UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

DISSERTAÇÃO DE MESTRADO

EVALUATION OF $p16^{Ink4a}$ EXPRESSION AND PRESENCE OF HPV-16 DNA BY REAL TIME PCR IN PATIENTS WITH ORAL LEUKOPLAKIA

Araçatuba - SP
2019

STEPHANYE PINTO BISS

EVALUATION OF $p16^{Ink4a}$ EXPRESSION AND PRESENCE OF HPV-16 DNA BY REAL TIME PCR IN PATIENTS WITH ORAL LEUKOPLAKIA

Dissertação apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP, para obtenção do título de Mestre em Odontologia, área de Concentração em Estomatologia.

Orientadora: Professora Doutora Kellen Cristine Tjioe
Coorientador: Professor Titular Glauco Issamu Miyahara

Araçatuba- SP
2019

Catalogação na Publicação (CIP)

Diretoria Técnica de Biblioteca e Documentação – FOA / UNESP

B623e

Biss, Stephanye Pinto.
Evaluation of p16Ink4a expression and presence of HPV-16 DNA by *Real time* PCR in patients with oral leukoplakia / Stephanye Pinto Biss. – Araçatuba, 2019
46 f. : il. ; 2 tab.

Dissertação (Mestrado) – Universidade Estadual Paulista, Faculdade de Odontologia, Araçatuba
Orientadora: Profa. Kellen Cristine Tjioe
Coorientador: Prof. Glauco Issamu Miyahara

1. Imunohistoquímica 2. Leucoplasia 3. Papillomaviridae 4. Reação em Cadeia da Polimerase em Tempo Real I. Título

Black D6
CDD 617.63

Claudio Hideo Matsumoto CRB-8/5550

Dedicatória

Dedico a conquista e a vitória deste momento...

À minha maravilhosa mãe, **Noeli,** que me deu à luz da vida e me dá forças todos os dias para aguentar as dificuldades encontradas pelo caminho. Uma pessoa que não mede esforços pra me ver bem, e mesmo estando tão longe de mim, sempre foi muito presente. Você é a minha inspiração de força, perseverança e amor incondicional. Eu espero poder retribuir todo esse amor e zelo que você tem por mim. Obrigada por ser essa mãezona maravilhosa, incrível e guerreira, literalmente uma 'super' mãe. Em meus momentos de fraqueza você sempre segurou a barra e com sábias palavras me dizia: "Filha, vai ficar tudo bem, mamãe tá aqui pra você". Você é tudo e mais um pouco pra mim! Agradeço todos os dias por sua existência! Te amo!!!

Ao meu pai, **Adilson**, que mesmo a distância que existe entre nós, você sempre se faz presente na minha vida. E desejo do fundo do meu coração muita paz e amor! Te amo!!

À minha tia-irmã, **Nelcili,** que faz o papel de tia legal e irmã chata. Já passamos por tantas coisas, desde você aumentando o som do rádio pra me assustar quando criança, pintar meu cabelo (oi?) e ser minha parceira nas baladas e na vida. Uma pessoa livre, aberta a conhecer coisas novas e que me ensinou muita coisa, mesmo à distância. Sempre disponível e podendo me ajudar no que fosse possível. Infelizmente você não estará presente fisicamente, mas sei que estará torcendo pela minha vitória. Acho que eu não poderia ter escolhido uma tia tão sensacional como você. E pode ter certeza que muitas coisas que eu sou hoje, você tem uma grande parcela de contribuição nesse meu crescimento. Te amo Shei!!!

Aos meus avós maternos, **Erci e Neuza,** que até hoje eu os chamo de ‘pai e mãe’. Obrigada por todo auxílio na minha educação, vocês foram essenciais. Amo vocês!

Aos meus irmãos, **Daniele e Felipe,** eu só tenho a agradecer à Deus por vocês estarem na minha vida. Não poderia ter pedido irmãos mais legais e loucos iguais a vocês, muita luz e sucesso e que nós estejamos juntos para sempre! Também gostaria de agradecer aos meus cunhados, **Ricardo e Daniele,** por estarem presentes na minha vida, os melhores cunhados que eu poderia ter. E não posso esquecer dos meus queridos sobrinhos, **Rafaela e Alexandre**, um dos melhores presentes que eu poderia ter tido na vida é ser tia. Amo vocês!

À todas as paixões da minha vida, **Piti, Polaco, Rabisco, Melo, Miau, Mário e o Vida.**

♥

Agradecimentos especiais

À Deus, que sem a vontade Dele eu nem estaria aqui neste momento. Ele que me preparou pra eu estar aqui e sem Ele, eu não teria conseguido vencer essa etapa. Obrigada meu Deus por ter sido tão bom comigo e ter me guiado e encontrado pessoas especiais que me ajudaram a percorrer esse caminho tão árduo. Quando eu achava que não tinha saída, Você vinha e mostrava o que eu não enxergava, e me guiou até eu vencer e mostrando que nunca, nada, é impossível aos olhos do Senhor.

À minha orientadora, **Prof. Dra. Kellen Cristine Tjioe**, que eu não tenho palavras por tudo que a senhora fez por mim. Acabei caindo de paraquedas na sua orientação e foi uma das melhores coisas que poderia ter me ocorrido no mestrado. Mesmo com todos os seus afazeres do pós doutorado e pra ajudar, estando fora do país, a senhora sempre foi disponível com qualquer coisa que eu precisasse. Gostaria de ter aproveitado mais a nossa convivência. Obrigada pelos ensinamentos e pelas palavras sábias, levo a senhora no meu coração. Obrigada!

Ao meu coorientador, **Prof. Tit. Glauco Issamu Miyahara,** que foi o motivo inicial de eu querer estar no mestrado na FOA/UNESP. Gostaria de agradecer por todas as palavras, os ensinamentos e a disponibilidade, mesmo o senhor sempre com tantos compromissos. Obrigada por ter permitido que eu tivesse a oportunidade de estar na sua equipe, aprendendo sobre a Estomatologia e fazer parte do Centro de Oncologia Bucal, do qual, era um dos meus sonhos. Obrigada!

Ao **Prof. Ass. Daniel Galera Bernabé,** que é um professor de um brilhantismo e uma dedicação à pesquisa e a docência imensurável. Me espelho em um dia tornar uma professora com uma paixão tão grande pelo o que faz e acredita. Obrigada por todos os momentos bons compartilhados.

Ao **Prof. Adj. Éder Ricardo Biasoli,** que é um cirurgião excepcional de extrema competência, de um convívio leve e harmonioso.

Ao **Prof. Adj. Marcelo Macedo Crivelini,** que me ajudou na realização e análise da imunohistoquímica. Obrigada pelos ensinamentos e por toda a disponibilidade.

À **Prof. Tit. Sandra Helena Penha de Oliveira**, agradeço por toda a disposição da utilização do laboratório, sem isso, não conseguiria ter desenvolvido minha pesquisa. Meus agradecimentos.

À minha primeira orientadora e idealizadora do meu sonho, **Prof. Dra. Estela Maris Losso,** que me introduziu na área da pesquisa e mostrou um mundo totalmente alheio aos meus conhecimentos. Sempre com muita paciência, me ensinou os desafios da pesquisa e suas recompensas também. Onde eu estou hoje, é resultado da sua dedicação por mim. Obrigada eternamente!

Agradecimentos

À minha amiga, **Glívia Queiroz Lima,** que foi a minha parceira nesses dois anos de mestrado. Obrigada por todos os momentos vividos, as festas, as brigas, os choros, as conquistas, tudo! Tenho certeza que Deus colocou você no meu caminho pra me segurar quando eu não conseguia ficar mais em pé, para me aconselhar e comemorar as vitórias. Também agradeço por ter sido acolhida pela Família **Queiroz Lima**. Você é uma pessoa iluminada e que ilumina todos ao redor, e hoje já nem consigo imaginar se você não tivesse presente na minha vida. Te desejo muita luz e sucesso, porque você é sensacional. Infelizmente nossa jornada juntas termina por aqui, mas com certeza nos reencontraremos muito ainda. Te amo incondicionalmente!

À minha amiga e colega de mestrado, **Giseli Mitsuy Kayahara,** eu não tenho palavras pra te agradecer por tudo, tudo mesmo. É uma profissional excepcional e uma amiga incrível. Acredito que as vezes eu te deixava meio louca (e eu sei que você vai sentir falta, já te disse), mas você com a sua calma nipônica, me colocava nos eixos e tudo dava certo. Sempre com a maior paciência do mundo pra me ajudar, me ensinar. Meu mestrado ficou muito mais leve quando começamos a conviver e sou muito agradecida por isso. Sentirei falta das nossas conversas e da nossa convivência. Você é maravilhosa Gi! Te desejo muito sucesso!

À minha amiga, **Daniela Brito Bastos,** obrigada por sua amizade, mesmo que tardia, deu match. Obrigada por toda sua dedicação, amizade, paciência e os momentos juntas. Uma amiga que não mede esforços para ver os outros bem e felizes. Também gostaria de agradecer ao **Leandro** por todos os momentos vividos. Foi maravilhoso ter sido sua

amiga, você é uma pessoa incrível, que me ensinou muita coisa e com certeza você estará comigo sempre. Te desejo muito sucesso! Te amo.

Aos meus queridos vizinhos, **Neusa e Eurides Montibeller,** obrigada por toda a amizade e carinho. Sentirei muita falta de vocês e também do Dick. Só tenho a agradecer por uma amizade tão pura, fazendo eu me sentir em casa, e isso é muito bom. Obrigada!

Aos meus amigos de graduação, **Kaíke Lessa e Nádia Militão**, que estão presentes na minha vida sempre e que eu os amo muito. Que a nossa amizade perdure os anos e que estejamos sempre 'presentes' nos momentos mais importantes das nossas vidas. Obrigada pela amizade, e por sempre estarem comigo quando eu precisei. Muita luz e sucesso a nós todos!

Ao meu psicólogo, **Rafael Baptista**, que sem você não sei como teria sido aguentar essa barra que se chama 'pós-graduação' e 'estar longe de casa' ao mesmo tempo. A sua presença foi imprescindível para o meu amadurecimento, compreensão e luta dos meus sonhos e objetivos. Obrigada eternamente!

À minha amiga, **Raiane Perez**, a melhor manicure do mundo. Obrigada por esses anos de amizade e estar sempre presente na minha vida. Só tenho a agradecer a nossa amizade ser tão sólida. Estarei sempre aqui para o que precisar. Muito sucesso! Te amo.

À minha amiga, **Luana Trevelin de Carvalho**, pela nossa amizade especial e por um dos maiores presentes que eu já recebi: o **Vida**. Sem você, a minha casa não teria tanta alegria como tem hoje. Muito sucesso 'dotôra'. Te amo.

À minha amiga, **Beatriz Alves Furtado**, pela nossa amizade especial, pelas caronas e por todos os favores. Você é uma amiga muito especial, sentirei saudades. Te amo.

Às minhas meninas, **Mariana Vieira de Souza e Fernanda Antunes**, por me darem uma 'palhinha' de como é ser uma orientadora. Vocês me ensinaram que o caminho é árduo, mas não tem nada melhor como o gosto da vitória e da conquista. Sou muito agradecida de vocês terem entrado na minha vida. Sucesso princesas!

Aos amigos da Pós-Graduação **(Saygo Tomo, Jéssica Figueira, Vitor Valente, Bruna Sarafim, Tamara Fernandes de Castro)**, agradeço por toda amizade e experiências compartilhadas no dia-a-dia. Cada um com sua personalidade, com seu método de trabalho e maneira de enxergar a vida, contribuiu para meu crescimento pessoal, profissional, além de trabalho em equipe. Sou privilegiada em ter tido pessoas tão maravilhosas ao meu lado durante esse percurso.

Ao departamento de Patologia e Propedêutica Clínica, e aos docentes P**rof. Cristiane Furuse, Prof. Ana Maria Soubhia, Prof. Renata Calestini**, por toda a paciência, conhecimento e tempo dedicado. À querida **Adriana Leal**, que tanto me ajudou em todos os momentos que precisei. Sentirei muito sua falta. Obrigada por todos os conselhos, uma mãezona mesmo. E a funcionária **Marli Santos**, por todos os cafés quentinhos e acolhedores. Obrigada.

Aos Funcionários do Centro de Oncologia Bucal – COB **(Anne Cocato, Jane Fátima, Gabrielle Duarte, Daniene Ribeiro, Regiane Nogueira, Francisco Collado, Sebastião Conrado)**. Gostaria de agradecer pela convivência durante essa caminhada, por sempre

estarem dispostos em me ajudar, por terem feito meus dias mais agradáveis. Agradeço por termos criado, além de um convívio profissional, termos construído laços de uma amizade tão sincera. Obrigada por terem contribuído, cada um à sua maneira, para minha formação.

À equipe do laboratório de farmacologia, **Beatriz Ballassoni, Ayná Barreto, Victor Balera e Sabrina Frasnelli,** por toda ajuda no laboratório e na realização dos experimentos.

À Faculdade de Odontologia de Araçatuba – UNESP, na pessoa do Diretor **Prof. Tit. Wilson Roberto Poi** e o Vice-Diretor, **Prof. Tit. João Eduardo Gomes Filho**, pelo acolhimento e oportunidade de realização do curso de Mestrado. Agradeço pelo empenho e dedicação para o crescimento não só da faculdade, mas também da pós-graduação em Odontologia da FOA.

Ao Programa de Pós-Graduação em Odontologia, da Faculdade de Odontologia de Araçatuba, da Universidade Estadual Paulista "Júlio de Mesquita Filho" com o atual coordenador **Prof. Adj. André Luiz Fraga Briso**.

Aos funcionários da Pós-Graduação da Faculdade de Odontologia de Araçatuba – UNESP, **Valéria Zagato, Lilian Mada e Cristiane Lui**, pela disponibilidade e gentileza em ajudar.

Aos pacientes, meu muito obrigada! A vocês que foram essenciais à minha formação. Vocês serão sempre razão e estímulo ao meu crescimento profissional.

À Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES), pela concessão da bolsa de mestrado durante meus dois anos de mestrado. Meus sinceros agradecimentos por promover o apoio financeiro durante a minha formação.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pelo auxílio regular concedido à nossa equipe (Processo no. 2016/12982-0) e por todo suporte financeiro indispensável para a realização deste estudo.

Epígrafe

"Sempre há outra chance, uma outra amizade, um outro amor. Para todo fim, um recomeço."

Antoine de Saint-Exupéry

Biss SP. Avaliação da expressão da p16^{Ink4a} e a presença do DNA do HPV-16 pela *Real time* PCR em pacientes com leucoplasia bucal. [dissertação]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2019.

Resumo

Objetivo: Avaliar a expressão do p16^{Ink4a} por imunohistoquímica e a presença do HPV-16 pela *Real time* PCR em tecido fresco, plasma e saliva de pacientes com leucoplasia bucal (LB) e hiperplasia fibrosa inflamatória (HFI). **Material e métodos:** Foram incluídos 67 pacientes com o diagnóstico de LB e 44 pacientes com diagnóstico de HFI no estudo. Foram coletados dados sociodemográficos, clinicopatológicos, amostras de tecido fresco, sangue e saliva, que foram armazenados em um freezer a -80ºC para realização de análise molecular. Também foi utilizado o tecido parafinizado para realização da imunohistoquímica para a p16^{Ink4a}. As amostras de materiais biológicos obtidos dos pacientes foram submetidas à detecção do DNA do HPV-16 pela *Real time* PCR. O tecido parafinizado dos mesmos pacientes foram utilizados para avaliar a expressão da p16^{Ink4a} pela imunohistoquímica. **Resultados:** Dos 67 pacientes incluídos de LB no estudo, 55,2% eram do sexo masculino com uma média de idade de 57,1 anos. Os pacientes idosos (>45 anos) compuseram 86,6% da amostra. As localizações anatômicas mais acometidas por LB foram a mucosa jugal (35,8%) e língua (20,9%). Sobre o tabagismo, 71,8% dos pacientes eram fumantes, onde a maioria (47,8%) foi classificada como tabagista leve. Em relação ao consumo de álcool, 47,4% eram alcoolistas, sendo a sua maioria classificada como alcoolista leve (59,3%). O grupo HFI foi composto por 54,5% pacientes do sexo masculino com uma média de idade de 57,3 anos. 90,9% dos pacientes eram idosos (>45 anos). A ocorrência das lesões foram em mucosa jugal (31,8%) e rebordo alveolar (25%). A expressão para p16^{Ink4a} na LB foi fraca (41,8%) e para o grupo HFI, majoritariamente a expressão foi forte (68,2%). *Real time* PCR não detectou HPV-16 em nenhumas das amostras analisadas. **Conclusão:** Os achados em nosso estudo mostraram que a detecção de HPV de alto risco em tecido fresco, plasma e saliva para LB e HFI não se correlacionaram com a superexpressão da p16^{Ink4a}.

Palavras-chave: Imunohistoquímica, Leucoplasia, Papillomaviridae, Reação em cadeia da polimerase em tempo real.

Biss SP. Evaluation of $p16^{Ink4a}$ expression and presence of HPV-16 DNA by *Real time* PCR in patients with oral leukoplakia. [dissertation] – Araçatuba: UNESP – São Paulo State University; 2019.

Abstract

Objective: To evaluate the expression of $p16^{Ink4a}$ by immunohistochemistry and the presence of HPV-16 by *Real time* PCR in fresh tissue, blood plasma, and saliva of patients with oral leukoplakia (OL) and inflammatory fibrous hyperplasia (IFH). **Material and methods**: Sixty-seven patients with the diagnosis of OL and 44 patients with the diagnosis of IFH were included in the study. Sociodemographic, clinicopathological, fresh tissue, blood, and saliva samples were collected and stored at - 80ºC for molecular analysis. Paraffinized-embedded tissue was also used to perform the immunohistochemistry for $p16^{Ink4a}$. Samples of biological material obtained from the patients were submitted to HPV-16 DNA detection by real time PCR, both with specific probes. Paraffinized-embedded tissue from the same patients were used to evaluate the expression of $p16^{ink4a}$ by immunohistochemistry. **Results:** Out of the 67 included OL patients, 55.2% were male with a mean age of 57.1 years. Elderly patients (> 45 years) comprised 86.6% of the sample. The prevalence of lesions was highest in the buccal mucosa (35.8%) followed by the mobile tongue (20.9%). 71.8% were smokers and the majority (47.8%) was classified as light smoker. Regarding alcohol consumption, 47.4% were alcoholics, most of whom were classified as light alcoholics (59.3%). The IFH group was composed of 54.5% of male patients with a mean age of 57.3 years. 90.9% of the sample were elderly patients (> 45 years). The highest occurrence of the lesions was in buccal mucosa (31.8%) and alveolar ridge (25%). The expression for $p16^{Ink4a}$ in LB was mostly weak (41.8%) and for the IFH group, the expression was mostly strong (68.2%). There was no positivity in any of the samples for HPV-16 by real time PCR**. Conclusion:** Our findings showed the HR-HPV detection in fresh tissue, plasma blood and saliva for OL and IFH does not correlate with $p16^{Ink4a}$ immunoexpression in paraffinized-embedded tissue.

Keywords: Immunohistochemistry, Leukoplakia, Papillomaviridae, Real-time polymerase chain reaction.

LISTA DE FIGURAS

.

LISTA DE TABELAS

LISTA DE ABREVIAÇÕES

DNA = Deoxyribonucleic acid

EDTA = Ethylenediamine tetraacetic acid

FFPE = Formalin-fixed, paraffin-embedded

HPV = Human Papillomavirus

HNSCC = Head and neck squamous cell carcinoma

IFH = Inflammatory fibrous hyperplasia

IHQ = Immunohistochemistry

IPC = Internal positive control

ISH = *in situ* hybridization

nPCR = Nested polymerase chain reaction

OPMD = Oral potentially malignant disorders

OL = Oral leukoplakia

OSCC = Oral squamous cell carcinoma

pRb = Retinoblastoma protein

qPCR = Quantitative polymerase chain reaction

SCC = Squamous cell carcinoma

UNESP = Universidade Estadual Paulista Júlio de Mesquita Filho

SUMÁRIO

Evaluation of p16^{Ink4a} expression and presence of HPV-16 DNA by *Real time* PCR in patients with oral leukoplakia*

Authors: Stephanye Pinto Biss[1], Saygo Tomo[1], Sandra Helena Penha de Oliveira[2], Marcelo Macedo Crivelini[3], Éder Ricardo Biasoli[1], Daniel Galera Bernabé[1], Kellen Cristine Tjioe[1], Glauco Issamu Miyahara[1*]

[1] Oral Oncology Center, São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil.

[2] Department of Basic Science, São Paulo State University (UNESP), School of Dentistry, Araçatuba, São Paulo, Brazil.

[3] Department of Pathology and Clinical Propaedeutic, São Paulo State University (UNESP), School of Dentistry, Araçatuba, São Paulo, Brazil.

Keywords: Immunohistochemistry, Leukoplakia, Papillomaviridae, Real-time polymerase chain reaction.

Correspondence to:

Glauco Issamu Miyahara
Oral Oncology Center
São Paulo State University (UNESP), Araçatuba School of Dentistry
Rua José Bonifácio, 1193, Araçatuba, São Paulo, Brazil 16015-050
glauco.miyahara@unesp.br

***Formatado de acordo com as normas do periódico Journal of Oral Pathology and Medicine (ISSN: 1600-0714)**

INTRODUCTION

Human papillomavirus (HPV) has been classically associated with the development of cervical cancer (1) and, more recently, with the onset of tumors arising in other mucosal sites (2). In oropharyngeal cancer, HPV has been demonstrated as a risk factor for its occurrence while in oral cancer this relationship remains unclear (3). HPV has an exclusively intraepithelial infectious cycle and infects both mucosal squamous epithelium and cutaneous squamous epithelium (4). Currently, over than 200 subtypes of HPV have been identified and they are grouped as high-risk (HR) and low-risk (LR) according to their oncogenic potential (5). HPV subtypes 16 and 18 are the most common HR subtypes (6-8). The association between HR-HPV and head and neck squamous cell carcinoma (HNSCC) is well established, mostly in oropharyngeal cancer (9, 10). Additionally, HPV+HNSCC seems to have better prognosis than HPV− HNSCC (11).

Oral leukoplakia is the most frequent oral potentially malignant disorder (OPMD) (12). The most affected sites by leukoplakia are the lateral tongue border and floor of the mouth (13). Leukoplakia has been observed mostly in middle-aged men, with an increasing prevalence in increasing age (14). The classic risk factors for development of OL are alcohol and tobacco (15). Interestingly, recent studies have been suggesting HPV as a possible risk factor for OL (16). HPV has been detected in samples of OL in variable indices. Saghravanian *et al*. (17) evaluated 20 samples of fresh tissue OL and in none of the samples HPV was found. On the other hand, we previously found HPV-16 detection in 68.75% of OL fresh tissue (22/32) (18). However, a recent study reported no association between OPMD and HPV (2). This reinforces the need of studies to aiming at establishing the relationship between OPMD and HPV.

The methods for the detection of HPV are also source of debate. Immunohistochemistry (IHC) for $p16^{Ink4a}$ (CDKN2A) in paraffin-embedded (FFPE) tissues is the most widely used method, mainly in the lower anogenital tract. Indeed, $p16^{Ink4a}$ expression is currently being explored as a prognostic marker in oropharyngeal SCC (19). Due to the absence of a consistent link between HPV infection and $p16^{Ink4a}$ expression by IHC, this method is not specific for HPV detection. Therefore, using other techniques with higher specificity is essential to detect accurately the HPV and, potentially, draw some conclusions (20).

Data mentioned above show that the role of HPV in the onset of OPMD, namely OL, remains unknown. Besides studies using unspecific methods for the detection of HPV added to the high variation of techniques used to detect HPV limit the interpretation of their results. Therefore, the aim of this study is to evaluate the presence of HPV-16 in fresh tissue, plasma and saliva by *Real time* PCR, and correlate these findings with the immunohistochemical expression of $p16^{Ink4a}$ from patients with OL.

MATERIALS AND METHODS

Study design and population

This cross-sectional study included 67 patients with the diagnosis of OL (case group) matched at age ± 3 years with 44 patients diagnosed with IFH (control group). A patient in the control group could be paired with more than one patient of the case group. The groups were selected at Oral Oncology Center, School of Dentistry, São Paulo State University (UNESP), Araçatuba, Brazil. The inclusion criteria were I. Patients with clinically and microscopically confirmed diagnosis of OL or IFH, II. Availability of fresh tissue, plasma, saliva and/or FFPE, III. patients who agreed to participate in the study. The exclusion criterion was patients who received previous treatment for OL or OSCC. This study was approved by the Research Ethics Committee in human studies (process number: FOA-01034/2011).

Data and material collection

Demographic and clinicopathological data such as age, sex, anatomical site, tobacco and alcohol consumption were collected from the medical records and summarized in a spreadsheet. Tobacco and alcohol consumption were graded as follows: abstinence, ex-smoker or ex-drinker, light use (up to 10 cigarettes and 1-2 drinks per day), moderate use (11-20 cigarettes and 3-4 drinks per day), and heavy use (more than 20 cigarettes and more than 4 drinks per day)(21). For diagnosis, an incisional biopsy was performed and the tissue was split into two fragments: the first one was fixed in 10% buffered formaldehyde for histopathologic processing and the second one was stored at -80ºC for biomolecular analysis. For saliva collection, patients were instructed to do not ingest

liquids or eating 30 minutes before collection. Each patient spit in a sterile 15 mL centrifugation tube for 10 minutes to obtain at least 5 mL of saliva. The samples were centrifuged at 4ºC, 3.000 rpm for 6 minutes. Subsequently it was stored at -80ºC for future analysis. Blood samples were collected after saliva collection. Twenty milliliters of blood were obtained by venipuncture, placed in a tube containing ethylenediamine tetra-acetic acid (EDTA) and centrifuged immediately at 4ºC, 1.500 rpm for 15 minutes. Plasma was collected and stored at -80ºC for future analysis. FFPE was submitted to hematoxylin and eosin staining and immunohistochemistry for $p16^{Ink4a}$ while fresh tissue, saliva and plasma were submitted to DNA extraction and *Real time* PCR for HPV-16.

DNA Extraction

DNA extraction was performed according to the QIAamp® mini kit DNA extraction kit manufacturer's instructions (QIAGEN, Hilden, Germany). The incubation time with the proteinase of the samples varied: 3 hours for tissues and 10 minutes for plasma/saliva at 56ºC. The remaining steps were the same. DNA obtained from all samples were analyzed at 260/280nm by spectrophotometry and samples with confirmed DNA presence and integrity were stored at -20ºC for posterior analysis.

Detection of HPV-16 by Real Time PCR

Real Time PCR technique was applied using the TaqMan® (Life Technologies®, Carlsbad, USA) in conjunction with the TaqMan® gene expression assays of HPV-16 and 18 (Life Technologies®, Carlsbad, USA) and Taqman® Master mix Universal (Life Technologies®, Carlsbad, USA) following the manufacturer's protocol. The reaction was performed in the standard time stipulated by the Step-One Plus equipment (Life

Technologies®, Carlsbad, USA). SiHa (cell line with HPV-16 integrated) was used as exogenous positive control.

$p16^{Ink4a}$ Immunohistochemistry

Four-μm-thick tissue sections were deparaffinized and rehydrated. Antigen retrieval was conducted with citrate buffer at 55ºC for 40 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 15 min. Sections were incubated with anti-CDKN2A/$p16^{Ink4a}$ antibody (Abcam, Cambridge, USA) at 1:2500 for 3h at room temperature. Host specie-specific Nichirei's N-Histofine® Simple Stain MAX PO (MULTI) [Nichirei Biosciences inc., Tokyo, Japan] was used as the detection system. Color was revealed by diaminobenzidine (Spring Bioscience, Pleasanton, EUA) and Mayer's hematoxylin was used as counter stain. Cervical squamous cell carcinoma was used as positive control. Immunostaining was analyzed by two independent observers (GM and MC). Nuclear as well as cytoplasmic staining was considered positive. The distribution of $p16^{Ink4a}$ positivity was evaluated by a semiquantitative score (22), as follows: 0: absent (<5% positive cells), 1: weak (5-25% positive cells) and 2: strong (>25% positive cells).

Statistical Analysis

All data were stored in a Microsoft Office Excel (Microsoft, Redmond, USA) spreadsheet and the statistical analyses were performed in GraphPad Prism 7 software (GraphPad Software, San Diego, USA). Normality was tested using the Shapiro-Wilk test. The Chi-square, Fisher exact, or Spearman's correlation tests were used to evaluate associations between groups and categorical variables (clinicopathological, sociodemographic and lifestyle). The results were presented in graphs and a p value of <0.05 was considered statistically significant.

RESULTS

Of the 67 patients diagnosed with OL (Figure 1) included in the study, 37 (55.2%) were male (Table 1). The mean age for men was 57.1 years and, for women, 60.5 years. Most of the patients were elderly (89.5%). The prevalence of OL was higher on buccal mucosa (35.8%) followed by tongue (20.8%). The majority of OL did not present epithelial dysplasia (73.1%). About lifestyle, most patients with OL were classified as current (71.8%) and light smokers (47.8%) smokers. Regarding the alcohol status, 47.4% of the patients were current drinkers and mostly were classified as light drinkers (59.3%, Table 1).

Most IFH (Figure 2) patients (54.5%) were male and 90.9% were elderly (Table 2). The prevalence of IFH was on buccal mucosa (31.8%) followed by alveolar ridge (25%). Mostly of the IFH samples presented low inflammatory infiltrate (52.3%).

p16 ^{Ink4a} expression was predominantly weak (41.8%) in OL (Figure 3) while most of IFH presented strong p16^{Ink4a} expression (68.2%, Figure 4).

DNA extraction from fresh tissue, saliva and plasma were performed in all 67 OL samples. For IFH, 23 fresh tissue, 20 saliva, and 16 plasma blood samples were available for the DNA extraction. HPV-16 DNA was not detected in any sample of OL and IFH (Tables 1-2 and Supplementary Figures 1-3).

Table 1. Sociodemographic, clinicopathologic and lifestyle data, p16 ^{Ink4a} and HPV-16 DNA detection results of patients with OL.

VARIABLES	N	%
Age	Mean: 57.1 (Male) 60.5 (Female)	
Gender		
Male	37	55.2%
Female	30	44.8%
Anatomic location		
Buccal mucosa	24	35.8%
Tongue	14	20.9%
Gingiva	11	16.4%
Mouth floor	5	7.4%
Labial mucosa	4	6%
Hard palate	4	6%
Lip	2	3%
Oropharynx	3	4.5%
Epithelial dysplasia		
Absent	49	73.2%
Mild	10	14.9%
Moderate	7	10.4%
Severe	1	1.5%
Tobacco smoking status*€		
Non-smoker	9	14.1%
Smoker	46	71.8%
Ex-smoker	9	14.1%
Tobacco smoking intensity		
Light	22	47.8%
Moderate	16	34.8%
Severe	8	17.4%
Alcohol drinking status*ŧ		
Non-drinker	17	29.8%
Drinker	27	47.4%
Ex-drinker	13	22.8%
Alcohol drinking intensity		
Light	16	59.3%
Moderate	9	33.3%
Severe	2	7.4%
p16 ^{Ink4a} Expression		
Absent	14	20.9%
Weak	28	41.8%
Strong	25	37.3%
Detection of HPV-16 DNA in fresh tissue		
Negative	67	100%
Positive	0	0%
Detection of HPV-16 DNA in saliva		
Negative	67	100%
Positive	0	0%
Detection of HPV-16 DNA in plasma		
Negative	67	100%
Positive	0	0%

*Excluding patients with missing data

Abbreviations: HPV, Human papillomavirus.

€ Smoking: each category equals (per day): Tobacco consumption were classified as: light (≤ 19 cigarettes/day), moderate (20-39 cigarettes/day) and severe (≥ 40 cigarettes/day).

ŧ Alcoholism: each category equals (per day) as: light (0-2 drinks/day), moderate (3-4 drinks/day) and severe (> 5 drinks/day).

Table 2. Sociodemographic, clinicopathologic and lifestyle data, p16 ^{Ink4a} and HPV-16 DNA detection results of patients with IFH.

VARIABLES	N	%
Age	Mean: 57.3 (Male) 61 (Female)	
Gender		
Male	24	54.5%
Female	20	45.5%
Anatomic location		
Buccal mucosa	14	31.8%
Alveolar ridge	11	25%
Fornix	8	18.2%
Tongue	6	13.6%
Gingiva	2	4.5%
Lip	2	4.5%
Hard palate	1	2.3%
Inflammatory infiltrate		
Absent	5	11.4%
Mild	23	52.3%
Moderate	6	13.6%
Intense	10	22.7%
p16 ^{Ink4a} Expression		
Absent	8	18.2%
Weak	6	13.6%
Strong	30	68.2%
Detection of HPV-16 DNA in fresh tissue		
Negative	24	100%
Positive	0	0%
Detection of HPV-16 in FFPE		
Negative	20	100%
Positive	0	0%
Detection of HPV-16 in saliva		
Negative	20	100%
Positive	0	0%
Detection of HPV-16 DNA in plasma		
Negative	16	100%
Positive	0	0%

*Excluding patients with missing data

Abbreviations: HPV, Human papillomavirus.

€ Smoking: each category equals (per day): Tobacco consumption were classified as: light (≤ 19 cigarettes/day), moderate (20-39 cigarettes/day) and severe (≥ 40 cigarettes/day).

ŧ Alcoholism: each category equals (per day) as: light (0-2 drinks/day), moderate (3-4 drinks/day) and severe (> 5 drinks/day).

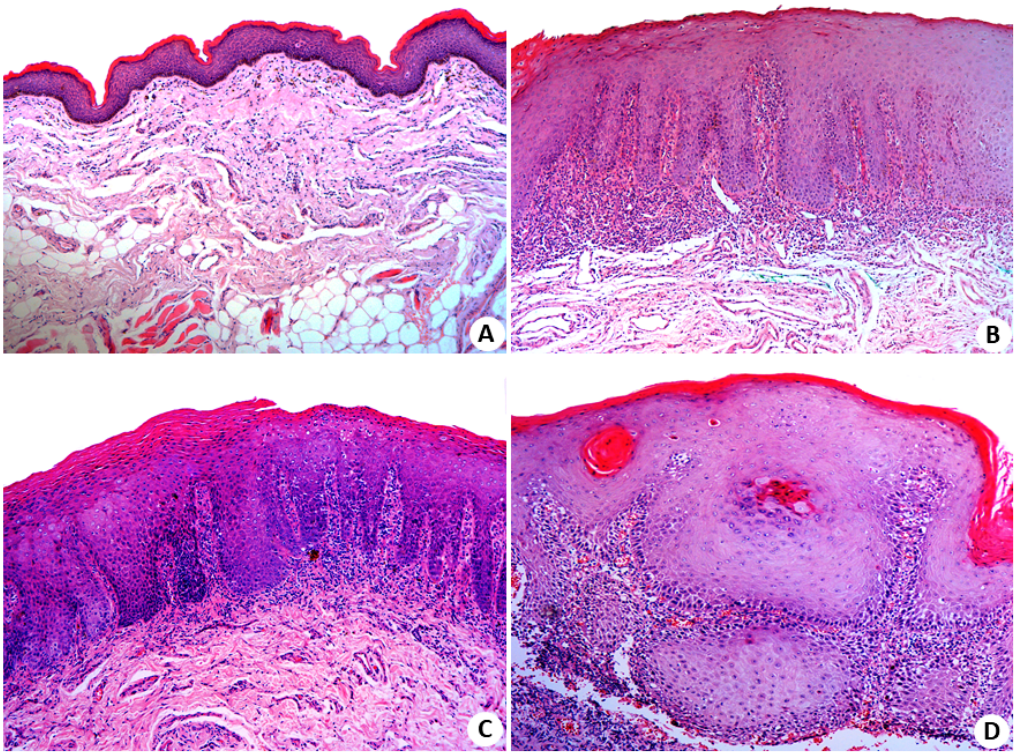


Figure 1. Oral leukoplakia A. absent, with B. mild, C. moderate, and D. severe epithelial dysplasia (H&E, 200X).

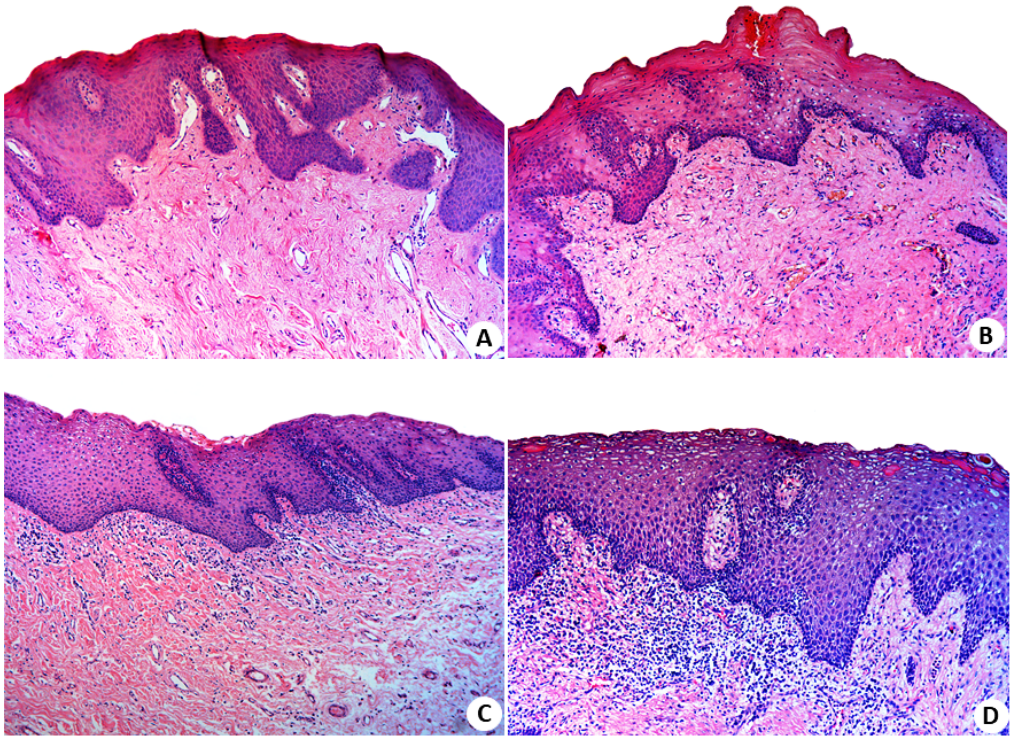


Figure 2. Inflammatory fibrous hyperplasia presenting A. absent, B. mild, C. moderate, and D. intense inflammatory infiltrate (H&E, 200X).

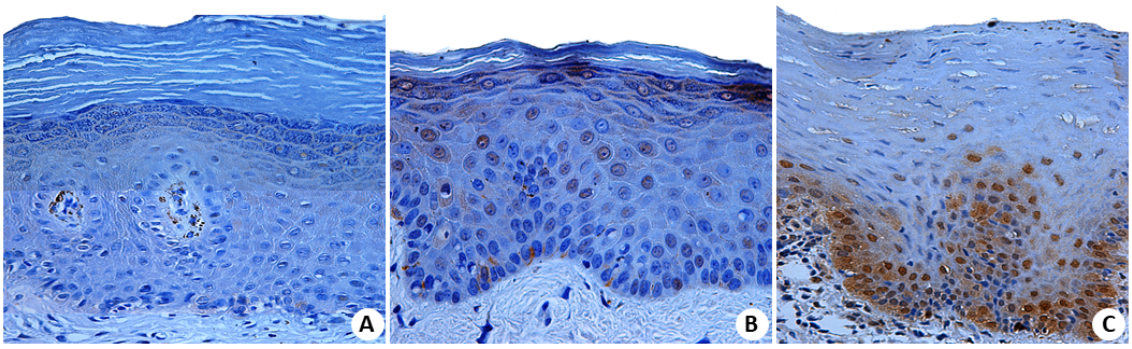


Figure 3. Oral leukoplakia presenting A. absent, B. weak, and C. strong immunostaining for $p16^{Ink4a}$ (IHQ, 200X).

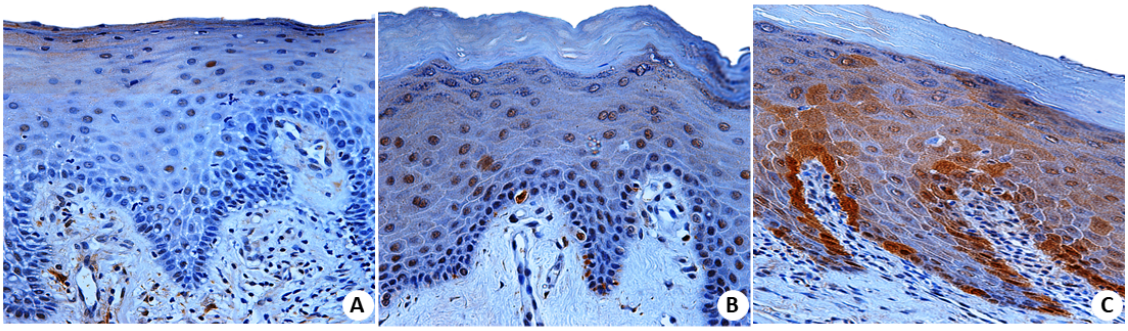


Figure 4. Inflammatory fibrous hyperplasia presenting A. absent, B. weak, and C. strong immunostaining for $p16^{Ink4a}$ (IHQ, 200X).

Most OLs were positive for p16^{Ink4a} (Figure 5). However, the intensity of the immunostaining proportionally increased in OLs with moderate epithelial dysplasia when compared to lesions with absent or mild epithelial dysplasia. The low number of OL with severe dysplasia impaired further analysis. Epithelial dysplasia in OL was not statistically correlated with p16^{Ink4a} intensity (p>0.05).

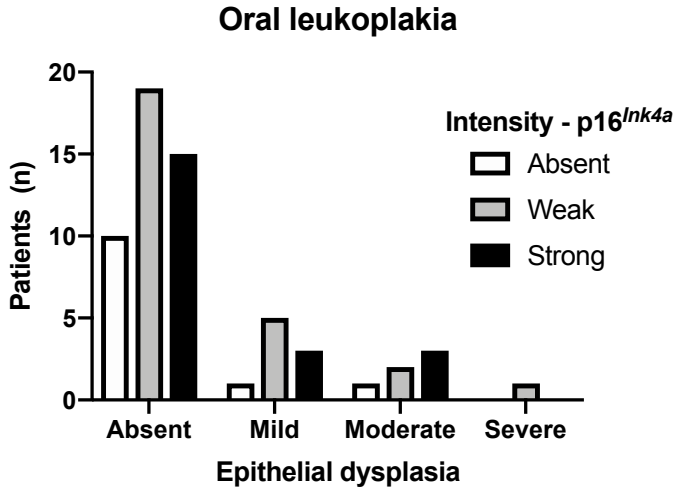


Figure 5. Correlation between epithelial dysplasia and p16 ^{Ink4a} expression in oral leukoplakia. Spearman's correlation test (p=0.569, r= 0.075).

p16^{Ink4a} expression was observed in most (68.1%) of the IFHs regardless the presence of inflammatory infiltrate (p=0.547, r=0.093, Figure 6).

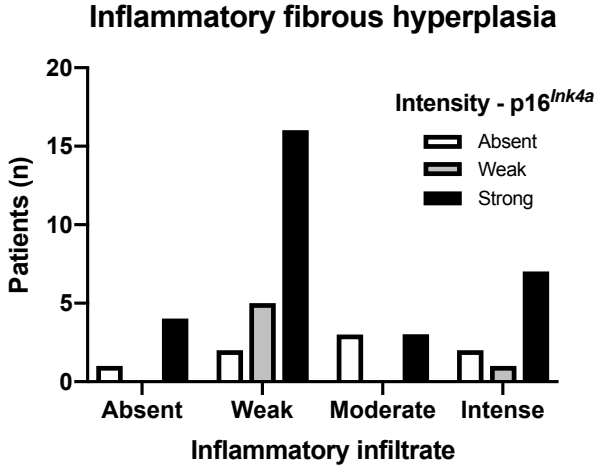


Figure 6. Correlation between the inflammatory infiltrate intensity and p16^{Ink4a} expression in inflammatory fibrous hyperplasia. Spearman's correlation test (p=0.547, r= -0.093).

Figure 7 shows an inverse correlation of $p16^{Ink4a}$ expression if OL and IFH (p=0.001, r=-0.466). While OL patients presented predominantly weak staining for the marker, most IFH patients exhibited strong expression of $p16^{Ink4a}$.

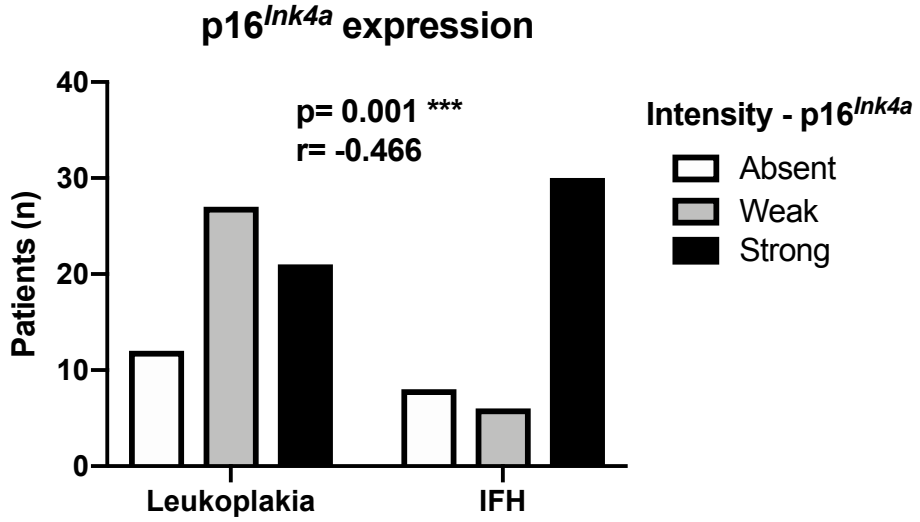


Figure 7. Correlation between the $p16^{Ink4a}$ expression in oral leukoplakia and inflammatory fibrous hyperplasia. Spearman's correlation test.

DISCUSSION

Oral leukoplakia is the most frequent oral potentially malignant disorder with a transformation rate ranging from 0.13 to 34% (23). Besides the classic risk factors associated to the malignant progression of OL, HPV has been suggested to participate in this process. In this study, we found *null* prevalence of HPV-16 in the 67 samples of OL and 20 samples of inflammatory fibrous hyperplasia (IFH) analyzed by real time PCR. Some investigations corroborate our results (2, 17, 24) while others do not (18, 25). The prevalence of HPV in OL is variable and ranges from 0% to 68.7% (18, 26). Thus, the association between HPV and OL is still controversial. It is questionable if HPV can be considered as an etiological or a malignancy risk factor in OPMD or malignant lesions (27). In this context, some research groups identified HR-HPV antigens and viral DNA in OPMD and malignant oral disorders (28).

Immunohistochemistry of $p16^{Ink4a}$ has been routinely employed to detect the presence of HPV-16 in different kinds of neoplasias (29). Since there is a connection between $p16^{Ink4a}$ overexpression and HPV-16 positivity, we also analyzed $p16^{Ink4a}$ expression in OL and IFH. Interestingly, 53/67 OL and 36/44 IFH were positive for $p16^{Ink4a}$.

A fact that may be associated with HPV negativity in OL is association between age (24). In our study, the mean was greater than 55 years (25-82 years). Studies have shown that the prevalence of HPV infection occurs in younger patients (28, 30).

Bhosale *et al.* (31) evaluated the presence of HR-HPV by nPCR and expression of $p16^{Ink4a}$ in leukoplakia samples and found a null prevalence in both analyses. These results match with qPCR results, but not with IHC. But another study, observed a null prevalence of HR-HPV, with low $p16^{Ink4a}$ overexpression (13/74) (24). A hypothesis is that $p16^{Ink4a}$ is involved with the cell cycle and apoptosis (32). In several tumors it can

have its loss or downexpressed to overexpression (33). The major function of $p16^{Ink4a}$ is inhibit cell cycle (phase G0 to S), trying to protect this cell from any type of stress: oxidative stress, contact with drugs (early senescence), inflammation and DNA damage. $p16^{Ink4a}$ overexpression should not directly associated with the presence of HPV (34, 35). Suggested with its location at 9p21 where occurs loss of heterozygosity, defined as allelic loss in a heterozygous locus for a given marker, is known to be related with allelic loss of various tumor suppressor gene in our case, $p16^{Ink4a}$ (36). Such possibility is corroborated by our findings in IFH. IFH is a chronic condition that is associated to long duration trauma (37). It is interesting to observe that the epithelium constantly exposed to a physical injury presented a high expression of $p16^{Ink4a}$, indicating this marker may have a role in the defense mechanisms triggered.

Although several studies document the association of HPV infection with OL, if HPV infection can promote malignant progression of OL, it is still unclear (38). Also, overexpression for $p16^{Ink4a}$ is not limited to HPV positive lesions and therefore is not a reliable marker for the presence of HPV (24, 39).

There are different sources of biological samples that are used to detect HPV: fresh tissue, FFPE tissue and exfoliated cells (40). The gold-standard biological material for HPV detection is fresh tissue but its limited availability due to its need for diagnostic and histological grading is an important (41). Thus, other sources of biological material have been investigated, such as saliva and plasma (42). A recent study by Qureishi *et al.* (43) found 72.2% sensitivity and 90% specificity for HPV detection in saliva by PCR when compared to IHC for p16 and HPV DNA in situ hybridization in oropharyngeal cancer. These results suggest the potential of a non-invasive method to detect HPV without surgical intervention.

For HPV detection, there is a variety of methods to detection (44). Abreu *et al.* (45) showed in a review molecular-biology techniques for HPV detection. From lowest to highest sensitivity and accuracy are: Nucleic acids hybridization assays (Sothern blot, *In situ* hybridization and Dot blot hybridization); signal amplification assays and nucleic acids amplification assays (Microarray, Conventional PCR, *Real time* PCR, Linear Array). Benefits using this technique are the high sensitivity as nucleic acids can be detected in very small concentrations, very reproducible and less false-positives results (46).

Farah *et al.* (47) related malignant transformation in lesions with epithelial dysplasia in 13.6% to 36.4% of the cases. In our study, 75% of the patients did not present epithelial dysplasia, and just one patient presented severe epithelial dysplasia. But we cannot correlate with the presence of HR-HPV with a weak $p16^{Ink4a}$ expression. And in our study, did not showed a tendency of the major degree of dysplasia, the higher expression of $p16^{Ink4a}$ (Figure 1). But another study observed an association between the major degree of dysplasia, the higher is $p16^{Ink4a}$ expression (48).

In this study, we compared the presence of HR-HPV and $p^{16Ink4a}$ expression with another lesion, which is not potentially malignant and not associated with the presence of HR-HPV and is the first study who reported HR-HPV detection in fresh tissue, plasma blood and saliva in OL and IFH, and comparing with FFPE for $p16^{Ink4a}$ IHC.

The findings in our study showed the HR-HPV was not identified in fresh tissue, plasma blood and saliva for OL and IFH. And the lack of presence of HR-HPV does not correlate with $p16^{Ink4a}$ immunoexpression in FFPE in both groups. Further studies are necessary to investigate the association between HR-HPV negativity and $p16^{Ink4a}$ overexpression.

ACKNOWLEDGMENTS

To CAPES (Coordination for the improvement of Higher Level -or Education- Personnel for my master's fellowship.

CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflict of interest regarding this work.

FUNDING

This study was supported by the São Paulo Research Foundation (FAPESP). Grant number: 2016/12982-0.

REFERENCES

1. Mahmoodi P, Fani M, Rezayi M, Avan A, Pasdar Z, Karimi E, et al. Early detection of cervical cancer based on high-risk HPV DNA-based genosensors: A systematic review. Biofactors. 2019;45(2):101-17.
2. Chen XJ, Sun K, Jiang WW. Absence of high-risk HPV 16 and 18 in Chinese patients with oral squamous cell carcinoma and oral potentially malignant disorders. Virol J. 2016;13:81.
3. Hübbers CU, Akgül B. HPV and cancer of the oral cavity. Virulence. 2015;6(3):244-8.
4. Flanagan CE, Rhodus NL, Cole KA, Szabo E, Ondrey FG. Correlation analysis of oral lesion sizes by various standardized criteria. Am J Otolaryngol. 2016;37(6):502-6.
5. So KA, Hong JH, Lee JK. Human Papillomavirus Prevalence and Type Distribution Among 968 Women in South Korea. J Cancer Prev. 2016;21(2):104-9.
6. Faridi R, Zahra A, Khan K, Idrees M. Oncogenic potential of Human Papillomavirus (HPV) and its relation with cervical cancer. Virol J. 2011;8:269.
7. Rantshabeng P, Kasvosve I, Ndlovu A, Gaseitsiwe S, Moyo S. Prevalence of high-risk human papilloma virus in women with high-grade squamous cell intraepithelial lesions in Botswana using Abbott RealTime HPV assay. PLoS One. 2019;14(1):e0211260.
8. Xu HH, Wang K, Feng XJ, Dong SS, Lin A, Zheng LZ, et al. Prevalence of human papillomavirus genotypes and relative risk of cervical cancer in China: a systematic review and meta-analysis. Oncotarget. 2018;9(20):15386-97.
9. LeConte BA, Szaniszlo P, Fennewald SM, Lou DI, Qiu S, Chen NW, et al. Differences in the viral genome between HPV-positive cervical and oropharyngeal cancer. PLoS One. 2018;13(8):e0203403.
10. Agalliu I, Gapstur S, Chen Z, Wang T, Anderson RL, Teras L, et al. Associations of Oral α-, β-, and γ-Human Papillomavirus Types With Risk of Incident Head and Neck Cancer. JAMA Oncol. 2016;2(5):599-606.
11. Dok R, Nuyts S. HPV Positive Head and Neck Cancers: Molecular Pathogenesis and Evolving Treatment Strategies. Cancers (Basel). 2016;8(4).

12. Warnakulasuriya S, Johnson NW, van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med. 2007;36(10):575-80.

13. Villa A, Woo SB. Leukoplakia-A Diagnostic and Management Algorithm. J Oral Maxillofac Surg. 2017;75(4):723-34.

14. Bewley AF, Farwell DG. Oral leukoplakia and oral cavity squamous cell carcinoma. Clin Dermatol. 2017;35(5):461-7.

15. Porter S, Gueiros LA, Leão JC, Fedele S. Risk factors and etiopathogenesis of potentially premalignant oral epithelial lesions. Oral Surg Oral Med Oral Pathol Oral Radiol. 2018;125(6):603-11.

16. Panneerselvam K, Rameshkumar A, Rajkumar K, Ramadoss R. Detection of human papillomavirus 16 and 18 in patients with oral squamous cell carcinoma and potentially malignant oral disorders in South Indian population: A pilot study. J Cancer Res Ther. 2019;15(3):571-5.

17. Saghravanian N, Ghazi N, Meshkat Z, Mohtasham N. Human Papillomavirus in Oral Leukoplakia, Verrucous Carcinoma, Squamous Cell Carcinoma, and Normal Mucous Membrane. Oman Med J. 2015;30(6):455-60.

18. Ferreira LL, Biasoli É, Bernabé DG, Nunes CM, Miyahara GI. Plasma HPV DNA is detectable in oral leukoplakia patients. Pathol Res Pract. 2017;213(7):759-65.

19. Duncan LD, Winkler M, Carlson ER, Heidel RE, Kang E, Webb D. p16 immunohistochemistry can be used to detect human papillomavirus in oral cavity squamous cell carcinoma. J Oral Maxillofac Surg. 2013;71(8):1367-75.

20. Awan MS, Irfan B, Zahid I, Mirza Y, Ali SA. Comparison of Polymerase Chain Reaction and Immunohistochemistry Assays for Analysing Human Papillomavirus Infection in Oral Squamous Cell Carcinoma. J Clin Diagn Res. 2017;11(6):XC10-XC3.

21. Harris SL, Kimple RJ, Hayes DN, Couch ME, Rosenman JG. Never-smokers, never-drinkers: unique clinical subgroup of young patients with head and neck squamous cell cancers. Head Neck. 2010;32(4):499-503.

22. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, et al. Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer. 2001;92(2):276-84.

23. Warnakulasuriya S, Ariyawardana A. Malignant transformation of oral leukoplakia: a systematic review of observational studies. J Oral Pathol Med. 2016;45(3):155-66.

24. Sundberg J, Korytowska M, Burgos PM, Blomgren J, Blomstrand L, DE Lara S, et al. Combined Testing of p16 Tumour-suppressor Protein and Human Papillomavirus in Patients With Oral Leukoplakia and Oral Squamous Cell Carcinoma. Anticancer Res. 2019;39(3):1293-300.

25. Pierangeli A, Cannella F, Scagnolari C, Gentile M, Sciandra I, Antonelli G, et al. Frequent detection of high human papillomavirus DNA loads in oral potentially malignant disorders. Clin Microbiol Infect. 2016;22(1):95.e9-.e15.

26. Bhargava A, Shakeel M, Srivastava AN, Raza TS, Rizvi S, Varshney P. Role of human papilloma virus in oral leukoplakia. Indian J Cancer. 2016;53(1):206-9.

27. Speight PM, Khurram SA, Kujan O. Oral potentially malignant disorders: risk of progression to malignancy. Oral Surg Oral Med Oral Pathol Oral Radiol. 2018;125(6):612-27.

28. Candotto V, Lauritano D, Nardone M, Baggi L, Arcuri C, Gatto R, et al. HPV infection in the oral cavity: epidemiology, clinical manifestations and relationship with oral cancer. Oral Implantol (Rome). 2017;10(3):209-20.

29. Mahajan A. Practical issues in the application of p16 immunohistochemistry in diagnostic pathology. Hum Pathol. 2016;51:64-74.

30. Gillison ML, Broutian T, Pickard RK, Tong ZY, Xiao W, Kahle L, et al. Prevalence of oral HPV infection in the United States, 2009-2010. JAMA. 2012;307(7):693-703.

31. Bhosale PG, Pandey M, Desai RS, Patil A, Kane S, Prabhash K, et al. Low prevalence of transcriptionally active human papilloma virus in Indian patients with HNSCC and leukoplakia. Oral Surg Oral Med Oral Pathol Oral Radiol. 2016;122(5):609-18.e7.

32. Roussel MF. The INK4 family of cell cycle inhibitors in cancer. Oncogene. 1999;18(38):5311-7.

33. Ayhan S, Isisag A, Saruc M, Nese N, Demir MA, Kucukmetin NT. The role of pRB, p16 and cyclin D1 in colonic carcinogenesis. Hepatogastroenterology. 2010;57(98):251-6.

34. Romagosa C, Simonetti S, López-Vicente L, Mazo A, Lleonart ME, Castellvi J, et al. p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene. 2011;30(18):2087-97.

35. LQ Y, X X, CX L, WY W, XM S, ZT Z, et al. Human papillomavirus genotypes and p16 expression in

oral leukoplakia and squamous cell carcinoma. Int J Clin Exp Pathol2019. p. 1022-8.
36. Kim JH, Choi KY, Lee DJ, Rho YS, Jo SJ. Loss of Heterozygosities in Five Tumor Suppressor Genes (FHIT Gene, p16, pRb, E-Cadherin and p53) in Thyroid Tumors. Clin Exp Otorhinolaryngol. 2014;7(1):53-8.
37. Shukla P, Dahiya V, Kataria P, Sabharwal S. Inflammatory hyperplasia: From diagnosis to treatment. J Indian Soc Periodontol. 2014;18(1):92-4.
38. Feller L, Lemmer J. Oral Leukoplakia as It Relates to HPV Infection: A Review. Int J Dent. 2012;2012:540561.
39. Stephen JK, Divine G, Chen KM, Chitale D, Havard S, Worsham MJ. Significance of p16 in Site-specific HPV Positive and HPV Negative Head and Neck Squamous Cell Carcinoma. Cancer Clin Oncol. 2013;2(1):51-61.
40. Venuti A, Paolini F. HPV detection methods in head and neck cancer. Head Neck Pathol. 2012;6 Suppl 1:S63-74.
41. Ahn SM, Chan JY, Zhang Z, Wang H, Khan Z, Bishop JA, et al. Saliva and plasma quantitative polymerase chain reaction-based detection and surveillance of human papillomavirus-related head and neck cancer. JAMA Otolaryngol Head Neck Surg. 2014;140(9):846-54.
42. Wang Y, Springer S, Mulvey CL, Silliman N, Schaefer J, Sausen M, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Sci Transl Med. 2015;7(293):293ra104.
43. Qureishi A, Ali M, Fraser L, Shah KA, Møller H, Winter S. Saliva testing for human papilloma virus in oropharyngeal squamous cell carcinoma: A diagnostic accuracy study. Clin Otolaryngol. 2018;43(1):151-7.
44. Burd EM. Human Papillomavirus Laboratory Testing: the Changing Paradigm. Clin Microbiol Rev. 2016;29(2):291-319.
45. Abreu AL, Souza RP, Gimenes F, Consolaro ME. A review of methods for detect human Papillomavirus infection. Virol J. 2012;9:262.
46. Hans R, Marwaha N. Nucleic acid testing-benefits and constraints. Asian J Transfus Sci. 2014;8(1):2-3.
47. Farah CS, Woo SB, Zain RB, Sklavounou A, McCullough MJ, Lingen M. Oral cancer and oral potentially malignant disorders. Int J Dent. 2014;2014:853479.
48. Angiero F, Berenzi A, Benetti A, Rossi E, Del Sordo R, Sidoni A, et al. Expression of p16, p53 and Ki-67 proteins in the progression of epithelial dysplasia of the oral cavity. Anticancer Res. 2008;28(5A):2535-9.

Anexo A

Parecer consubstanciado do CEP- FOA/UNESP

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

COMITÊ DE ÉTICA EM PESQUISA

CERTIFICADO

Certificamos que o Projeto ***"Detecção do HPV por nPCR em leucoplasias bucais: Estudo caso-controle"***, sob a responsabilidade do Pesquisador **GLAUCO ISSAMU MIYAHARA**, está de acordo com os Princípios Éticos em Pesquisa e foi aprovado em 27/05/2011, de acordo com o Processo FOA-01034/2011.

Araçatuba, 06 de junho de 2011.

ALESSANDRA MARCONDES ARANEGA
Vice-Coordenadora do CEP

AMA/abro.

Faculdade de Odontologia e Curso de Medicina Veterinária
Rua José Bonifácio, 1193 CEP 16015-050 Araçatuba – SP
Tel (18) 3636-3214 E-mail: cepfoa@unesp.br

Anexo B

Periódico de interesse para submissão

Journal of Oral Pathology and Medicine

Edited By: Peter Brennan

Impact factor:2.237

ISI Journal Citation Reports © Ranking: 2017:22/91 (Dentistry, Oral Surgery & Medicine)

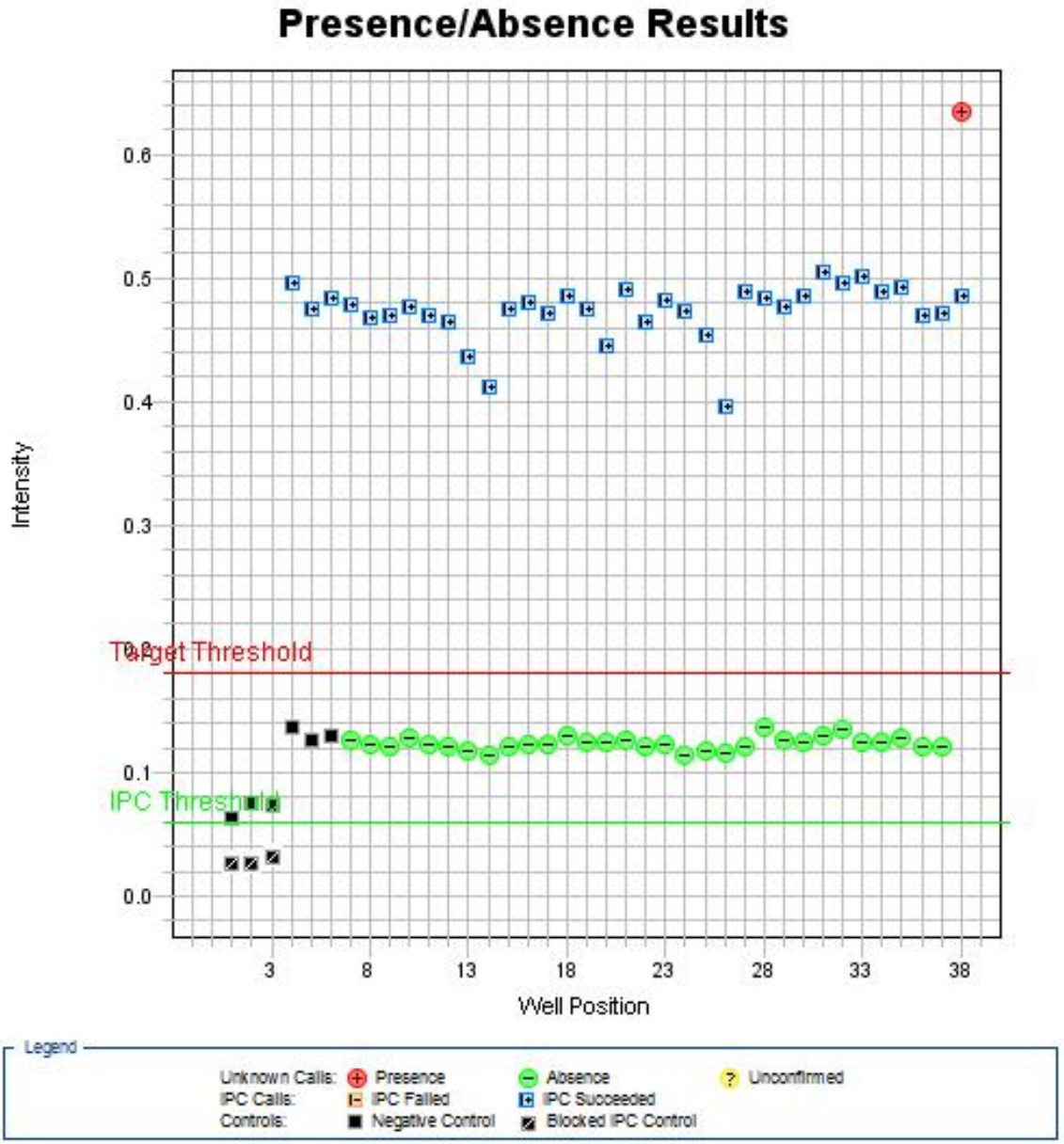
ISI Journal Citation Reports © Ranking: 2017:34/79 (Pathology)

Online ISSN:1600-0714

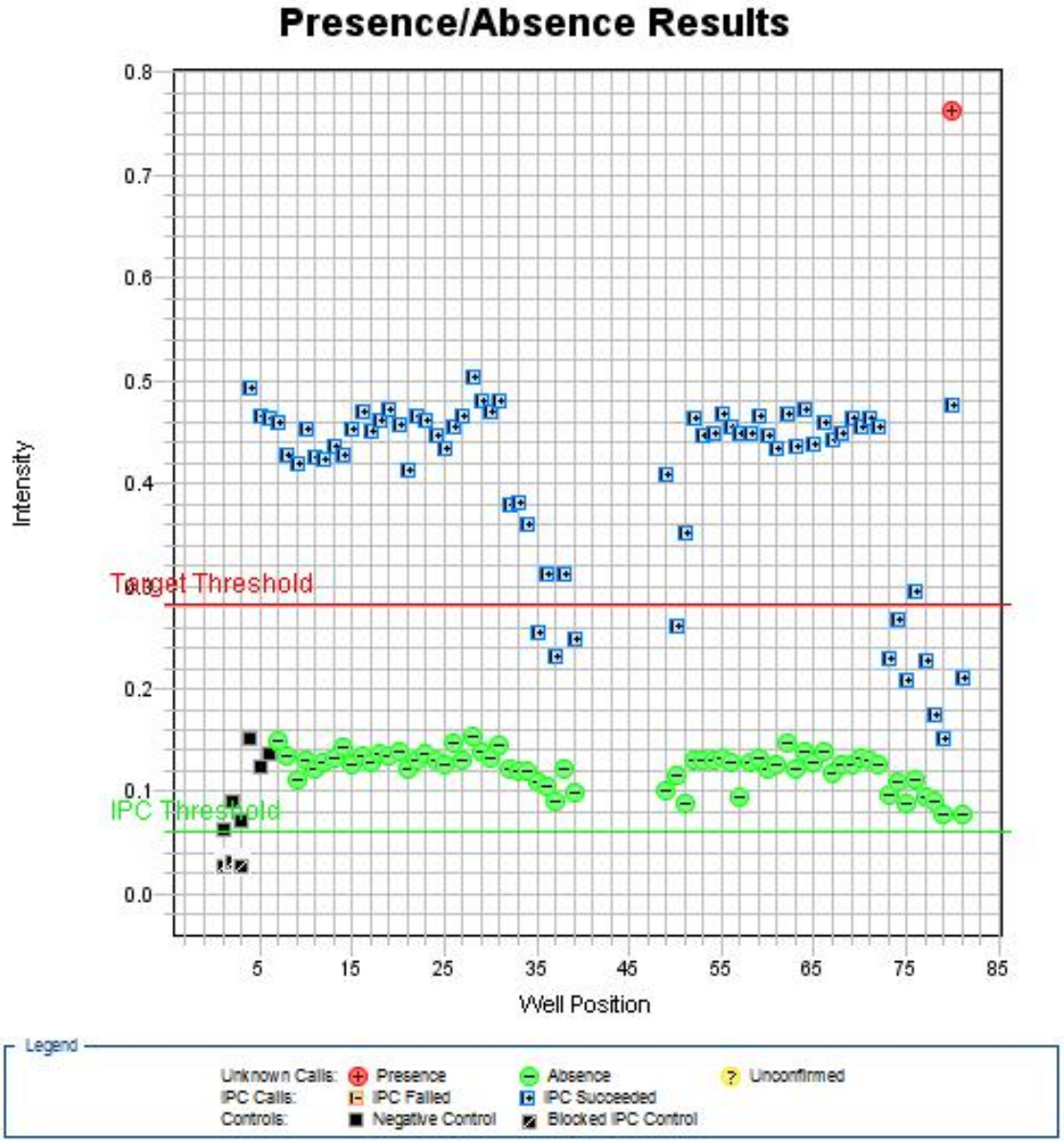
Qualis CAPES: A2

Anexo C

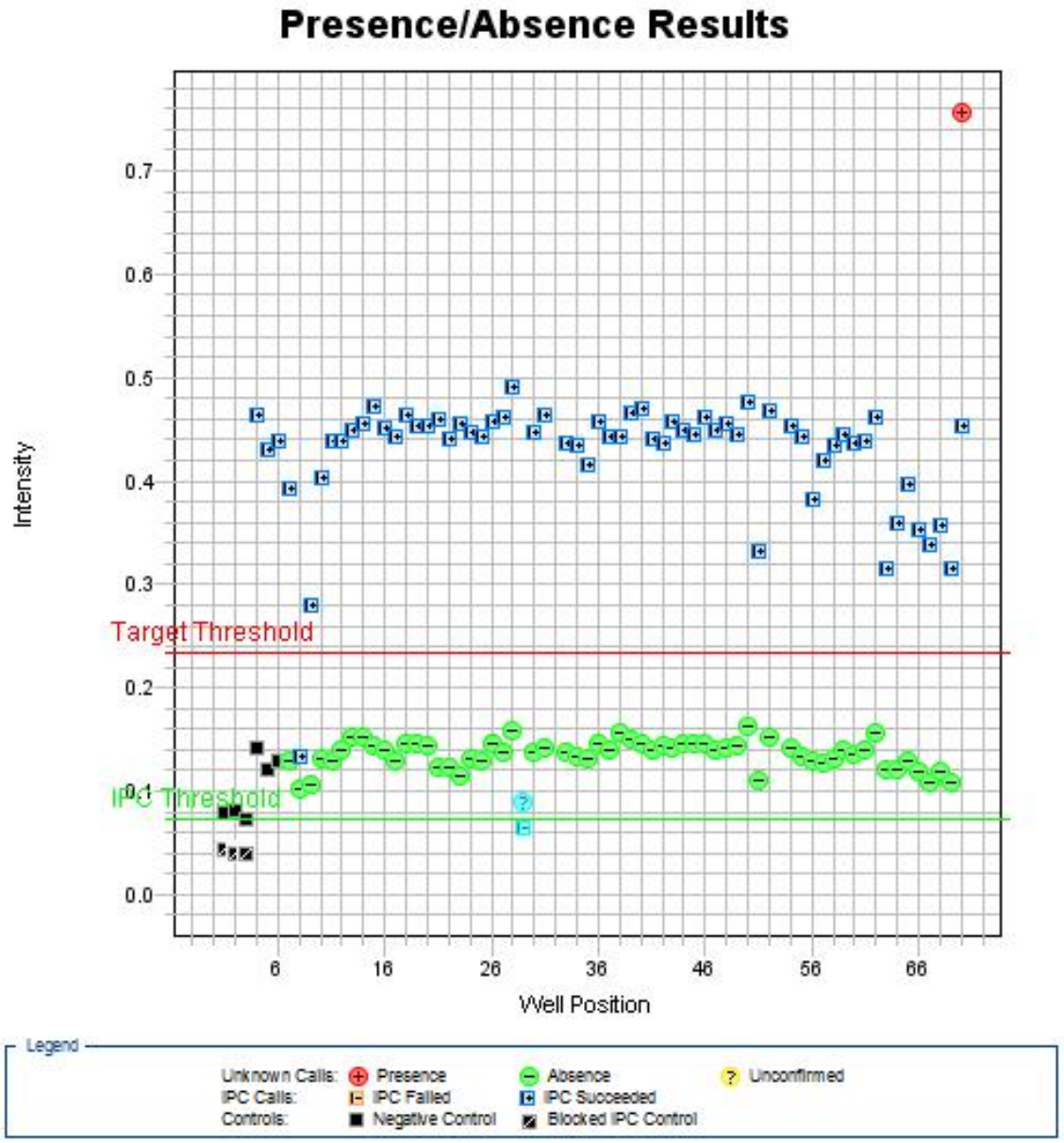
Gráficos dos ensaios de presença e ausência para o DNA do HPV-16 pela *Real time* PCR



Supplementary Figure 1. Qualitative *Real Time* PCR results in fresh tissue of patients with OL. Positive control (red) SiHa cell line sample, absence of viral DNA (green), positive internal control (IPC) for each sample (blue) and negative control (black).



Supplementary Figure 2. Qualitative *Real Time* PCR results in saliva and plasma blood of patients with OL. Positive control (red) SiHa cell line sample, absence of viral DNA (green), positive internal control (IPC) for each sample (blue) and negative control (black).



Supplementary Figure 3. Qualitative *Real Time* PCR results in fresh tissue, FFPE, saliva and plasma of patients with IFH. Positive control (red) SiHa cell line sample, absence of viral DNA (green), positive internal control (IPC) for each sample (blue) and negative control (black).