PVL patients with a fold change of -12,5 suggesting that an expression loss this molecule can be a high risk factor to PVL indicating a possible role of AGT as diagnostic marker. Nevertheless, no difference was observed in expression and abundance inside the PVL group in relation to patients that developed oral cancer and these results were expected since patients with PVL probably will develop cancer during the follow-up.

In turn, DPP1 (51kDa) also known as cathepsin c, cathepsin J, dipeptidyl transferase and dipeptidyl aminopeptidase (UNIPROT P53634) is a lysosomal cysteine protease that participates of the intracellular process of proteins degradation (Thong et al., 2011). This activity of DPP1 is crucial in the differentiation of precursor promyelocytes into mature neutrophils and in the production of neutrophil elastase, proteinase-3, and cathepsin G (Owen et al., 2008, Thong et al., 2011). The most common pathology involved with DPP1 mutations is Papillon-Lefèvre syndrome, an autosomal recessive condition associated with the loss of the enzymatic activities of these three serine proteases in neutrophils causing palmoplantar keratosis and severe precece periodontal disease (Toomes et al., 1999).

In addition, to their involvement in physiological processes, an intrinsic relation among squamous carcinogenesis and DPP1 expression was demonstrated in relation to tumor growth and angiogenesis (Ruffell et al., 2013). Among the tumors involved, squamous cell carcinoma of the oral cavity, nasopharynx and thyroid exhibit increased expression when compared to control samples (Ruffell et al., 2013). Nevertheless, DPP1 did not show effect in the formation or progression in pancreatic endocrine tumor presenting expression in innate immune cells (Gocheva et al., 2006). Interestingly, dipeptidyl peptidase IV (DPPIV) is another cathepsin expressed in normal epithelial cells with loss of expression in various cancers, such as melanoma, lung and prostatic cancer suggesting that loss of DPPIV expression can be a critical event during cancer progression (Bogenrieder et al., 1997, Wesley et al., 1999, Wesley et al., 2004, Wesley et al., 2005).

In the present study, the protein DPP1 was found with loss of expression in PVL patients and although some articles demonstrated the up-expression of this molecule and its association with tumor progression, one study showed a loss of expression DPP1 related with cancer (Gocheva et al., 2006). DPPIV also presented a similar behavior suggesting a variable action of cathepsins depending of type of lesion being that an association of DPP1 with AGT showed a potential protector effect associated with suppression activity in PVL patients. Nevertheless, a deeper investigation of this protein to analyse the role in PVL disease will add more safety to clinical use. Therefore, this study revealed evidences about the possible diagnosis role of the proteins AGT and DPP1 in PVL



being that the model created as from these biomarkers can be used in the future to screening patients with risk to PVL contributing to establishment of a continuous clinical follow-up. However, the logit formula validation with a new saliva sample is an important step to confirm the high reliability values found for the studied sample.

In conclusion, no effective screening test so far exists for aid in the clinical diagnosis of PVL patients besides the routine oral examination. The proximity of saliva to the PVL lesions is undoubtedly an important factor in facilitating detection of possible biomolecules through the salivary proteome examination. Thus, the minimally invasive strategy used in the present study led to the identification of a panel of biomarkers with two proteins that may be an useful biological tool for prediction of patients with high risk of developing PVL so contributing to early detection of this challenging oral entity.

## **CONFLIT OF INTEREST**

The authors declare there are no conflicts of interest.

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## TABLES

**Table I.** Demographic aspects of 30 patients (15 control and 15 PVL) with salivary proteome profile assessed by LC-MS/MS.

	CONTROL		PVL	
	n	%	n	%
<b>Age (Years)</b>				
50-59	5	33.33	4	26.66
60-69	3	20.00	3	20.00
70-79	7	46.66	7	46.66
>80	0	0	1	6.66
<b>Sex</b>				
Female	15	100	15	100
Male	0	0	0	0
<b>Smoking/ alcohol</b>	0	0	0	0

n=number



**Table II.** Clinicopathological data of 15 patients with PVL included in the study.

<b>Case</b>	<b>Anatomic Location</b>	<b>Histopathologic findings</b>	<b>Follow-up time (years)</b>
1	1a, 3a, 3b, 4, 5,6b	Epithelial dysplasia (3a, 6b) OSCC (3b)	11
2	2, 3b, 5a, 6a, 8, 9	Epithelial dysplasia (2, 9) OSCC (2, 8)	5
3	2, 3b, 4, 10	Acanthosis/hiperkeratosis (2, 10) Epithelial dysplasia (2)	10
4	2, 5a, 6a, 7a	Acanthosis/hyperkeratosis (2) Epithelial dysplasia (2, 5a)	20
5	2, 5a, 5b, 6b	Acanthosis/dysplasia (6b)	2
6	2, 3a, 4, 5a, 9	Epithelial dysplasia (3a)	1
7	2, 3b	-	2
8	2, 6a, 7b	Acanthosis/hyperkeratosis (7b) Epithelial dysplasia (7b)	4
9	1b, 2, 3a, 3b, 4, 6a, 6b	Epithelial dysplasia (1b, 2)	1
10	2, 3b, 6a	Epithelial dysplasia (3b)	3
11	2, 3a, 3b, 4, 5a, 6b,8	Epithelial dysplasia (8) Verrucous carcinoma (5a) OSCC (4)	6
12	2, 4, 5b, 6b, 8	Acanthosis/hiperkeratosis (5b) Epithelial dysplasia (2)	3
13	2, 3b	Epithelial dysplasia (3b) OSCC (3b)	3
14	1a, 1b, 2, 3a, 3b, 4, 6a, 6b	Epithelial atrophy/ hyperkeratosis (3b)	1
15	1a, 1b, 2, 3a, 3b, 5a, 5b, 6a, 6b, 8	Epithelial dysplasia (5a,5b)	10

1: a) inferior labial mucosa b) superior labial mucosa; 2: buccal mucosa; 3: a) tongue (ventral) b) tongue (lateral border) 4: Floor of mouth; 5:a) inferior alveolar ridge b) superior alveolar ridge; 6: a) inferior fórnix b) superior fórnix; 7: a) inferior gengiva b) superior gengiva 8: hard palate; 9: soft palate; 10: tonsil pillar.

**Table III.** List of 39 proteins statistically significant in relation to expression and group according to Chi-Square test (degree of freedom = 1; value > 3.84).

Protein (39)	Likelihood ratio Chi-Square	
	Value	Prob
UPF0762 protein C6orf58	4.49	0.03
Glucose-6-phosphate isomerase	7.94	0.00
Profilin-1	6.16	0.01
6-phosphogluconate dehydrogenase, decarboxylating	4.49	0.03
Small proline-rich protein 3	4.49	0.03
Keratin, type II cytoskeletal 1	6.16	0.01
Leukocyte elastase inhibitor	6.16	0.01
Heat shock 70 kDa protein 1A/1B	6.16	0.01
Leukotriene A-4 hydrolase	6.72	0.01
Moesin	4.14	0.04
Adenylyl cyclase-associated protein 1	5.68	0.02
Fibrinogen beta chain	6.72	0.01
Isoform 2 of Acyl-CoA-binding protein	3.69	0.05
Alpha-1-acid glycoprotein 1	4.49	0.03
Protein S100-A11	6.72	0.01
Cofilin-1	6.79	0.01
Isoform Cytoplasmic+peroxisomal of Peroxiredoxin-5, mitochondrial	8.58	0.00
ERO1-like protein alpha	6.79	0.01
Histone H2B type 1-D	5.68	0.02
Complement C4-B	10.65	0.00
Calreticulin	5.68	0.02
Nucleobindin-1	6.79	0.01
Actin-related protein 3	4.14	0.04
Ig lambda chain V-III region LOI	3.69	0.05
Isoform Short of 14-3-3 protein beta/alpha	4.49	0.03
<b>Dipeptidyl peptidase 1</b>	<b>11.87</b>	<b>0.0006</b>
Peroxiredoxin-6	5.06	0.02
Nicotinamide phosphoribosyl transferase	6.72	0.01
<b>Angiotensinogen</b>	<b>12.99</b>	<b>0.0003</b>
Isoform 3 of Nucleoside diphosphate kinase B	14.07	0.00
Ras GTPase-activating-like protein IQGAP1	5.06	0.02

Transforming protein RhoA	4.14	0.04
Chitinase-3-like protein 2	6.72	0.01
Ubiquitin-like modifier-activating enzyme 1	7.94	0.00
Interleukin-36 alpha	7.94	0.00
Kallikrein-6	4.49	0.03
Isoform 3 of Pyridoxal kinase	4.49	0.03
Protein S100-A14	4.49	0.03
Hexokinase-3	4.49	0.03

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**Table IV.** Average of spectral counts (SC), standard deviation (SD), p value and fold change of 31 proteins with difference in the abundance levels in PVL saliva compared to the control group according to ANOVA test.

Protein (31)	Control		PVL		<i>p value</i>	<i>FC</i>
	SC	SD	SC	SD		
Histone H2B type 1-D	0.12	0.32	1.09	1.73	0.0413	9.08
Keratin, type II cytoskeletal 1	2.33	2.45	5.24	4.67	0.0418	2.24
Ig lambda chain V-III region LOI	0.45	0.50	1.01	0.66	0.0154	2.24
Lipocalin-1	7.03	4.46	13.44	6.84	0.005*	1.91
Cystatin-B	1.67	1.38	3.19	2.25	0.0338	1.91
Fatty acid-binding protein, epidermal	2.22	1.36	3.99	2.04	0.009*	1.79
Actin, alpha cardiac muscle 1	9.66	3.79	6.78	3.71	0.0446	-1,42
Submaxillary gland androgen-regulated protein 3B	18.26	9.40	11.05	5.19	0.0148	-1.66
Glucose-6-phosphate isomerase	4.21	2.89	2.12	2.05	0.0309	-2.00
Leukocyte elastase inhibitor	3.93	1.57	1.87	1.75	0.002*	-2.12
Peroxiredoxin-1	2.11	1.41	0.87	1.04	0.0106	-2.43
Thymosin beta-4	1.09	1.01	0.40	0.64	0.0323	-2.77
ERO1-like protein alpha	0.93	0.67	0.32	0.59	0.0126	-2.94
Cofilin-1	1.66	1.79	0.54	0.99	0.0423	-3.12
Nucleobindin-1	0.82	0.56	0.27	0.47	0.007*	-3.12
Rho GDP-dissociation inhibitor 2	1.61	1.76	0.49	0.79	0.0317	-3.33
Adenylyl cyclase-associated protein 1	3.18	2.86	0.83	0.97	0.005*	-3.84

Moesin	2.44	2.52	0.60	0.63	0.0107	-4.16
Transforming protein RhoA	0.53	0.63	0.13	0.36	0.0453	-4.16
Calreticulin	0.61	0.65	0.13	0.35	0.0172	-4.76
Isoform 2 of Glycogen phosphorylase, liver form	1.70	2.11	0.34	0.63	0.0239	-5.00
Actin-related protein 3	0.97	1.33	0.13	0.35	0.0268	-7.69
Nicotinamide phosphoribosyl transferase	0.53	0.64	0.07	0.26	0.0139	-7.69
Chitinase-3-like protein 2	0.61	0.75	0.07	0.28	0.0143	-9.09
Peroxiredoxin-6	0.60	0.87	0.05	0.21	0.0255	-12.5
<b>Angiotensinogen</b>	<b>0.83</b>	<b>0.73</b>	<b>0.07</b>	<b>0.26</b>	<b>0.0006*</b>	<b>-12.5</b>
Complement C4-B	0.93	1.02	0.07	0.28	0.004*	-14.28
<b>Dipeptidyl peptidase 1</b>	<b>0.66</b>	<b>0.98</b>	<b>0.00</b>	<b>0.00</b>	<b>0.0150</b>	<b>NC</b>
Isoform 3 of Nucleoside diphosphate kinase B	0.58	0.60	0.00	0.00	0.000*	NC
Ubiquitin-like modifier-activating enzyme 1	0.38	0.59	0.00	0.00	0.0189	NC
Interleukin-36 alpha	0.43	0.68	0.00	0.00	0.0197	NC

FC: Fold change. Up-expression:  $FC \geq +1.00$ ; downexpression:  $FC < +1.00$ . \*  $p \leq 0.01$ : highly significant; NC: Not calculated

**Table V.** Stepwise method parameters selected by logistic regression test among proteins identified by LC-MS/MS.

<b>Parameter</b>	<b>Estimate</b>
Intersection	2.5647
P192 (Dipeptidyl peptidase 1)	-12.4678
P206 (Angiotensinogen)	-4.3839

**Table VI.** Values regarding to reliability of the model built by the logistic regression test with two potential protein biomarkers to PVL identified by LC-MS/MS.

<b>Percent concordant</b>	<b>C value•</b>	<b><i>p</i></b>	<b>Correct event</b>		<b>Sensitivity</b>	<b>Specificity</b>
91.1	0.942	0.0187	14*	14*	93.3%	93.3%

• area under Roc curve \*control and PVL

## FIGURES AND LEGENDS

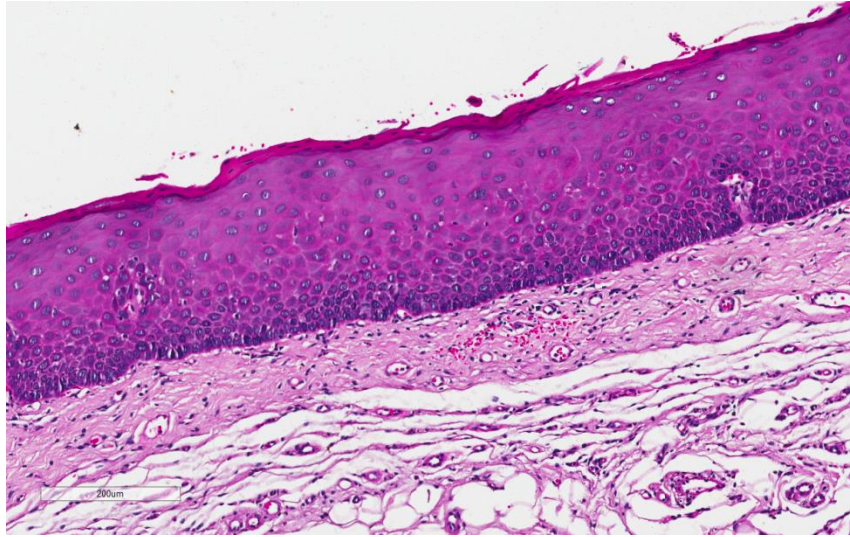


**Figure 1.** Asymptomatic thin white plate in left lateral border of the tongue and buccal floor.

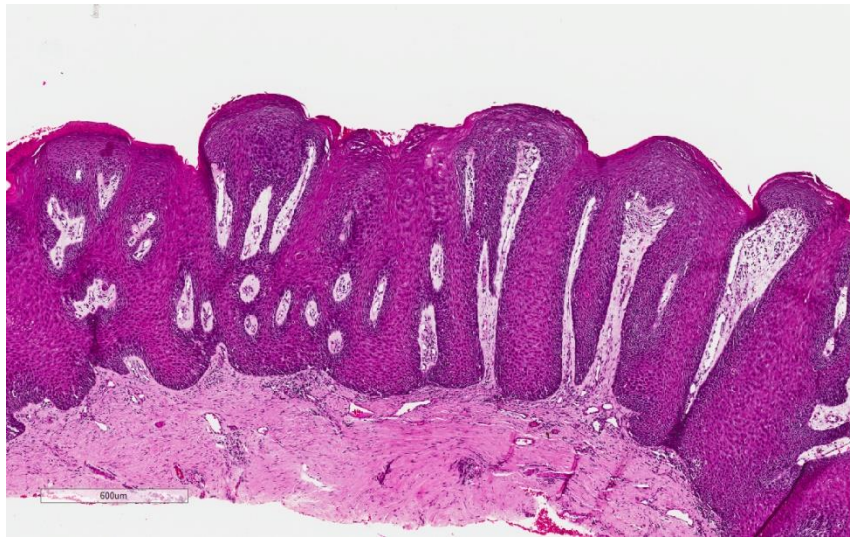


**Figure 2.** Extensive fissured white plates in the bilateral inferior alveolar ridge, bilateral inferior fornix and bilateral buccal floor. Note a lesion also in the left lateral border of the tongue. All lesions were asymptomatic.





**Figure 3.** Hyperparakeratosis and acanthosis (H&E; 100X).



**Figure 4.** Papillary projections with severe epithelial dysplasia (H&E; 100X).

## CONCLUSÕES

- A determinação do perfil do proteoma salivar em pacientes com LVP pela utilização da MS apresentou-se como uma importante ferramenta para o descobrimento de biomoléculas que podem desvendar aspectos ainda não compreendidos e contribuir para o diagnóstico dessa entidade.
- As pacientes com LVP apresentaram significativa perda de expressão das proteínas angiotensinogênio e dipeptidil peptidase 1, podendo ser consideradas no futuro como importantes marcadores para diagnóstico de LVP.
- A seleção de uma proteína salivar como biomarcadora da evolução da LVP para malignidade depende de estudos prospectivos adicionais em amostras de pacientes no qual possa ser observada diferenças na expressão e abundância nas várias etapas da doença.

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\* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors. Abreviatura dos periódicos em conformidade com o Medline.

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# APÊNDICE

**Supplemental Table 1.** List of 283 proteins identified by LC-MS/MS with their normalized spectral counts according ScaffoldQ+ software.

Identified Protein (2SD)	CONTROL														PVL															
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	PVL 1	PVL 2	PVL 3	PVL 4	PVL 5	PVL 6	PVL 7	PVL 8	PVL 9	PVL10	PVL11	PVL12	PVL13	PVL14	PVL15
Adiponectin (ADI1, UniProt)	175.17	273.06	496.20	284.77	209.28	241.52	159.23	230.02	430.92	104.80	442.11	249.38	315.99	146.42	241.21	255.96	309.63	452.88	353.47	140.86	340.11	121.20	334.96	284.61	306.59	187.19	246.43	268.76	250.53	413.12
Secreted phospholipase A2 (PLA2G6, UniProt)	72.73	87.62	117.91	115.14	143.36	162.83	117.38	131.44	149.32	227.78	138.00	125.62	41.17	212.48	164.02	64.24	112.77	69.34	108.92	80.64	152.43	53.21	211.34	175.61	168.10	116.15	176.90	220.73	55.23	96.00
Mucin-9 (MUC9, UniProt)	156.73	90.68	77.38	95.55	78.46	94.08	106.15	48.32	63.14	76.22	88.17	117.30	371.58	41.07	42.45	116.61	167.24	76.92	128.14	126.88	98.96	237.48	100.69	81.75	50.62	249.59	104.30	154.31	158.48	132.67
Polyoma virus noncoding RNA (PNC1, UniProt)	184.39	152.83	100.71	128.51	127.86	86.84	108.19	89.88	93.20	131.65	109.89	126.54	85.43	84.81	128.32	155.15	133.79	151.68	147.37	59.14	103.51	93.61	99.69	103.95	120.35	78.72	100.21	72.56	62.43	85.21
Proteinase 3 (PR3, UniProt)	59.41	75.40	34.39	62.71	71.68	56.99	68.39	45.42	47.10	79.68	44.72	95.14	53.52	64.28	76.22	105.74	109.90	91.01	87.57	30.11	67.11	75.88	45.86	82.76	79.28	62.40	57.26	76.64	36.82	81.98
Cystatin A (CYST1, UniProt)	59.41	52.98	49.13	54.24	58.80	128.61	62.82	60.13	48.50	49.83	72.97	21.62	41.07	41.49	78.07	59.25	39.00	56.60	25.81	75.08	95.58	52.71	56.52	41.07	40.32	49.08	10.22	46.42	46.38	
Cystatin D (CYST4, UniProt)	62.49	42.79	36.85	90.47	49.40	59.70	94.92	63.79	80.17	30.31	40.81	65.52	51.78	53.07	48.42	62.12	30.34	54.46	26.88	34.13	101.50	74.77	64.59	19.10	43.20	36.81	15.33	36.02	45.30	
Cystatin E (CYST5, UniProt)	75.80	42.81	35.27	116.17	100.74	109.45	150.04	92.78	71.15	35.51	56.22	112.08	58.67	70.53	53.67	82.02	92.70	69.34	80.83	43.01	83.84	163.58	86.73	90.83	37.25	69.21	83.85	25.55	89.65	73.25
β-ketoacid CoA synthase (KCS1, UniProt)	91.17	61.13	24.56	46.26	52.31	28.04	32.66	29.06	41.69	77.05	23.00	60.04	30.88	47.32	52.10	94.87	62.12	68.26	46.99	80.44	36.40	25.62	38.88	53.49	51.58	46.08	48.06	39.85	24.01	31.28
Cathepsin D (CTSD, UniProt)	5.12	38.72	57.72	34.95	36.81	18.09	25.52	16.43	31.07	3.46	53.67	21.24	45.29	8.93	22.19	37.55	20.47	44.42	39.51	12.90	26.16	43.36	29.91	23.21	7.64	19.20	25.56	38.55	24.01	57.17
Actin cap (ACTC1, UniProt)	17.41	44.83	40.53	18.51	61.02	27.14	20.41	81.18	31.07	61.49	44.72	28.86	4.12	68.74	58.85	23.72	3.82	6.50	5.34	47.31	37.54	9.85	19.94	40.37	21.01	16.32	69.53	43.94	20.01	10.79
Zonula occludens protein 1 (ZO1, UniProt)	36.88	50.94	54.04	28.79	36.81	24.42	61.24	18.36	28.06	62.13	46.00	19.40	18.53	8.03	14.47	38.54	21.98	33.59	47.78	18.28	15.93	46.31	28.91	19.18	27.70	30.72	32.72	24.55	41.62	31.28
Albumin (ALB, UniProt)	13.32	32.60	25.79	11.31	40.68	19.90	18.37	56.05	15.03	58.89	33.22	29.56	10.29	53.57	44.38	10.87	15.29	13.00	7.48	20.41	34.13	9.85	17.94	23.21	63.04	18.24	38.86	31.68	12.81	11.87
Cystatin B (CYST2, UniProt)	17.41	10.19	23.34	42.15	31.00	32.57	39.81	28.03	35.08	12.99	29.39	26.79	9.26	20.53	23.16	17.79	47.78	23.84	29.90	12.90	22.75	41.39	39.88	38.35	20.06	16.32	16.36	7.15	13.61	17.26
Semaphorin 6D (SEMA6D, UniProt)	17.41	16.30	12.28	18.51	17.44	41.61	15.31	24.16	31.07	39.84	24.28	24.01	4.12	34.82	22.49	8.89	16.25	3.25	13.88	9.68	21.61	3.94	31.90	26.24	32.47	9.60	28.63	34.75	1.60	15.10
RII1 catalytic domain (RIM1A1, UniProt)	15.37	21.40	13.51	11.31	17.44	14.47	21.43	5.80	8.02	9.53	17.89	12.93	39.11	11.61	15.44	22.73	21.02	14.95	17.20	20.48	63.07	19.94	15.14	12.42	26.88	11.25	13.28	6.00	9.71	
Interleukin-23 receptor (IL23R, UniProt)	9.22	12.23	33.16	27.76	10.66	12.66	20.41	24.16	29.06	2.60	33.22	10.16	13.38	18.75	16.40	11.86	8.60	17.34	12.81	12.90	13.65	20.69	16.95	10.09	6.69	4.80	8.18	0.00	10.41	10.79
Rita-1 (RIT1, UniProt)	0.0	0.0	0.0	0.0	55.18	3.06	0.00	0.00	5.01	2.60	0.00	23.67	16.07	1.93	0.0	0.0	0.0	0.0	0.0	133.33	0.00	0.00	1.00	0.0	6.69	30.72	4.09	2.04	119.26	26.97
Immortal cell necrosis-inducing protein 2 (ICIN2, UniProt)	20.63	9.17	7.37	12.34	8.72	6.33	18.37	6.77	13.03	20.79	3.83	20.32	16.47	13.39	12.54	15.81	22.94	23.84	20.29	6.45	5.69	29.56	6.98	15.14	24.83	10.56	11.25	18.39	14.41	23.73
Lysosomal protein-3 (LAMP1, UniProt)	8.19	9.17	30.71	11.31	9.69	6.33	10.21	14.50	14.03	2.60	21.72	11.08	8.23	7.14	10.61	12.85	3.82	18.42	11.75	2.15	10.24	19.71	12.96	7.06	5.73	3.84	5.11	6.13	6.40	12.94
Lysosomal protein-2 (LAMP2, UniProt)	15.37	2.04	3.68	14.39	4.84	23.52	19.39	2.90	25.05	0.87	6.39	8.31	11.32	12.50	3.86	29.65	9.56	18.42	9.61	6.45	1.14	2.96	20.93	3.03	12.42	55.68	6.14	8.18	3.20	19.42
Ornithine decarboxylase (ODC1, UniProt)	25.61	13.25	3.68	6.17	5.81	9.95	16.33	3.87	11.02	2.60	6.39	4.62	8.23	8.03	4.42	18.90	32.50	23.49	23.91	7.53	10.24	3.94	2.99	8.07	10.51	19.20	10.23	8.18	5.60	19.42
Ornithine decarboxylase-like 2 (ODCL2, UniProt)	5.12	6.11	12.28	4.11	25.18	11.76	13.27	19.33	9.02	18.19	7.67	1.85	0.0	25.00	16.40	8.89	19.1	6.50	2.14	18.28	17.06	1.97	8.97	14.13	11.46	7.68	12.27	17.37	7.20	3.24
RII2 catalytic domain (RIM1B1, UniProt)	5.12	26.49	15.51	3.08	3.87	19.00	4.08	0.97	3.01	0.87	2.56	3.69	18.53	0.89	0.0	17.79	14.33	7.58	3.20	4.30	6.83	9.85	1.99	5.05	12.42	40.48	43.97	15.53	12.81	19.42
RII2 catalytic domain (RIM1B1, UniProt)	4.10	15.28	9.83	7.20	2.91	9.95	6.12	1.93	4.01	13.86	12.78	4.62	8.23	2.68	4.93	12.85	22.94	8.67	12.81	8.00	12.51	11.82	11.00	16.15	13.37	31.88	9.20	14.31	12.81	12.94
β-tubulin class III (TUBB3, UniProt)	7.17	8.15	8.00	10.28	7.75	7.24	7.14	8.70	12.03	12.99	10.22	7.39	3.09	16.96	12.54	7.91	4.78	5.42	9.41	7.53	11.38	3.94	9.07	8.07	13.37	9.60	15.34	6.11	4.80	15.10
β-tubulin class II (TUBB2B, UniProt)	84.00	67.25	34.39	52.43	59.09	47.04	57.16	38.66	42.09	68.42	40.89	71.12	43.23	54.66	65.61	90.92	87.92	80.17	77.95	20.43	55.74	74.89	41.87	74.68	69.72	50.88	50.10	74.60	28.81	72.27
Zinc-alpha-2-glycoprotein (ZAG, UniProt)	6.15	10.19	7.37	9.25	6.78	4.52	4.08	6.77	6.01	2.60	5.11	4.62	11.32	3.57	9.65	4.94	11.47	10.83	10.68	4.30	7.96	23.05	6.98	7.06	6.69	4.80	10.23	4.09	5.60	9.71
Thrombospondin-2 (THSD2, UniProt)	13.32	11.21	1.23	4.11	4.84	0.90	5.10	9.66	3.01	12.13	7.67	12.01	8.23	19.64	14.47	0.99	4.78	8.07	6.68	12.90	12.51	5.91	2.99	3.03	14.33	5.76	15.34	16.35	8.00	12.94
Alpha-2-macroglobulin (A2M, UniProt)	3.07	7.13	11.05	5.14	9.69	15.38	2.04	8.70	7.02	17.32	3.83	12.01	0.0	11.61	6.75	8.89	3.82	0.00	3.20	2.53	6.83	0.0	9.97	6.06	11.46	0.96	7.16	18.39	0.0	0.00
RII1 catalytic domain (A member 2) (RIM1A2, UniProt)	4.10	12.23	9.83	8.22	2.91	6.33	14.29	2.90	5.01	2.60	10.22	5.54	9.26	1.79	4.82	3.95	3.82	11.92	8.54	2.15	4.55	30.55	11.96	5.05	0.00	8.64	5.11	1.02	10.41	14.02
Immortal cell necrosis-inducing protein 1 (ICIN1, UniProt)	12.29	9.17	6.14	4.11	6.78	2.71	6.12	4.83	4.01	6.06	3.83	3.69	5.15	3.57	8.68	10.87	7.65	10.83	9.61	0.00	2.28	2.96	5.98	6.06	4.78	4.80	9.20	3.07	2.40	6.47
Cystatin D (CYST4, UniProt)	1.02	0.00	3.68	13.36	11.42	5.43	6.12	9.66	6.01	0.87	3.83	10.16	13.38	3.57	1.93	10.87	6.69	6.50	4.27	0.00	6.83	32.52	5.98	9.08	8.27	12.48	3.07	0.00	2.40	2.16
UPP1/2 protein (UPP1, UniProt)	7.17	3.06	2.46	10.28	2.91	6.33	18.37	2.90	9.02	6.93	3.83	11.08	4.12	3.57	2.89	7.91	7.65	7.58	9.61	0.00	7.96	19.71	18.94	6.06	0.0	6.72	4.09	0.0	0.80	6.47
Immortal cell necrosis-inducing protein 3 (ICIN3, UniProt)	0.0	4.08	0.00	0.00	0.97	1.81	1.02	5.80	1.00	73.62	11.50	0.92	0.0	0.89	42.45	0.0	0.00	0.00	4.27	1.08	2.28	0.0	8.97	2.02	3.82	0.96	0.00	0.0	0.0	0.0
Alpha-2-macroglobulin variant 1 (A2MV1, UniProt)	12.29	8.15	2.46	6.17	1.94	0.90	7.14	2.90	2.00	8.66	5.11	4.62	5.15	7.14	3.86	3.95	0.96	6.50	6.41	1.08	12.51	0.00	1.99	4.04	14.33	2.88	6.14	8.18	7.20	8.63
Complement C7 (C7, UniProt)	2.05	7.13	4.91	8.22	5.81	7.24	1.02	12.56	7.02	8.66	2.56	4.62	3.09	13.39	5.79	0.99	0.96	1.08	7.48	4.30	4.55	0.99	5.05	3.82	2.88	3.07	14.31	0.0	3.24	
Cystatin C (CYST3, UniProt)	3.07	6.11	6.14	11.31	6.78	2.71	6.12	5.80	3.01	1.73	1.28	8.31	11.32	2.68	6.75	1.98	5.73	8.67	4.27	0.00	1.14	13.80	11.9							

3.07	3.06	3.68	5.14	0.97	1.81	3.06	0.97	2.00	1.73	3.83	4.62	4.12	7.14	2.89	2.96	0.00	5.42	2.14	2.15	5.69	0.0	0.0	1.01	3.82	0.96	1.02	3.07	4.00	3.24	
5.12	3.06	3.68	4.11	0.97	3.62	2.04	1.93	5.01	0.87	3.83	2.77	4.12	2.68	0.00	2.96	4.78	5.42	5.34	2.15	3.41	1.97	100	0.0	0.96	7.68	1.02	7.15	2.40	3.24	
4.10	3.06	0.00	6.17	1.94	0.0	0.0	2.90	1.00	4.33	2.56	4.62	1.03	1.79	1.93	4.94	6.69	2.17	2.14	1.08	0.0	5.91	2.99	3.03	2.87	1.92	2.05	1.02	1.60	4.31	
9.22	7.13	1.23	4.11	1.94	0.00	3.06	1.93	3.01	8.66	2.56	3.69	0.0	8.03	7.72	5.93	3.82	1.08	1.07	8.60	3.41	2.96	2.99	4.04	6.69	2.88	6.14	5.11	0.80	1.08	
8.19	4.08	0.0	1.03	1.94	3.62	6.12	0.97	2.00	0.00	0.0	0.92	4.12	0.0	1.93	2.96	3.82	3.25	5.34	17.20	5.69	2.96	3.99	1.01	1.91	5.76	2.05	3.07	15.21	4.31	
5.12	4.08	4.91	4.11	2.91	0.90	3.06	4.83	2.00	6.93	5.11	5.54	2.06	3.57	3.86	2.96	0.00	2.17	1.07	0.0	2.28	1.97	1.07	1.00	0.00	6.69	0.00	3.07	3.07	1.60	2.16
2.05	4.08	1.23	0.00	1.94	0.90	3.06	3.87	1.00	3.46	2.56	1.85	0.00	7.14	4.82	1.98	0.96	3.25	0.00	2.15	3.41	0.99	1.00	1.01	5.73	0.96	4.09	1.02	1.60	2.16	
1.02	3.06	0.0	3.08	5.81	1.81	1.02	2.90	0.00	1.73	0.00	1.85	1.03	2.68	5.79	1.98	0.96	0.00	1.07	2.15	3.41	0.0	100	0.00	6.06	1.92	4.09	4.09	0.80	1.08	
1.02	4.08	0.00	1.03	1.94	4.52	2.04	3.87	3.01	3.46	1.28	3.69	1.03	4.46	1.93	1.98	1.91	2.17	1.07	0.0	2.28	0.99	1.99	2.02	2.87	1.92	3.07	2.04	0.80	1.08	
1.02	4.08	4.91	7.20	2.91	7.24	5.10	4.83	5.01	7.79	7.67	6.47	0.00	7.14	5.79	2.96	2.87	3.25	2.14	5.38	5.69	0.99	6.98	3.03	6.69	4.80	5.11	6.13	0.0	6.47	
1.02	2.04	1.23	4.11	3.87	5.43	0.00	5.80	3.01	5.30	1.28	7.39	0.0	7.14	0.96	2.96	0.96	0.0	1.07	2.15	3.41	0.0	100	4.04	2.82	0.96	10.25	1.02	0.0	1.08	
2.05	1.02	4.91	4.11	3.87	1.81	2.04	1.93	2.00	0.87	5.11	2.77	2.06	6.25	4.82	1.98	0.96	2.17	1.07	2.15	4.55	1.97	0.00	1.01	4.76	1.92	2.05	7.16	0.80	2.16	
2.05	1.02	1.23	0.0	0.97	1.81	2.04	2.90	0.00	1.73	0.0	0.92	2.06	6.25	4.82	0.99	0.96	2.17	1.07	1.08	2.28	0.99	1.99	3.03	2.87	1.92	2.05	6.13	0.80	0.0	
3.07	0.0	0.0	1.03	0.97	0.90	2.04	0.97	1.00	2.40	1.28	1.85	1.03	5.36	2.89	3.95	3.82	1.08	1.07	6.45	2.28	1.97	0.0	3.03	5.73	0.0	7.16	5.11	4.00	2.16	
1.02	1.02	6.14	2.06	7.75	1.81	5.10	9.66	2.00	3.46	5.11	0.92	1.03	6.25	1.93	0.00	0.0	0.00	2.14	1.08	3.41	0.0	100	2.02	1.91	0.96	6.14	8.18	1.60	1.08	
2.05	2.04	2.46	1.03	0.0	0.90	0.00	0.0	0.00	0.0	0.00	8.31	1.03	0.00	5.79	0.00	0.0	1.08	2.14	0.0	3.41	12.81	0.00	1.01	0.0	2.88	1.02	2.04	4.00	1.08	
1.02	1.02	0.00	1.03	3.87	0.90	0.00	0.00	1.00	7.79	0.00	3.69	1.03	5.36	1.93	0.00	0.96	2.17	1.07	2.15	7.96	0.99	0.0	2.02	4.78	0.00	5.11	6.13	1.60	2.16	
2.05	4.08	1.23	2.06	3.87	1.81	3.06	6.77	1.00	4.33	1.28	3.69	0.0	5.36	5.79	1.98	0.00	0.0	0.0	2.15	5.69	0.0	1.99	7.06	4.78	0.0	4.09	3.07	0.0	0.00	
2.05	1.02	4.91	1.03	0.00	0.90	4.08	9.66	2.00	0.00	7.67	0.00	1.03	0.89	0.96	1.98	0.96	0.0	2.14	4.30	3.41	0.0	100	1.01	0.00	3.84	1.02	1.02	1.60	0.0	
1.02	3.06	1.23	2.06	1.94	0.00	2.04	0.97	0.00	0.87	3.83	1.85	2.06	0.89	0.96	3.95	0.96	3.25	2.14	0.0	0.00	3.94	1.00	5.05	0.0	2.88	1.02	2.04	1.60	5.39	
3.07	3.06	2.46	1.03	1.94	1.81	3.06	7.73	1.00	1.73	0.00	3.69	2.06	2.68	4.82	0.99	0.96	2.17	0.00	3.23	3.41	0.0	100	3.03	2.87	0.0	3.07	5.11	0.80	1.08	
0.0	4.08	0.00	0.00	6.78	4.52	3.06	1.93	1.00	3.46	3.83	0.0	0.0	0.89	2.89	0.99	0.0	0.0	0.00	2.28	0.0	2.99	2.02	0.0	0.0	2.05	6.13	0.0	0.0		
2.05	0.00	1.23	2.06	0.97	0.90	3.06	1.93	1.00	1.73	1.28	1.85	1.03	0.89	1.93	0.96	1.91	1.08	1.07	0.00	0.99	1.00	0.00	0.00	0.96	0.96	0.0	0.00	1.60	3.24	
2.05	3.06	0.00	3.08	2.91	0.00	3.06	0.00	2.00	0.00	0.00	0.00	1.03	0.00	0.00	0.99	0.00	0.00	2.24	2.15	1.14	3.94	0.00	0.00	1.92	0.00	0.00	1.60	2.16		
8.19	6.11	3.68	5.14	3.87	1.81	2.04	1.93	2.00	1.73	3.83	2.77	6.18	5.36	5.79	3.95	0.96	2.17	3.20	8.60	4.55	2.96	1.99	5.05	2.87	1.92	4.09	5.11	0.00	3.24	
3.07	3.06	0.0	0.0	1.94	0.90	1.02	0.97	1.00	6.93	1.28	5.54	1.03	7.14	2.89	1.98	1.91	2.17	1.07	0.00	2.28	1.97	0.0	1.01	4.78	0.96	6.14	4.09	1.60	3.24	
2.05	1.02	2.46	2.06	3.87	5.43	5.10	0.97	4.01	0.00	3.83	0.92	3.09	0.89	1.93	0.99	0.96	5.42	1.07	1.08	3.41	1.97	4.98	0.00	0.96	0.0	0.0	0.00	3.20	2.16	
1.02	0.0	0.0	0.0	0.0	13.57	0.0	0.97	2.00	1.73	0.0	0.0	0.0	17.50	2.68	0.96	0.0	0.0	0.00	40.31	0.0	0.0	0.0	0.00	0.96	20.16	1.02	3.07	47.22	1.08	
3.07	2.84	1.23	1.03	0.97	0.00	0.00	0.97	2.00	1.73	1.28	0.92	1.03	1.79	0.96	0.00	0.00	0.00	2.14	0.0	0.00	0.99	1.00	1.81	0.96	0.00	1.05	2.04	0.80	2.16	
0.00	2.04	4.91	5.14	1.94	6.33	4.08	1.93	7.02	1.73	0.00	0.00	0.00	2.68	2.89	4.94	0.0	0.00	0.0	2.15	2.28	0.00	8.97	1.01	0.00	0.0	6.14	2.04	0.00	1.08	
0.00	3.06	1.23	2.06	3.87	1.81	3.06	10.63	0.0	1.73	2.56	1.85	1.03	2.68	0.96	1.98	0.00	1.08	0.00	0.00	1.14	0.0	100	1.01	0.00	0.96	1.02	0.00	0.80	0.0	
0.00	2.04	1.23	1.03	4.84	3.62	3.06	2.90	2.00	3.46	1.28	1.85	1.03	0.89	2.89	6.92	2.87	2.17	5.34	1.08	0.0	4.93	1.00	3.03	0.96	2.88	0.0	2.04	0.80	1.08	
0.00	4.08	7.37	3.08	3.87	0.90	3.06	10.63	2.00	0.87	2.56	0.92	0.0	3.57	4.82	1.98	0.0	0.0	0.0	2.15	1.14	0.00	1.00	2.02	0.00	0.0	2.05	2.04	0.00	0.00	
5.12	1.02	1.23	0.0	0.97	0.90	0.00	0.00	0.00	0.0	1.28	0.92	3.09	0.0	4.0	0.0	0.0	0.0	1.07	26.88	0.0	0.0	1.99	0.0	0.0	0.96	0.0	2.04	19.21	5.39	
2.05	1.02	1.23	6.17	1.94	1.81	1.02	3.87	1.00	3.46	1.28	0.92	0.0	3.57	0.96	0.00	0.0	0.00	2.15	1.14	0.0	2.99	6.06	2.87	0.96	2.05	2.04	0.00	0.00		
3.07	4.08	1.23	3.08	0.97	0.90	3.06	1.93	3.01	2.60	2.56	0.00	1.03	0.89	0.96	1.98	0.96	2.17	4.27	1.08	1.14	1.97	1.00	1.91	0.0	0.00	0.00	0.80	0.0	0.0	
0.00	0.00	3.68	1.03	0.97	5.43	2.04	1.93	3.01	5.20	0.0	0.00	0.0	4.46	0.96	0.99	0.0	0.0	0.00	3.23	0.00	0.0	1.99	4.04	1.91	0.96	0.0	2.04	0.0	0.0	
1.02	3.06	1.23	3.08	1.94	2.71	3.06	2.90	1.00	1.73	0.0	0.0	2.77	1.03	0.00	2.89	0.00	2.87	1.08	2.14	1.08	1.14	2.96	2.99	1.01	0.0	2.88	0.00	0.0	1.60	2.16
2.05	1.02	0.0	2.06	0.97	0.0	2.04	0.00	0.00	1.73	0.0	3.69	0.0	2.68	2.89	0.99	0.96	1.08	0.00	4.30	1.14	0.99	0.00	2.02	3.82	0.00	1.02	6.13	1.60	2.16	
1.02	0.0	1.23	1.03	1.94	0.00	0.00	0.00	1.00	1.73	0.00	1.85	3.09	0.00	0.96	0.99	1.91	0.00	0.00	0.0	2.28	0.0	1.00	0.00	0.96	1.92	1.02	3.07	0.00	0.0	
11.27	8.15	14.74	9.25	6.78	4.52	10.21	13.53	12.03	7.79	14.06	7.39	1.03	12.50	11.58	4.94	3.82	5.42	4.27	7.53	13.65	0.99	7.98	11.10	6.69	2.88	13.29	9.20	5.60	4.31	
1.02	2.04	2.46	0.0	1.94	3.62	0.00	5.80	3.01	1.73	2.56	0.92	0.0	2.68	2.89	0.99	0.0	1.08	0.0	0.00	1.14	0.0	1.00	0.0	4.78	0.00	0.0	0.80	1.08	0.00	
6.15	2.04	2.46	3.08	0.97	1.81	1.02	1.93	0.0	3.46	0.0	1.85	1.03	1.79	2.89	0.99	0.96	4.33	4.27	2.15	2.28	0.00	1.00	5.05	1.91	1.92	1.02	1.00	1.60	0.00	
1.02	0.00	0.0	0.00	0.00	2.71	2.04	0.00	2.00	0.97	0.00	0.92	0.00	0.00	1.93	0.99	0.00	0.00	1.07	3.23	1.14	0.00	0.0	0.0	1.91	0.0	0.00	2.04	4.00	1.08	
0.00	3.06	1.23	1.83	2.81	2.71	1.02	8.30	0.00	6.30	0.0	0.0	0.00	2.68	1.93	0.99	0.00	0.00	1.07	8.68	0.80	0.0	0.00	2.87	0.00	0.0	0.00	2.05	2.04	0.0	1.08
2.05	1.02	0.00	1.03	0.97	0.00																									

Inducible long of basic protein 439kDa	2.05	0.0	0.0	1.03	0.97	0.00	1.02	0.0	0.0	0.00	0.00	1.85	0.0	2.68	0.00	0.0	0.00	0.00	1.08	1.07	0.00	1.14	0.99	0.00	0.0	1.91	0.00	1.02	1.02	0.80	1.08		
Basic valency protein/239kDa protein 210kDa	0.0	2.04	0.0	1.03	0.0	1.81	2.04	0.97	0.0	0.0	0.0	3.09	0.00	0.00	0.00	0.99	0.96	0.0	2.14	4.30	0.0	3.94	3.99	1.01	0.0	0.0	0.0	0.0	0.0	3.24			
Katidin type B/239kDa protein 210kDa	0.0	0.0	0.0	1.03	0.0	9.95	0.0	0.0	4.01	0.00	1.28	0.92	16.47	1.79	0.00	0.0	0.96	0.0	1.07	36.56	1.14	0.99	0.00	0.191	3.84	1.02	1.02	24.81	1.08				
Inducible long of basic protein 439kDa	2.05	2.04	1.23	1.03	0.97	0.00	3.06	1.93	1.00	0.87	1.28	0.92	0.179	2.89	0.99	0.00	0.0	1.08	1.14	0.0	1.99	2.02	0.00	0.0	4.09	1.60	0.0	0.0	0.0	0.0			
Inducible long of basic protein 439kDa	0.00	2.04	0.00	0.0	0.97	0.0	0.0	3.87	0.0	0.00	0.00	0.0	0.0	0.89	4.82	0.0	0.0	0.0	1.08	1.14	0.00	0.0	0.00	0.00	0.0	0.0	0.0	0.0	0.0	0.0			
Inducible long of basic protein 439kDa	0.0	0.00	0.0	0.00	0.0	0.00	0.00	0.0	1.00	0.00	0.0	0.0	1.03	0.00	0.0	0.0	0.0	0.00	0.00	1.08	1.14	0.00	0.0	0.00	0.00	0.0	0.0	0.0	10.41	0.0			
Inducible long of basic protein 439kDa	1.02	1.02	1.23	1.03	1.94	0.00	2.04	1.93	1.00	0.00	1.28	0.92	0.179	2.89	0.99	0.96	0.00	0.0	1.08	1.14	0.00	0.0	2.15	0.0	1.99	1.01	1.91	0.0	1.02	0.00	0.0		
Inducible long of basic protein 439kDa	3.07	1.02	0.0	0.0	0.00	0.00	0.00	0.00	0.00	2.60	1.28	0.92	0.179	2.89	0.00	0.0	0.0	1.08	1.00	0.0	2.28	0.99	0.00	0.0	0.96	0.0	1.05	1.02	0.80	0.0	0.0		
Inducible long of basic protein 439kDa	2.05	0.0	0.0	0.00	0.0	0.0	0.0	0.0	0.0	0.87	0.0	0.0	0.0	1.79	1.93	0.99	0.96	1.08	1.07	0.00	1.14	0.0	0.0	0.96	0.96	0.00	0.00	0.00	0.00	0.0	0.0		
Inducible long of basic protein 439kDa	0.0	0.0	0.0	0.0	1.94	0.0	0.0	0.0	0.0	0.00	0.0	0.00	0.0	5.36	0.96	0.0	0.00	0.0	1.08	1.14	0.00	0.0	0.99	0.00	2.02	0.96	0.00	1.02	1.02	0.0	0.0		
Inducible long of basic protein 439kDa	0.0	0.00	0.0	0.0	0.0	0.0	0.0	0.0	1.00	0.00	0.0	0.0	11.22	0.00	0.0	0.0	0.0	0.00	15.05	0.0	0.00	0.00	0.00	0.00	0.96	1.02	0.00	22.41	0.00	0.00			
Inducible long of basic protein 439kDa	1.02	1.02	1.23	1.03	0.00	0.00	0.0	0.97	1.00	0.00	1.28	1.85	0.0	1.79	1.53	0.0	0.0	1.08	0.0	0.00	0.99	0.0	0.0	1.91	0.0	0.0	0.0	0.0	0.80	0.0	0.0		
Inducible long of basic protein 439kDa	0.0	0.0	0.0	2.08	2.91	0.0	1.02	2.90	0.00	2.60	0.0	0.0	0.0	1.79	0.96	0.99	0.0	0.0	2.22	0.0	1.00	1.01	0.00	0.96	1.02	3.07	0.80	0.0	0.0	0.0	0.0		
Inducible long of basic protein 439kDa	0.0	0.00	1.23	2.06	2.91	2.71	1.02	4.83	0.0	0.87	0.0	0.0	0.0	0.89	0.0	0.0	0.0	1.08	0.0	0.0	0.0	3.03	0.0	0.00	2.05	1.02	1.02	0.00	0.0	0.0	0.0		
Inducible long of basic protein 439kDa	1.02	1.02	1.23	2.06	1.94	0.0	0.0	0.0	1.00	0.87	0.0	0.185	1.03	1.79	0.96	1.98	0.0	2.17	1.07	0.00	1.14	0.0	0.0	0.0	0.0	1.92	1.02	1.02	0.80	1.08	0.0	0.0	
Inducible long of basic protein 439kDa	0.0	0.0	0.0	0.0	0.0	0.0	1.02	0.0	1.00	2.60	0.0	1.85	1.03	1.79	0.96	0.0	0.96	2.17	0.0	1.08	1.14	0.0	0.0	0.0	2.87	2.88	1.02	2.04	2.40	1.08	0.0	0.0	
Inducible long of basic protein 439kDa	6.15	0.0	0.0	1.03	0.0	0.00	0.00	0.0	0.00	0.87	0.0	0.0	4.12	0.0	0.0	0.0	0.96	0.0	1.07	1.08	1.14	3.94	1.00	0.0	0.0	1.92	0.0	2.04	0.80	0.0	0.0	0.0	
Inducible long of basic protein 439kDa	2.05	1.02	1.23	0.0	0.00	0.0	0.00	1.00	1.00	0.00	1.85	2.06	0.89	0.96	0.00	0.96	1.08	1.07	1.08	1.14	0.00	0.0	0.0	0.96	0.0	0.0	1.60	0.0	0.0	0.0	0.0		
Inducible long of basic protein 439kDa	1.02	4.08	1.23	0.0	0.97	1.02	0.97	1.00	0.0	2.56	1.85	1.03	0.0	0.0	0.99	0.00	1.08	0.0	2.28	1.97	0.0	1.01	0.0	0.96	0.0	0.0	1.60	0.0	0.0	1.08	0.0	0.0	
Inducible long of basic protein 439kDa	1.02	1.02	1.23	0.0	1.94	0.90	1.02	3.87	0.0	1.73	0.0	1.85	0.0	2.68	2.89	0.99	0.0	0.0	1.14	0.0	1.00	3.03	0.00	0.0	3.07	3.07	0.0	0.0	0.0	0.0	0.0	0.0	
Inducible long of basic protein 439kDa	2.05	2.04	1.23	0.00	2.91	0.0	2.04	2.90	1.00	0.87	0.0	0.0	0.0	0.89	0.00	0.99	0.0	1.08	1.14	0.0	0.00	1.00	1.00	0.96	0.00	1.02	0.0	1.02	0.00	0.0	0.00	0.00	
Inducible long of basic protein 439kDa	0.00	0.00	1.23	1.03	0.00	0.0	3.06	0.00	0.00	0.00	0.0	0.92	0.0	0.00	0.99	0.00	1.08	0.0	0.0	0.00	0.99	1.00	0.00	0.0	0.0	1.02	0.0	1.02	0.00	0.0	1.08	0.0	0.0
Inducible long of basic protein 439kDa	0.00	2.04	1.23	0.0	0.0	0.0	0.0	2.90	2.00	0.0	1.28	0.92	0.00	0.0	0.00	0.00	1.91	1.08	0.00	1.08	1.14	0.0	0.0	1.91	0.0	2.05	1.02	0.00	0.0	0.0	0.0	0.0	
Inducible long of basic protein 439kDa	0.0	3.06	1.23	1.03	0.0	0.90	2.04	0.97	2.00	1.73	2.56	0.0	0.0	0.89	0.99	0.0	0.0	0.0	0.00	0.00	1.00	1.01	0.96	0.0	0.0	2.04	0.0	2.04	0.0	0.0	0.0	0.0	
Inducible long of basic protein 439kDa	4.10	2.04	0.0	0.0	0.97	0.0	0.0	0.0	0.0	2.60	0.0	2.77	1.03	1.79	0.0	0.99	0.00	0.0	0.0	1.08	0.0	0.99	0.0	0.0	3.82	1.02	1.02	0.0	0.0	0.0	0.0	0.0	
Inducible long of basic protein 439kDa	0.00	0.00	0.0	0.0	0.00	0.90	0.00	0.0	0.0	0.0	0.92	0.0	0.0	0.0	0.0	0.0	0.96	0.0	0.0	6.45	0.00	0.0	1.00	1.01	0.0	3.07	2.04	0.80	1.08	0.0	0.0		
Inducible long of basic protein 439kDa	0.0	1.02	0.0	2.06	0.0	0.0	2.04	5.80	1.00	0.87	1.28	0.00	0.0	0.0	0.96	0.99	0.0	0.0	6.00	1.08	2.28	0.0	0.0	2.02	0.0	0.0	1.02	0.00	1.08	0.0	0.0	0.0	
Inducible long of basic protein 439kDa	1.02	1.02	1.23	2.06	1.94	0.00	0.0	2.00	1.85	1.28	0.92	0.0	2.57	0.0	0.00	0.0	1.98	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Inducible long of basic protein 439kDa	0.00	1.02	0.00	0.0	0.97	0.00	0.0	0.0	0.0	2.60	0.0	0.00	1.03	0.89	0.0	0.0	0.96	0.0	2.14	0.0	1.11	0.0	0.0	1.81	0.0	0.96	1.02	1.02	0.0	0.0	0.0	0.0	0.0
Inducible long of basic protein 439kDa	0.0	0.0	1.23	1.03	0.00	0.0	0.0	0.0	0.0	1.28	0.0	0.0	0.0	0.00	0.00	0.00	0.0	0.0	0.00	1.97	1.00	0.00	0.96	0.00	0.00	0.00	0.80	0.00	0.00	0.00	0.00	0.00	
Inducible long of basic protein 439kDa	3.07	4.08	1.23	0.0	2.91	0.90	1.02	0.97	1.00	1.73	1.28	0.92	11.38	2.68	3.86	0.99	4.78	2.17	4.27	2.15	0.0	3.94	4.98	3.03	3.82	10.56	2.05	5.11	2.40	2.16	2.16	2.16	2.16
Inducible long of basic protein 439kDa	1.02	1.02	2.46	1.03	1.94	2.71	2.04	6.77	1.00	2.60	2.56	2.77	2.06	1.79	0.0	3.95	0.96	2.17	0.00	2.15	1.14	0.99	1.00	1.01	0.96	1.92	1.02	0.00	0.80	0.0	0.0	0.0	
Inducible long of basic protein 439kDa	0.0	2.04	0.00	1.03	0.97	0.90	1.02	0.97	1.00	0.0	1.28	0.0	0.0	0.00	0.00	0.00	0.0	0.0	0.00	0.0	0.0	0.00	0.0	0.0	0.96	0.0	0.00	0.00	0.00	0.00	0.00	0.00	
Inducible long of basic protein 439kDa	0.0	2.04	1.23	0.0	0.97	0.00	0.0	4.83	0.0	0.0	0.0	0.00	1.79	0.96	0.0	0.0	0.0	1.08	1.14	0.0	0.0	4.04	0.0	0.0	1.02	1.02	1.02	0.00	0.00	0.00	0.00	0.00	0.00
Inducible long of basic protein 439kDa	0.0	1.02	0.0	1.03	0.00	0.90	1.02	3.87	1.00	0.87	0.0	0.0	0.0	0.0	0.96	0.99	0.0	0.0	0.00	2.28	0.00	0.00	2.02	0.96	0.0	1.02	1.02	0.0	0.0	0.0	0.0	0.0	0.0
Inducible long of basic protein 439kDa	0.0	0.0	1.23	0.0	3.87	0.00	0.0	3.87	1.00	0.87	0.0	0.0	0.00	0.89	0.96	0.99	0.0	0.0	0.0	1.14	0.0	0.0	2.02	0.0	0.0	0.00	2.05	2.04	0.0	0.0	0.0	0.0	0.0
Inducible long of basic protein 439kDa	1.02	1.02	1.23	1.03	0.97	0.00	1.02	0.97	1.00	0.0	1.28	1.85	0.0	0.89	0.0	0.99	0.96	0.0	0.0	1.14	0.99	0.0	0.0	0.0	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Inducible long of basic protein 439kDa	4.10	5.09	4.91	7.20	4.84	4.52	5.10	4.83	6.01	4.33	5.11	7.39	2.06	9.82	4.82	5.93	2.87	4.33	5.34	3.23	7.96	5.99	5.98	3.03	9.55	10.56	10.23	4.00	4.00	8.63	8.63	8.63	8.63
Inducible long of basic protein 439kDa	0.00	1.02	0.0	1.03	0.0	0.0	0.0	0.0	1.00	0.0	1.85	2.06	0.0	0.00	0.99	0.96	1.08	0.00	0.0	0.0	0.99	1.99	0.0	0.0	0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Inducible long of basic protein 439kDa	0.00	0.0	0.00	1.03	0.00	0.00	0.0	0.0	1.73	0.0	0.0	0.0	0.0	0.89	0.96	0.0	0.96	0.0	0.00	1.14	0.00	0.00	2.02	0.0	0.0	0.0	1.02	0.0	0.0	0.0	0.0	0.0	0.0
Inducible long of basic protein 439kDa	0.0	0.0	0.0	0.0	0.90	0.0	0.0	0.0	0.0	0.0	0.0	2.06	0.00	0.0	0.0	0.0	0.0	0.0	5.38	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00	0.00	11.21	0.0	0.0	0.0	
Inducible long of basic protein 439kDa	3.07	1.02	0.00	1.03	0.00	0.0	0.0	0.97	1.00	0.0	0.92	1.03	0.89	1.93	0.00	0.96	0.0	1.07	0.00	0.0													

Table with 28 columns (0-27) and 575 rows. Each row contains numerical values representing data for a specific category, such as 'Ruralized C3' or 'Actin-related protein 2'. The values are mostly 0.0, with some non-zero values like 1.23, 2.90, 1.81, etc., distributed across the columns.



**COMITÊ DE ÉTICA EM PESQUISA**  
**FACULDADE DE ODONTOLOGIA DE PIRACICABA**  
**UNIVERSIDADE ESTADUAL DE CAMPINAS**



## CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Avaliação do perfil imunológico, imunoistoquímico e do proteoma salivar de pacientes com Leucoplasia Verrucosa Proliferativa**", protocolo nº 105/2013, dos pesquisadores Isadora Luana Flores e Marcio Ajudarte Lopes, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 10/10/2013.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Immunological and immunohistochemistry profile evaluation and salivary proteomic assessment of patients with Proliferative Verrucous Leukoplakia**", register number 105/2013, of Isadora Luana Flores and Marcio Ajudarte Lopes, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 10/10/2013.

**Prof. Dr. Felipe Bevilacqua Prado**  
Secretário  
CEP/FOP/UNICAMP

**Profa. Dra. Lívia Maria Andaló Tenuta**  
Coordenadora  
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.  
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO