Andressa Fernanda Paza Miguel

# ANÁLISE DO INFILTRADO INFLAMATÓRIO E DOS MARCADORES IMUNOISTOQUÍMICOS MMP-9, TIMP-1, TIMP-2 E VIMENTINA NA PROGRESSÃO DAS DISPLASIAS EPITELIAIS ORAIS.

Dissertação submetida ao Programa de Pós-Graduação em Odontologia da Universidade Federal de Santa Catarina para a obtenção do Grau de Mestre em Odontologia.

Orientador: Prof. Drª. Elena Correa Riet Rivero

Florianópolis

2018

Ficha de identificação da obra elaborada pelo autor
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Miguel, Andressa Fernanda Paza
ANÁLISE DO INFILTRADO INFLAMATÓRIO E DOS MARCADORES IMUNOISTOQUÍMICOS MMP-9, TIMP-1, TIMP
2 E VIMENTINA NA PROGRESSÃO DAS DISPLASIAS EPITELIAIS ORAIS. / Andressa Fernanda Paza Miguel ; orientador, Elena Correa Riet Rivero, 2018.
111 p.

Dissertação (mestrado) - Universidade Federal de Santa Catarina, Centro de Ciências da Saúde, Programa de Pós-Graduação em Odontologia, Florianópolis, 2018.

Inclui referências.

1. Odontologia. 2. Displasia epitelial. 3. Inflamação. 4. Vimentina. 5. Metaloproteinases. I. Rivero, Elena Correa Riet. II. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Odontologia. III. Título.

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**ANÁLISE DO INFILTRADO INFLAMATÓRIO E DOS MARCADORES IMUNOISTOQUÍMICOS MMP-9, TIMP-1, TIMP-2 E VIMENTINA NA PROGRESSÃO DAS DISPLASIAS EPITELIAIS ORAIS.**

Esta Dissertação foi julgada adequada para obtenção do Título de "Mestre em Odontologia" e aprovada em sua forma final pelo Programa de Pós-Graduação em Odontologia.

Florianópolis, 13 de setembro de 2018.

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Prof. Drª. Elena Correa Riet Rivero

Coordenador do Curso

**Banca Examinadora:**

_____

Prof.ª Dr.ª Elena Correa Riet Rivero
Orientadora
Universidade Federal de Santa Catarina

_____

Prof.ª Dr.ª Kamile Leonardi Dutra
Universidade Federal de Santa Catarina

_____

Prof. Dr. Filipe Ivan Daniel
Universidade Federal de Santa Catarina

Este trabalho é dedicado à
minha amada família.

*AGRADECIMENTOS*

A Deus, por seu amor incondicional e sua eterna bondade, que me permitiram conquistar este sonho.

A minha orientadora e mestre, Profª. Elena, por ter me acolhido e me ensinado tanto nesse período. Com certeza não teria chegado tão longe sem o seu apoio.

Aos membros da banca examinadora, por terem aceitado o convite e disponibilizado seu tempo. A qualidade final deste trabalho não seria possível sem a valorosa contribuição de vocês.

A minha família, em especial meu marido Paulo André e minha mãe Nely, por todo o amor e apoio em todos os momentos da minha vida.

Aos colegas da Pós-Graduação, por estarem sempre prontos a ajudar, sem qualquer julgamento, criando um ambiente seguro e prazeroso de trabalho.

# RESUMO

A presença de inflamação no tecido conjuntivo de lesões potencialmente malignas da cavidade bucal cria um ambiente rico em mediadores químicos da inflamação, os quais poderiam contribuir para a progressão maligna. Além disso, durante a carcinogênese o infiltrado inflamatório poderia promover invasão e metástase ao induzir a expressão de enzimas degradadoras de matriz, como a MMP-9, e a ocorrência de transição epitélio-mesenquimal. Diante disso, o objetivo deste trabalho foi avaliar se a intensidade do infiltrado inflamatório e a expressão imunoistoquímica de MMP-9, TIMP-1 e 2 e de Vimentina (VIM) teriam influência na progressão de displasias epiteliais orais (DE) para carcinoma epidermoide bucal (CEB). A amostra foi composta de 66 casos de DE, 28 CEB e 29 de epitélio não neoplásico (ENN), as quais foram submetidas à análise imunoistoquímica para a detecção de anticorpos anti-MMP-9, TIMP-1 e 2 e VIM. A intensidade de infiltrado inflamatório foi avaliada no tecido conjuntivo adjacente ao epitélio. A expressão de MMP-9 foi maior no tecido conjuntivo e no epitélio de DE e CEB do que em ENN, e teve correlação com a expressão de TIMP-1. A inflamação aumentou com a progressão de DE para CEB e teve correlação com a expressão de VIM ($p=0.000$). A inflamação parece estar envolvida na progressão de DE para CEB, o que pode estar relacionado com a indução de transição epitélio-mesenquimal.

**Palavras-chave:** Leucoplasia; Carcinoma de Células Escamosas; Metaloproteinase 9 de matriz; Inibidor tecidual de Metaloproteinase; Inflamação; Vimentina.

## ABSTRACT

The aim of this study was to determine if the intensity of the inflammatory infiltrate and the immunohistochemical expression of MMP-9, TIMP-1, TIMP-2, and vimentin correlate with the progression of oral potentially malignant disorders (OPMD) towards oral squamous cell carcinoma. The sample was composed of 66 cases of epithelial dysplasia, 27 of oral cancer and 28 of non-neoplastic epithelium. Samples were subjected to immunohistochemical detection of MMP-9, TIMP-1, TIMP-2, and vimentin. The number of inflammatory cells was assessed in the underlying connective tissue. The expression of MMP-9 was higher in the connective tissue of epithelial dysplasia and oral cancer when compared to non-neoplastic epithelium. TIMP-2 expression in the epithelium and connective tissue increased from non-neoplastic epithelium to epithelial dysplasia. None of the markers correlated with the severity of dysplasia. Inflammation increased with the progression of dysplasia to oral cancer and this difference was statistically significant ($p=0.004$). Moreover, the intensity of inflammatory infiltrate positively correlated with the expression of vimentin ($p=0.000$). Inflammation seems to be involved in the progression of OPMD to cancer, and this could be through the triggering of epithelial-mesenchymal transition.

**KEYWORDS:** Oral Potentially Malignant disorders; Oral Squamous cell carcinoma; Inflammation; MMP-9; Vimentin; Tissue Inhibitor of Metalloproteinases.

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## LISTA DE ABREVIATURAS E SIGLAS

**AC:** Actinic cheilitis

**CA:** California

**CEB:** Carcinoma epidermoide de boca

**CEL:** Carcinoma epidermoide de lábio

**CEPSH:** Comitê de ética em Pesquisa com Seres Humanos

**DAB:** Diaminobenzidina

**DE:** Displasia epitelial

**ECM:** Extracellular matrix

**ED:** Epithelial dysplasia

**EMT:** Transição epitélio-mesenquimal (Epithelial mesenchymal transition)

**EN:** Epitélio normal

**ENN:** Epitélio não neoplásico

**EUA:** Estados Unidos da América

**H2O2:** Peróxido de Hidrogênio

**HPV***:* Vírus do papiloma humano

**HR:** High Risk

**LR:** Low Risk

**LSCC:** Lip squamous cell carcinoma

**MD:** Maryland

**MEC:** Matriz extracelular

**MMP-1:** Metaloproteinase 1 de matriz

**MMP-2:** Metaloproteinase 2 de matriz

**MMP-3:** Metaloproteinase 3 de matriz

**MMP-9:** Metaloproteinase 9 de matriz

**MMPs:** Metaloproteinases de matriz (Matrix metalloproteinases)

**MO:** Missouri

**MT1-MMP**: Metaloproteinase 1 de matriz associada a membrana

**NNE:** Non-neoplastic epithelium

**NF-κβ:** Nuclear factor kappa beta

**NIH:** National Institute of Health

**OMS:** Organização Mundial da Saúde

**OPMD:** Oral potentially malignant disorder

**OSCC:** Oral squamous cell carcinoma

**PBS:** Solução Tampão Salina (Phosphate buffered saline)

**QA:** Queilite actínica

**SCC:** Squamous cell carcinoma

**SD:** Standard Deviation

**TAM:** Macrófagos associados a tumores (Tumor associated macrophages)

**TGF-ß1**: Fator de crescimento transformador beta 1 (Transforming growth fator beta 1)

**TIMP:** Inibidor tecidual de metaloproteinase de matriz (Tissue inhibitor of metalloproteinases)

**TIMP-1:** Inibidor tecidual de metaloproteinase de matriz-1

**TIMP-2:** Inibidor tecidual de metaloproteinase de matriz-2

**TIMP-3:** Inibidor tecidual de metaloproteinase de matriz-3

**TIMP-4:** Inibidor tecidual de metaloproteinase de matriz-4

**TNF-α:** Tumor necrosis factor alpha

**UFSC:** Universidade Federal de Santa Catarina

**USA:** United States of America

**VIM:** Vimentina

**WHO:** World Health Organization

## SUMÁRIO

# 1 INTRODUÇÃO

O microambiente tumoral é uma rede de comunicação complexa caracterizada por um *crosstalk* entre células neoplásicas e células não neoplásicas do estroma. A população celular do estroma inclui fibroblastos, células endoteliais, pericitos, células-tronco e células inflamatórias. Estas células não neoplásicas dão condições para a progressão tumoral (Feller *et al.*, 2013). As células inflamatórias associadas ao ambiente tumoral incluem linfócitos T e B, neutrófilos, células dendríticas e macrófagos (Coussens e Werb, 2002; Feller *et al.*, 2013; Bhome *et al.*, 2015). Estas células produzem um arsenal de citocinas, quimiocinas, fatores de crescimento e proteinases que mantém um estado inflamatório prolongado, que pode promover crescimento, invasão e metástase tumoral (Feller *et al.*, 2013).

Em 1863, pela primeira vez, Virchow associou a presença de infiltrado inflamatório com neoplasias, afirmando que lesões malignas se desenvolveriam em sítios com inflamação crônica. Atualmente, sabe-se, de fato, da associação da inflamação crônica com o desenvolvimento de certos tipos de câncer, como o carcinoma cervical e gástrico (Balkwill e Mantovani, 2001) O câncer já foi descrito como

uma “ferida que não cicatriza”, uma vez que apresentaria em seu estroma características semelhantes às de um tecido que está em processo de cicatrização. A diferença seria que um processo de reparo em condições normais é autolimitado, enquanto que as células neoplásicas e do estroma de uma lesão maligna seriam capazes de perpetuar esse processo, por meio da produção de um arsenal próprio de citocinas (Dvorak, 1986).

A presença de mediadores da inflamação, como citocinas e fatores de crescimento, dá condições para o desenvolvimento tumoral por estimular a angiogênese e a proliferação celular. As quimiocinas, que são responsáveis pela quimiotaxia, permitem a chegada de mais células inflamatórias que, por sua vez, produzirão mais citocinas, fatores de crescimento e enzimas, amplificando a resposta inflamatória. Dentre as células inflamatórias, os macrófagos associados a tumores (do inglês,TAM) são a principal fonte de citocinas e fatores de crescimento no ambiente tumoral. Eles são responsáveis também pela produção de enzimas envolvidas na degradação da matriz extracelular (MEC) (Coussens e Werb, 2002). A MEC representa uma barreira física contra a migração celular, cuja quebra é uma das primeiras etapas importantes para a invasão e metástase. As

metaloproteinases (MMPs) são as principais enzimas responsáveis por essa tarefa (Hua *et al.*, 2011)

Além dos macrófagos, as próprias células neoplásicas são capazes de produzir MMPs, como foi descrito pela primeira vez por Jones e DeClerck em 1980 (Jones e Declerck, 1980). As MMPs são enzimas que exercem múltiplas funções no processo de carcinogênese. Seu papel na progressão de displasias epiteliais para carcinoma ainda não está bem estabelecido e têm sido objeto de estudo (Jordan *et al.*, 2004; Barros *et al.*, 2011; Ayva *et al.*, 2013; Lee e Kim, 2013; Tamamura *et al.*, 2013; Bajracharya *et al.*, 2014; Abdelazeem e El-Sayed, 2015; Bianco *et al.*, 2015; De Oliveira Poswar *et al.*, 2015). Para que ocorra a invasão do tecido conjuntivo, momento que marca a progressão para carcinoma, precisa haver o rompimento da membrana basal do epitélio, e as MMP-2 e 9 desempenham um papel importante neste evento (Hua *et al*., 2011). Após a remoção da barreira entre epitélio e conjuntivo, as células epiteliais precisam migrar em direção à lâmina própria, iniciando o processo de invasão e marcando a transição para carcinoma invasivo. Para que este processo ocorra, as células epiteliais precisam passar por mudanças em seu fenótipo, como perda da adesão e ganho

de motilidade, evento conhecido como transição epitélio-mesenquimal (do inglês, EMT) (Hua *et al*., 2011).

A presença de células inflamatórias e citocinas na lâmina própria de displasias poderiam influenciar as células epiteliais, estimulando sua proliferação e fornecendo condições para que células displásicas invadam o tecido conjuntivo. Entretanto, a influência do processo inflamatório na progressão das displasias epiteliais ainda não está clara.

## 1.2 FUNDAMENTAÇÃO TEÓRICA

Na carcinogênese, alguns agentes como o tabaco são capazes de ativar fatores de transcrição nuclear, os quais dentre muitas funções, irão ativar genes reguladores da inflamação. Dessa maneira, as células neoplásicas são capazes de induzir a resposta inflamatória no ambiente tumoral liberando mediadores da inflamação. Estes irão atrair células inflamatórias, que, por sua vez, irão produzir seu próprio arsenal de citocinas, quimiocinas e fatores de crescimento, amplificando, assim, a resposta inflamatória. Este *crosstalk* entre células neoplásicas e inflamatórias promove a progressão tumoral, pois fornece condições para a proliferação celular, angiogênese, migração e invasão (Feller *et al*., 2013)

Sobre a influência da inflamação no desenvolvimento de carcinomas, Liu *et al*. (2015), estudaram a relação entre HPV e inflamação em amostras de tecido normal, displásico e carcinoma epidermoide bucal. Os autores concluíram que a presença de inflamação crônica, associada à infecção por HPV, poderia contribuir para a carcinogênese orofaríngea, e que a elevada expressão de células progenitoras derivadas da linhagem mielóide, teriam um papel importante durante o processo (Liu *et al.*, 2015). Estas células são precursoras das células dendríticas, macrófagos e granulócitos e são induzidas por citocinas pró-inflamatórias (Feller *et al.*, 2013). Durante a carcinogênese elas promoveriam a progressão tumoral, inibindo a resposta imune inata e específica, responsáveis pela eliminação de células neoplásicas (Ostrand-Rosenberg e Sinha, 2009).

Autores como Jakobsson (1973) e Bryne (1998), atribuíram à inflamação um papel de proteção quando em seus sistemas de gradação de carcinomas, consideraram que quanto maior o grau histológico, menor o grau de inflamação encontrado (Jakobsson *et al.*, 1973; Bryne, 1998). Em contrapartida, segundo Piva *et al.* (2011), apesar de existirem evidências de que a falta de inflamação possa estar associada à um perfil mais agressivo de tumores, o contrário não é verdadeiro. Dessa maneira, a inflamação poderia exercer

diferentes papéis, em diferentes estágios da carcinogênese (Piva *et al.*, 2011). Nas displasias, por exemplo, a presença de infiltrado inflamatório poderia, a princípio, desempenhar uma função protetora. Entretanto, durante a progressão das displasias para carcinoma, a manutenção prolongada do estimulo inflamatório provocaria mudanças no perfil de citocinas presentes, favorecendo a transformação maligna (Piva *et al.*, 2013).

Sobre a influência da inflamação em displasias epiteliais bucais, Mashhadiabbas e Fayazi-Boroujeni (2017) investigaram se alterações na densidade vascular e a intensidade de infiltrado inflamatório em leucoplasias estariam relacionadas com o grau de severidade de displasia epitelial. Eles reportaram que lesões com inflamação intensa (>125 células inflamatórias) apresentaram um risco quatro vezes maior de serem severas, do que lesões com leve infiltrado inflamatório (<25 células inflamatórias) (Mashhadiabbas e Fayazi-Boroujeni, 2017). Similarmente, Gannot *et al.* (2002) reportaram um aumento nos linfócitos B em lesões com displasia moderada/severa quando comparadas com lesões apresentando hiperceratose/displasia leve. Logo, a presença de infiltrado inflamatório em lesões potencialmente malignas da boca poderia estar associada com a progressão para carcinoma (Gannot *et al.*, 2002).

Agrawal, Rai e Jain (2011) avaliaram as mudanças ultraestruturais da matriz extracelular (MEC) e sua associação com metástase em carcinoma epidermoide de boca (CEB). Eles estudaram os componentes fibrosos da MEC por meio de microscopia óptica e eletrônica. Para a avaliação das fibras elásticas eles utilizaram o método de Verhoeff-Van Gieson e para as fibras colágenas a coloração tricromo de Masson. Houve uma correlação entre mudanças estruturais na MEC e metástase. Além disso, eles avaliaram a intensidade de infiltrado inflamatório e encontraram uma correlação positiva entre redução de fibras colágenas, inflamação e metástase. Este resultado poderia ser atribuído à capacidade dos linfócitos de produzirem citocinas responsáveis pelo aumento da expressão de enzimas associadas à degradação dos componentes da MEC (Agrawal *et al.*, 2011).

A interação das células neoplásicas com a MEC é crucial na progressão tumoral. Dessa maneira, enzimas envolvidas em sua remodelação desempenham um papel importante na carcinogênese. A MEC representa uma barreira física contra a migração celular, assim, a quebra dessa barreira é uma etapa importante para invasão e metástase. Além de seu papel na degradação da MEC, as MMPs possuem outras atividades envolvidas na progressão

tumoral, como a produção de fragmentos com atividade biológica, gerados a partir da degradação da MEC; estímulo de outras enzimas que degradam MEC, como o ativador de plasminogêneo tipo uroquinase; liberação de fatores pró-angiogênicos; amplificação da atividade inflamatória e regulação da transição epitélio-mesenquimal (Hua *et al*., 2011).

Diante disso, o estudo de inibidores dessas enzimas mostrou-se uma alternativa promissora na terapia contra o câncer. Entretanto, as pesquisas clínicas falharam em confirmar a eficácia terapêutica de tais medicamentos (Moore *et al.*, 2003; Sparano *et al.*, 2004; Bissett *et al.*, 2005; Leighl *et al.*, 2005). Um dos motivos para tais resultados poderia ser o papel ambíguo que as MMPs muitas vezes desempenham. Sabe-se que elas liberam fatores pró-angiogênicos, mas que em determinadas circunstâncias podem também exercer a função oposta, por meio da liberação de angiostatinas e endostatinas, potentes inibidores da angiogênese (Patterson e Sang, 1997; Heljasvaara *et al.*, 2005). Dessa maneira, o resultado final da ação das MMPs sobre alguns processos importantes para a progressão tumoral, irá depender de qual metaloproteinase está envolvida, assim como no equilíbrio das ações pró e anti-tumorais de uma mesma MMP. Portanto, entender a maneira

como as MMPs estimulam ou inibem a progressão tumoral é um fator importante para definir como e quando é o melhor momento para usá-las como alvo em terapias anti-tumorais (Hua *et al*., 2011).

O papel das MMPs nas primeiras etapas da progressão tumoral tem sido muito estudado. Neste aspecto, sua possível participação na regulação do processo da transição epitélio-mesenquimal (do inglês, EMT) é de especial relevância. A EMT é um processo fisiológico importante, presente na embriogênese e no reparo tecidual, que envolve a perda de adesão, polarização e mudança da arquitetura celular epitelial, resultando em um fenótipo mesenquimal. Esse processo também está ativo durante a progressão tumoral, levando a invasão e metástase (Hua *et al*., 2011). Quando as células epiteliais alteram seu fenótipo para mesenquimal, elas mudam a expressão de algumas proteínas, que estão associadas às suas novas características. A proteína de superfície E-caderina é a principal envolvida na ancoragem das células epiteliais umas às outras. Células epiteliais que passam pela EMT deixam de expressar E-caderina, o que resulta na perda de adesão celular (Scanlon *et al.*, 2013). Em seguida, as células epiteliais reorganizam seu citoesqueleto, a fim de adquirir motilidade e migrar através dos tecidos. A proteína Vimentina (VIM), da família

dos filamentos intermediários, em tecidos normais, é expressa apenas em células de origem mesenquimal e está associada à locomoção (Scanlon *et al.*, 2013). Em carcinomas, a expressão de VIM em células epiteliais tem sido relacionada ao potencial metastático (Paccione *et al.*, 2008; Zhou *et al.*, 2015).

Em displasias epiteliais bucais, a EMT foi estudada por Chaw *et al.* (2012) que avaliaram a expressão de E-caderina, VIM e β-catenina nestas lesões. Eles encontraram uma redução dos níveis de E-caderina e aumento de VIM e β-catenina, à medida que as displasias tornavam-se mais severas (Chaw *et al.*, 2012). Do mesmo modo, Sawant *et al.* (2013) reportaram que em leucoplasias a expressão de VIM acompanhou a progressão de displasia. Além disso, leucoplasias não homogêneas apresentaram maior expressão de VIM do que as homogêneas. Segundo os autores, esse resultado poderia estar relacionado à diferença no comportamento clínico entre estas lesões (Sawant *et al.*, 2014). Estes resultados sugerem que a ocorrência de EMT estaria envolvida na progressão maligna das displasias.

Qiao *et al.* (2010) estudaram a EMT induzida pelo fator de crescimento transformador beta 1(TGF-ß1) em linhagens celulares de carcinoma epidermoide. Os autores

encontraram que o TGF-ß1 foi capaz de induzir alterações mesenquimais em todas as linhagens celulares estudadas. Além disso, eles mostraram uma associação positiva entre a expressão das MMP-2 e 9 com SNAIL, um importante fator de transcrição associado à EMT (QIAO *et al.*, 2010). Diante destes resultados, seria possível inferir que a EMT e a expressão de MMPs teriam uma via de ativação em comum, envolvendo a atuação de TGF-ß1.

A desorganização da membrana basal também representa uma etapa importante no processo da EMT, pois ela permite que as células epiteliais migrem para o tecido conjuntivo. A integridade dessa barreira entre epitélio e conjuntivo pode ser perdida de três maneiras: 1) afastamento por meio de forças mecânicas, 2) desorganização localizada em consequência da perda de componentes estruturais e 3) proteólise de seus constituintes. As MMPs, especificamente MMP-2, 9, 3 e MT1-MMP participam deste último processo (Horejs, 2016).

Tamamura *et al.* (2013) buscaram relacionar a expressão das MMP-2 e 9 com a perda de cadeias de colágeno da membrana basal durante a progressão de displasias epiteliais para CEB. As lesões sem invasão não apresentaram perda das cadeias de colágeno ao longo da

membrana basal, que se manteve íntegra. Ao contrário das lesões invasivas, nas quais a membrana basal mostrou fragmentação e redução na expressão das cadeias de colágeno. Além disso, observou-se que a expressão de MMP-2 e 9 foi maior em lesões invasivas, coincidindo com a perda das cadeias de colágeno. A partir disso, os autores sugeriram que a perda da continuidade da membrana basal possa estar associada com o aumento da atividade das MMP-2 e 9, o que é um processo crítico na progressão das lesões potencialmente malignas (Tamamura *et al.*, 2013).

Em lesões potencialmente malignas da boca a expressão de MMP-9 foi estudada por He *et al.* (2016), que encontraram um aumento progressivo em sua expressão entre tecido normal, displásico e CEB (He *et al.*, 2016). Similarmente, Chandolia *et al.* (2016) reportaram que a expressão de MMP-9 foi maior em CEB do que em displasias e tecido normal. Além disso, a expressão aumentou progressivamente de displasias moderadas para severas e de severas para CEB (Chandolia *et al.*, 2016). Entretanto, ao comparar tecido normal com displásico, a expressão de MMP-9 não aumentou de maneira significativa. Resultados semelhantes foram relatados por De Carvalho *et al.* (2014), nos quais a expressão de MMP-9 foi significativamente maior em CEB do que em displasia, mas

não entre displasia e tecido normal (De Carvalho Fraga *et al.*, 2014). Estes resultados suportam a teoria de que a MMP-9 é importante na degradação da membrana basal e invasão do tecido conjuntivo, uma vez que sua expressão aumentaria significativamente em lesões invasivas.

Bianco *et al.* (2015) avaliaram a expressão das MMP-1, 2 e 9 separadamente no epitélio e estroma de lesões de lábio potencialmente malignas (queilite actínica- QA), carcinioma epidermoide de lábio (CEL) e lesões não neoplásicas (mucocele). Seus resultados apontaram para uma maior expressão dessas proteinases no epitélio de QA, enquanto em CEL ela foi maior no tecido conjuntivo. A diferença na localização da expressão das MMPs entre QA e CEL, poderia indicar papéis diferentes conforme o estágio de carcinogênese. Em lesões potencialmente malignas elas seriam necessárias no epitélio, participando da transformação maligna. Em contrapartida, em lesões malignas elas atuariam principalmente no estroma por serem necessárias para invasão e metástase (Bianco *et al.*, 2015).

Por outro lado, em um estudo de Barros *et al.* (2011) a expressão das MMP-2 e 9 em carcinomas de lábio e língua variou de fraca a moderada. Os autores sugeriram que possivelmente resultados diferentes fossem encontrados em

estágios iniciais desses carcinomas, em que a degradação da membrana basal é necessária (Barros *et al.*, 2011).

Os principais reguladores da atividade das MMPs são os inibidores teciduais de metaloproteinases (do inglês, TIMP). Existem quatro tipos de TIMP, a saber, TIMP-1, TIMP-2, TIMP-3 e TIMP-4. De maneira geral, eles podem regular a atividade de todas as MMPs, mas existem diferenças na afinidade entre tipos específicos de TIMP e MMP (Verstappen e Von Den Hoff, 2006). TIMP-1 é o principal regulador da atividade de MMP-9, e a correlação destes em CEB foi reportada como negativa, de forma que altas expressões de MMP-9 coincidiram com expressão reduzida de TIMP-1 (Nanda *et al.*, 2014). Por outro lado, a expressão de TIMP-2 foi relatada como positivamente correlacionada com a severidade de displasia em lesões potencialmente malignas e com metástase em CEB (Bajracharya *et al.*, 2014; Li *et al.*, 2014).

Entender qual é o processo por trás da progressão de displasias e seus mediadores é importante para o desenvolvimento de novas terapias contra o câncer, assim como o estabelecimento de marcadores que permitirão identificar lesões com maior potencial de transformação. O método de diagnóstico mais utilizado em casos de displasia

epitelial é o histopatológico, segundo os critérios da Organização Mundial da Saúde (OMS, 2017). Entretanto, sabe-se que é um método subjetivo, que está ligado à experiência do examinador (Kujan *et al.*, 2006; Warnakulasuriya *et al.*, 2017). Kujan *et al.* (2006) propuseram um novo sistema binário, usando os mesmos critérios morfológicos estabelecidos pela OMS (alterações arquiteturais e citológicas), dividindo as lesões em "baixo risco" e "alto risco". O novo sistema binário provou ser um bom preditivo de mudanças malignas em displasias epiteliais orais, com bons valores de sensibilidade e especificidade (85% e 80% respectivamente). Os autores sugeriram que o novo sistema binário poderia complementar a classificação da OMS, além de ajudar clínicos na tomada de decisões, particularmente em casos de displasia moderada.

Além da dificuldade em se conseguir reprodutibilidade, o valor prognóstico do grau de displasia é outro problema relacionado à avaliação histológica dessas lesões. Apesar do consenso de que lesões com graus mais severos de displasia apresentam maior risco de transformação maligna (Warnakulasuriya e Ariyawardana, 2016), existem relatos de displasias que não progrediram para carcinoma ou mesmo que regrediram (Holmstrup *et al.*, 2006). Dessa maneira, estabelecer marcadores que permitam

diferenciar lesões de alto e baixo risco seria um caminho para tornar o diagnóstico mais objetivo e reproduzível. Porém, o uso de marcadores como método diagnóstico requer um entendimento dos processos mediados por eles, assim como o estabelecimento de correlações clínicas entre a progressão de lesões displásicas com seus possíveis mediadores.

O processo inflamatório regula a atividade das MMPs ao produzirem citocinas que elevam sua expressão, o que resulta na degradação da MEC. Em contrapartida, as MMPs, ao degradarem os componentes da matriz, liberam fragmentos ativos que amplificam a resposta inflamatória. A MMP-9 participa da degradação da membrana basal do epitélio, abrindo o caminho para a invasão do tecido conjuntivo pelas células neoplásicas. Estas, por sua vez, diante do estimulo de TGF-β1, entram em EMT e invadem o tecido conjuntivo. Dessa maneira, durante a complexa rede de eventos que determinam a carcinogênese, a inflamação parece exercer um papel fundamental, regulando a presença de MMPs e a ocorrência de EMT. Entretanto, ainda não existem estudos analisando a correlação destes eventos na progressão de lesões potencialmente malignas para CEB.

## 1.3 PERGUNTA NORTEADORA

A intensidade do infiltrado inflamatório em displasias epiteliais bucais está associada com a progressão maligna e correlacionada com a expressão de MMP-9, TIMPs (1 e 2) e Vimentina?

# 2 OBJETIVOS

## 2.1 GERAL:

- Avaliar a influência da intensidade do infiltrado inflamatório e a expressão imunoistoquímica dos marcadores de MMP-9, TIMP-1 e 2 e Vimentina na progressão das displasias epiteliais bucais para CEB.

## 2.2 ESPECÍFICOS:

- Classificar as displasias epiteliais segundo os critérios da OMS e do sistema binário de Kujan (Kujan *et al.*, 2006);

- Avaliar a intensidade do infiltrado inflamatório presente na lâmina própria de amostras de epitélio não neoplásico, displasia epitelial e CEB;

- Relacionar a intensidade do infiltrado inflamatório na lâmina própria com o grau histológico das displasias epiteliais, segundo o sistema binário e a classificação da OMS;

- Avaliar a expressão imunoistoquímica de MMP-9, TIMP-1 e TIMP-2 no epitélio e no estroma de amostras de epitélio não neoplásico, displasias epiteliais e CEB;

- Avaliar a expressão imunoistoquímica de Vimentina no epitélio de amostras de tecido não neoplásico, displasia epiteliais e CEB;

- Relacionar expressão imunoistoquímica de MMP-9, TIMP-1, TIMP-2 e Vimentina com o grau histológico das displasias epiteliais segundo o sistema binário e a classificação da OMS;

- Correlacionar a intensidade do infiltrado inflamatório na lâmina própria com a expressão imunoistoquímica de MMP-9, TIMP-1, TIMP-2 e Vimentina;

- Coletar dados clínicos referentes à idade, sexo, localização e hábitos, de todos os grupos estudados.

# 3 METODOLOGIA

## 3.1 DESENHO DO ESTUDO

Este foi um estudo do tipo observacional descritivo, com aprovação do Comitê de Ética em Pesquisa com Seres Humanos (CEPSH) da Universidade Federal de Santa Catarina (Plataforma Brasil- CAAE: 42976715.3.0000.0121; Parecer: 1.005.587- 30/03/2015) (Anexo I).

## 3.2 SELEÇÃO DA AMOSTRA

A amostra deste estudo foi composta por três grupos: epitélio não neoplásico (ENN), displasia epitelial (DE) e epitélio neoplásico (EN).

O grupo DE foi estabelecido com base em uma casuística realizada nos arquivos do Laboratório de Patologia Bucal da Universidade Federal de Santa Catarina (UFSC) (Mello *et al.*, 2018). Foram selecionados 66 casos diagnosticados como displasia epitelial com localização intra-bucal. O grupo foi constituído de 34 homens (idade média 57.8 ± 12.8) e 32 mulheres (idade média 57.6 ± 12.6); a localização destas lesões foi distribuída em: assoalho bucal (8), língua (21), gengiva (9), trígono retromolar (6),

retrocomissura labial (3), palato (8) e mucosa jugal (12). Para o grupo EN foram selecionados 27 casos de CEB, dos quais 24 eram homens (idade média 56.4 ± 8.6) e três eram mulheres (idade média 56.4 ± 8.5). Finalmente, no grupo ENN foram incluídos 28 casos de Hiperplasia Fibrosa, dos quais 13 eram homens (idade média 49.2 ± 14.5) e 15 eram mulheres (idade média 49.6 ± 13.9). As demais características da população estão descritas na tabela 2 do artigo.

## 3.3 CLASSIFICAÇÃO DAS DISPLASIAS EPITELIAIS

O grau de displasia epitelial foi classificado usando dois sistemas:

- Organização Mundial da Saúde (OMS 2017): displasias leve, moderada e severa;

- Sistema binário proposto por Kujan (KUJAN *et al.*, 2006): lesões de “alto-risco” e de “baixo-risco”.

A classificação das displasias foi realizada por meio de imagens capturadas com câmera fotográfica (Cannon, A620, San Jose, CA, EUA) acoplada a microscópio de luz

(Axiostar Plus, Carl Zeiss, Oberkochen, Alemanha), com magnitude de 400X. Os campos escolhidos para avaliação representavam a área de maior grau de displasia encontrado nas lâminas dos casos selecionados. Dois avaliadores realizaram a classificação cegada para as características histológicas, e discordâncias foram resolvidas após consultar um terceiro avaliador.

## 3. 4 CLASSIFICAÇÃO DO INFILTRADO INFLAMATÓRIO

A avaliação da intensidade do infiltrado inflamatório foi feita a partir da contagem do número de células inflamatórias presente no tecido conjuntivo, em cinco campos microscópicos consecutivos. No grupo DE as áreas selecionadas para contagem foram as mesmas áreas utilizadas na classificação das displasias. A média de células inflamatórias por caso foi categorizada da seguinte maneira: (0) ausência de inflamação, 0-10 células; (1) inflamação leve, 11-25 células inflamatórias; (2) inflamação moderada, 26-65 células inflamatórias; (3) inflamação severa, mais de 65 células inflamatórias (Bosio *et al.*, 2014).

## 3. 5 PROCEDIMENTOS LABORATORIAIS

Os casos selecionados correspondem às lesões fixadas em formol e emblocadas em parafina, de onde se obtiveram cortes teciduais de 3µm de espessura, montados em lâmina preparada com solução de ATPS (3-aminopropyltriethoxysilene) (Sigma-Aldrich, Saint. Louis, MO, EUA). As lâminas foram submetidas à técnica de imunoistoquímica para avaliação das proteínas MMP-9, TIMP-1, TIMP-2 e Vimentina, utilizando-se anticorpos primários específicos para esses antígenos (consultar tabela 1 do artigo). Para tanto, procedeu-se a desparafinação das lâminas em xilol e hidratação por passagens sucessivas em etanol de concentrações decrescentes (100%, 90%, 85%). O bloqueio da atividade da enzima peroxidase foi realizado após imersão em solução de H2O2/metanol a 6%. A recuperação antigênica foi feita por meio de um banho-maria a 96ºC, com tampão citrato 0.01M (pH 6.0), durante 40 minutos. Em seguida, as lâminas foram imersas durante 40 minutos em solução de leite desnatado em pó diluído em solução salina tamponada 0.05M, pH 7.4 (PBS), para bloqueio das reações inespecíficas. Entre cada processo foram realizadas lavagens com PBS. As lâminas foram então encubadas com os anticorpos primários para as proteínas

MMP-9, TIMP-1, TIMP-2 e VIM em câmara úmida a 4ºC durante a noite.

Para amplificação da reação as lâminas foram encubadas durante 1 hora com o sistema EnVision (Dako Corporation, Carpinteria, CA, EUA). Após lavagem com PBS, a revelação da reação foi realizada com solução cromógena, contendo diaminobenzidina (DAB) (Dako Corporation, Carpinteria, CA, EUA), durante três minutos. Em seguida, foi feita a contra-coloração das lâminas com hematoxilina de Harris durante dois minutos. Finalmente, as lâminas passaram por uma desidratação em cadeias de concentração crescentes de etanol (85% e 100%), diafanização em xilol e montagem com o adesivo Permount®.

## 3. 6 ANÁLISE IMUNOISTOQUÍMICA

A avaliação das reações foi realizada com o software NIH ImageJ 1.52a (National Institute of Health, Maryland, EUA) a partir de imagens capturadas com câmera fotográfica (Cannon, A620, San Jose, CA, EUA) acoplada a microscópio de luz (Axiostar Plus, Carl Zeiss, Oberkochen, Alemanha), com magnitude de 400X. No grupo DE, as imagens foram obtidas a partir dos mesmos campos

escolhidos para a classificação do grau de displasia. As imagens para avaliação do grupo EN foram obtidas em regiões de *hot spot.*

A imunorreatividade dos anticorpos anti-MMP-9, TIMP-1 e TIMP-2 foi analisada no citoplasma das células epiteliais e no tecido conjuntivo adjacente. A marcação foi avaliada por meio da porcentagem da área marcada (pixels positivos) em relação à área total do epitélio ou estroma em cada campo (pixels totais), em 10 campos consecutivos para cada caso. A expressão de Vimentina foi analisada contando-se o número de células positivas em relação ao número de células negativas, em cinco campos microscópicos, em regiões de *hot spot*.

**Quadro 1:** Distribuição dos anticorpos e parâmetros em estudo conforme o tecido nos quais foram analisados.

| **Critério/Marcardor** | **Epitélio** | **Conjuntivo** |
|---|---|---|
| MMP-9 | x | x |
| TIMP-1 | x | x |
| TIMP-2 | x | x |
| Vimentina | x | |
| Inflamação | | x |

## 3. 7 ANÁLISE ESTATÍSTICA

Após aplicação do teste de normalidade Shapiro-Wilk e a constatação da distribuição anormal dos dados avaliados, optou-se pela realização do teste não paramétrico de Kruskal-Wallis, quando mais de três grupos estavam sendo comparados e o teste de Mann-Whitney , quando apenas dois grupos estavam sendo comparados. Para comparações múltiplas entre os grupos foi utilizado o teste post hoc de Dunn- Bonferroni. A correlação entre a marcação das proteínas em estudo foi feita por meio da comparação de Spearman. O nível de significância foi estabelecido em $P < 0.05$.

# 4 ARTIGO

Artigo formatado de acordo com as normas da revista "Oral Diseases" (Anexo II, normas da revista).

**Title:** The role of inflammation in oral carcinogenesis

**Authors:** Andressa Fernanda Paza Miguel[a], Bubacar Embaló[b], Helison Bertoli Alves Dias[c], Elena Riet Correa Rivero[d]

[a]Postgraduate program in dentistry, Federal University of Santa Catarina - Florianópolis, Santa Catarina, Brazil. andressapaza@gmail.com

[b]Postgraduate program in dentistry, Federal University of Santa Catarina - Florianópolis, Santa Catarina, Brazil. abuembalo@gmail.com@gmail.com

[c]Postgraduate program in business, Federal University of Paraná, Curitiba, Paraná, Brazil. helison.bertoli@gmail.com

[d]Department of Pathology, Health Sciences Center, Federal University of Santa Catarina - Florianópolis, Santa Catarina, Brazil. E-mail: riet.elena@gmail.com

**Corresponding author:** Andressa Fernanda Paza Miguel
Department of Pathology, Health Sciences Center, Federal University of Santa Catarina. University Campus - Trindade, Florianópolis, Santa Catarina, Brazil. Zip code: 88040900. e-

mail: andressapaza@gmail.com. Phone number: +5548988052650.

## 4.1 ABSTRACT

**Objective:** The aim of this study was to determine if the intensity of the inflammatory infiltrate and the immunohistochemical expression of MMP-9, TIMP-1, TIMP-2, and vimentin correlate with the progression of oral potentially malignant disorders towards oral squamous cell carcinoma. **Material and Methods**: The sample was composed of 66 cases of epithelial dysplasia, 27 of oral cancer and 28 of non-neoplastic epithelium. Samples were subjected to immunohistochemical detection of MMP-9, TIMP-1, TIMP-2, and vimentin. The number of inflammatory cells was assessed in the underlying connective tissue. **Results:** The expression of MMP-9 was higher in the connective tissue of epithelial dysplasia and oral cancer when compared to non-neoplastic epithelium. TIMP-2 expression in the epithelium and connective tissue increased from non-neoplastic epithelium to epithelial dysplasia. None of the markers correlated with the severity of dysplasia. Inflammation increased with the progression of dysplasia to oral cancer and this difference was statistically significant (p=0.004). Moreover, the intensity of inflammatory infiltrate positively correlated with the expression of vimentin (p=0.000). **Conclusion:** Inflammation seems to be involved in the progression of oral potentially malignant disorders to cancer, and this could be through the triggering of epithelial-mesenchymal transition.

**KEYWORDS:** Oral Potentially Malignant disorders; Oral Squamous cell carcinoma; Inflammation; MMP-9; Vimentin; Tissue Inhibitor of Metalloproteinases.

## 4.2 INTRODUCTION

Squamous cell carcinoma of the oral cavity (OSCC) has shown an increased tendency in incidence worldwide (Ng, Iyer, Tan, & Edgren, 2017). In Brazil, it is the 5$^{th}$ most common cancer in men (INCA, 2018). According to the National Cancer Institute of Brazil, in 2015 there were 5.539 deaths by oropharyngeal cancer registered. In order to change these numbers, early diagnosis is imperative.

Most OSCC will progress from oral potentially malignant disorders (OPMD), such as leukoplakia and erythroplakia. According to a recent meta-analysis, the prevalence of these lesions worldwide was 4.47% and males were the most affected gender (Mello, Miguel, et al., 2018). The detection of these lesions before they progress to cancer is challenging, as well as determining the risk of malignization.

Currently, the risk of malignization of OPMD is accessed by the grade of epithelial dysplasia (ED) present. The classification from World Health Organization (WHO) is the standard system used to grade the severity of ED, although it is a subjective system with reported inter and intra-observer variability (Warnakulasuriya, Reibel, Bouquot, & Dabelsteen, 2017). Therefore, the use of biomarkers as a tool to accesses the risk of malignization has been studied. There are several biomarkers for ED reported in the literature (Smith, Rattay, McConkey, Helliwell, & Mehanna, 2009), some of them being related to inflammation (Piva et al., 2011) (Piva et al., 2013).

Inflammation in the underlying connective tissue of OPMD was recently associated with the degree of ED (Mashhadiabbas & Fayazi-Boroujeni, 2017). The presence of

inflammatory cells would create an environment reach in cytokines and chemokines, which would result in cell proliferation, tissue vascularization and cell migration (Coussens & Werb, 2002). Moreover, inflammatory infiltrate was associated with ultrastructural changes in the extracellular matrix (ECM) in OSCC, which was related to the risk of metastasis (AGRAWAL, RAI, & JAIN, 2011). These changes in the ECM may be attributed to the presence of matrix-degrading enzymes, such as matrix-metalloproteinases (MMPs).

MMPs are a family of zinc-dependent enzymes that degrade components of the ECM. The presence of these enzymes in the tumor environment has been associated with invasion, cell proliferation, epithelial-mesenchymal transition (EMT), angiogenesis and tumor immunity (Gialeli, Theocharis, & Karamanos, 2017). In OSCC, MMP-9 was correlated with poorer overall survival, lymph node metastasis and advanced T-stage (Zheng, Zhang, Yang, & Li, 2015). In OPMD, the expression of MMP-9 was found elevated in serum, tissue, and saliva, when compared to healthy controls (Venugopal & Uma Maheswari, 2016). MMP-9, also known as gelatinase B, is responsible for the breakdown of collagen type IV, an important component of the basement membrane. Thus, this enzyme is involved in the breakdown of the first barrier between the epithelium and lamina propria, which marks the progression of OPMD to invasive carcinoma (Tamamura et al., 2013).

The expression of MMPs has been linked to the occurrence of EMT (Orlichenko & Radisky, 2008). Furthermore, it was suggested that MMP-9 is correlated with the induction of EMT in squamous cell carcinoma cell lines (C. Y. Lin et al., 2011). EMT is a process in which epithelial cells acquire a mesenchymal phenotype, including loss of cell adhesions and ability to migrate. The latter is associated with

the expression of Vimentin (VIM), an intermediate filament protein associated with changes in cell shape and motility (Satelli & Li, 2011). In OPMD the expression of VIM was found to be increased as the epithelial dysplasia became more severe and progressed to cancer (Chaw et al., 2012).

The evidence suggests that the processes of inflammation, EMT, and expression of MMP-9 are all involved in the progression of oral epithelial dysplasia to cancer, although there are no studies that have evaluated the correlation of these processes with the severity of dysplasia in OPMD. In this study, we have hypothesized that the intensity of inflammation and the expression of MMP-9 and its inhibitors, and Vimentin are associated with the degree of ED in OPMD. In order to test this hypothesis, this study was designed to evaluate the association between the expression of MMP-9 and its inhibitors TIMP-1 and TIMP-2, and Vimentin (VIM), with the intensity of inflammatory infiltrate in samples of OPMD and compare with OSCC and non-neoplastic epithelium.

## 4.3 METHODOLOGY

*Sample selection*

The samples were selected based on previous screening for intraoral potentially malignant disorders from the Oral Pathology Laboratory at the Federal University of Santa Catarina (Mello, Melo, Meurer, & Rivero, 2018). A total of 66 cases of OPMD were selected. Inclusion criteria were cases with a diagnosis of epithelial dysplasia. Cases with insufficient tissue material were excluded. Hematoxylin and eosin slides from the selected cases of OPMD were analyzed by three

calibrated observers (one experienced pathologist and two master's students) and graded according to the WHO classification system for epithelial dysplasia as mild, moderate and severe (El-Naggar, Chan, Grandis, Takata, Slootweg, 2017). The cases were also grated according to the binary system of Kujan as "high-risk" and "low-risk" lesions (Kujan et al., 2006). Additionally, 27 cases of OSCC and 28 samples of non-neoplastic epithelium from fibrous hyperplasia (NNE) were included in this study. Furthermore, patient's charts were reviewed in order to obtain clinical and demographic variables. This study was reviewed and approved by the Ethics Committee in Human Research (protocol: CAAE 42976715.3.0000.0121).

*Immunohistochemistry*

Tissue samples were fixed in 10% buffered formalin, processed and embedded in paraffin. Time of storage of the samples in the University's archives was up to 11 years.

Immunohistochemistry was performed in three-micrometer sections, mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA). Deparaffinized slides were hydrated in decreasing alcohol concentrations and then immersed in a 6% $H_2O_2$/methanol solution to inhibit endogenous peroxidase activity. Antigen retrieval was performed using a water bath with 0.01M citrate buffer (pH 6.0), at 96°C (Merck, Darmstadt, Hessen, Germany). The blocking of non-specific binding sites was carried out with 5% skim milk in phosphate-buffered saline solution (PBS) for 40 min. Incubation with primary antibodies against MMP-9, TIMP-1, TIMP-2 and VIM (Sources, concentration, dilutions and positive controls are shown in Table 1) was done at 4º C overnight. Immunodetection was performed with the EnVision system (Dako Corporation, Carpinteria, CA, USA) for one hour. After two rinses with PBS, slides were incubated with diaminobenzidine (DAB) (Dako

Corporation, Carpinteria, CA, USA) for three minutes. Counterstaining was performed with Harris's hematoxylin. Negative controls were included in all reactions by omitting the primary antibody and replacing it with PBS solution. Positive controls were also included (Table 1).

**Table 1:** Details of the antibodies and positive controls used in the study

| Antibody | Clonality | Source | Dilution | Positive controls |
|---|---|---|---|---|
| MMP-9 | Polyclonal | IMGENEX | 1:200 | Breast Carcinoma |
| TIMP-1 | Monoclonal | Santa Cruz | 1:50 | Adenoid cystic carcinoma |
| TIMP-2 | Monoclonal | Santa Cruz | 1:100 | Salivary Gland |
| VIM | Monoclonal | Santa Cruz | 1:800 | Salivary Gland |

*Immunohistochemical analysis*

The immunohistochemical analysis was performed using NIH ImageJ 1.52a (National Institute of Health, Bethesda, MD, USA) to examine the images captured with a camera (Canon A620, Beijing, China) attached to a light microscope (Axiostar Plus, Carl Zeiss, Oberkochen, Germany) at 400x magnification. The fields chosen for immunohistochemycal analysis were the same ones used to grade the degree of epithelial dysplasia in the ED group. For the OSCC group, the field choice for immunohistochemycal analysis was based on *hot spot* areas.

Positive immunoreactivity for MMP-9, TIMP-1, TIMP-2, and VIM was defined by a brown cytoplasmic and inter-cellular staining. The expression of MMP-9, TIMP-1, and TIMP-2 was evaluated individually in the stroma and

epithelium, after image segmentation, in 10 consecutive fields. The result was expressed as the mean percentage staining of the demarcated area (positive pixels were equal to the area of diaminobenzidine staining) related to the total area (total pixels) of the epithelium or stroma (Dutra, Cordeiro, Vieira, & Rivero, 2016). The immunohistochemical staining for VIM was quantified by the number of positive cells on the epithelium, at 400x in five fields, and expressed as the percentage of positively-stained cells. Immunoreactivity for VIM was defined as more than 5% of positive cells (Sawant et al., 2014).

*Inflammatory Infiltrate analysis*

The inflammatory infiltrate was analyzed in H&E slides and the number of inflammatory cells was counted in five consecutive fields, the same ones used for immunohistochemycal analysis. The mean count of inflammatory cells by case was categorized as: (0) inflammation absent, 0–10 inflammatory cells; (1) mild inflammation, 11– 25; (2) moderate inflammation, 26-65 cells; and (3) severe inflammation, more than 65 inflammatory cells (BOSIO et al., 2014).

*Statistical analysis*

The Kruskal-Wallis and Mann-Whitney statistical tests ware used to compare the results between the groups and Dunn-Bonferroni post-Roc test for multiple comparisons. Furthermore, Spearman correlation was used to test the correlation between the antibodies. Statistical significance was set at $p<0.005$.

## 4.4 RESULTS

*Clinicopathological features*

Clinicopathological parameters by groups in the sample are described in Table 2. Furthermore, the distribution of cases according to the degree of dysplasia is presented in Figure 1.

**Table 2:** Clinical parameters of NNE, ED and OSCC.

| | NNE | | ED | | OSCC | |
|---|---|---|---|---|---|---|
| | Male | Female | Male | Female | Male | Female |
| Age (Mean±SD) | 49.2 ± 14.5 | 49.6 ± 13.9 | 57.8 ± 12.8 | 57.6 ± 12.6 | 56.4 ± 8.6 | 56.4 ± 8.5 |
| Smoking | 4 (30.8%) | 2 (13.3%) | 27 (79.4%) | 16 (48.5%) | 18 (75%) | 2 (66.7%) |
| Alcohol | 2 (15.4%) | 0 | 16 (47.1%) | 0 | 15 (62.5%) | 0 |
| Smoking + Alcohol | 1 (7.7%) | 0 | 16 (47.1%) | 0 | 14 (58.3%) | 0 |
| Location | | | | | | |
| Floor of the mouth | 0 | 0 | 5 (14.7%) | 3 (9.4%) | 5 (20%) | 0 |
| Tongue | 1 (7.7%) | 6 (40%) | 5 (14.7%) | 15 (46.9%) | 6 (24%) | 0 |
| Gingiva | 0 | 0 | 5 (14.7%) | 4 (12.5%) | 6 (24%) | 1 (33.3%) |
| Buccal mucosa | 9 (69.2%) | 8 (53.3%) | 5 (14.7%) | 7 (21.9%) | 4 (16%) | 2 (66.7%) |
| Retromolar | 0 | 0 | 5 (14.7%) | 1 (3.1%) | 1 (4%) | 0 |
| Lip | 1 (7.7) | 0 | 3 (8.8%) | 0 | 0 | 0 |
| Palate | 2 (15.4%) | 1 (6.7%) | 6 (17.6%) | 2 (6.2%) | 0 | 0 |
| TOTAL | 13 (46.4%) | 15 (53.6%) | 34 (51.5%) | 32 (48.5%) | 24(88.9%) | 3 (11.1%) |

NNE, Non-neoplastic epithelium; ED, Epithelial Dysplasia; OSCC, Oral Squamous Cell Carcinoma.

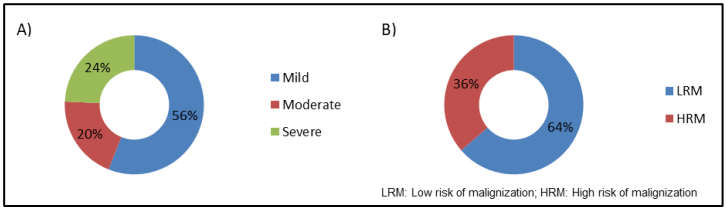


**Figure 1:** Distribution of epithelial dysplasia (ED) according to the degree of dysplasia: A) Distribution according to the WHO classification system; B) Distribution according to the binary system.

*Immunohistochemical analysis of markers*

The expression of all markers and the intensity of the inflammatory infiltrate are described in Table 3 and Figures 2 to 5.

*MMP-9*

Positive expression of MMP-9 was detected in all samples of NNE and ED, and in 26/27 (96.3%) of OSCC samples. In the epithelium, the overall expression of MMP-9 was located in the cytoplasm, intercellular space and nuclei of epithelial cells. In the connective tissue, MMP-9 expression was located mainly in blood vessels and in inflammatory cells. In the epithelium, the median expression was higher in the ED group, followed by NNE and OSCC, but there wasn't statistical difference (Figure 2a). In the connective tissue, median expression was higher in the OSCC group, followed by ED and NNE. There was statistical difference in the median expression of MMP-9 between OSCC and NNE group ($p= 0.03$), and between ED and NNE ($p= 0.015$) (Figure 2b). The expression of MMP-9 did not differ between the grades of dysplasia (Figure 3a, b; Table 3).

*TIMP-1*

Positive expression of TIMP-1 was detected in 22/28 (78.6%) in the NNE group, 58/66 (86.6%) in the ED group, and 24/27 (88.9%) in the OSCC group. In the epithelium, TIMP-1 median expression was higher in the ED group, and similar between OSCC and NNE. However, there wasn't statistical difference (Figure 2c). In the connective tissue, median expression was also higher in the ED group, followed by OSCC

and NNE group, tough without statistical difference (Figure 2d). The expression of TIMP-1 did not differ between the grades of dysplasia (Figure 3c, d; Table 3).

*TIMP-2*

Positive expression of TIMP-2 was present in 5/28 (17.9%) in the NNE group, 29/66 (43.9%) in the ED group and 5/27 (18.5%) in the OSCC group. In the epithelium, there was a statistical difference in the expression of TIMP-2 between the ED and the NNE group (p=0.037) (Figure 2e). In the connective tissue, the same result between the ED and the NNE group was observed (Figure 2f). Among the different degrees of epithelial dysplasia, the expression of TIMP-2 was higher in cases with severe dysplasia and in cases classified as "high-risk" lesions, although this wasn't statistically different (Figure 3e, f; Table 3).

*Vimentin*

The expression of VIM was detected in the cytoplasm of epithelial cells and located mostly in the basal and parabasal layer. VIM expression was negative in all NNE cases. The expression of this marker was positive in 22/66 (33.3%) cases of the ED group, and in 10/27 (37%) of the OSCC group. The expression of VIM was higher in the OSCC group, followed by ED and NNE. There was statistical difference in the expression of VIM between the OSCC and NNE groups (p=0.001) and between the ED and the NNE groups (p=0.000) (Figure 2g). Among the different degrees of epithelial dysplasia, VIM expression was higher in severe dysplasia and "high-risk" lesions, although this wasn't statistically different (Figure 3g; Table 3). Furthermore, there were 44 cases of ED that didn't

express VIM (<5%), among them 35/44 (79.5%) were graded as mild/moderate dysplasia and 29/44 (65.9%) as “low-risk” lesions.

*Analysis of inflammatory infiltrate*

The intensity of inflammatory infiltrate was higher in the OSCC group, followed by ED and NNE groups. The median count of inflammatory cells revealed that inflammation was considered absent in the NNE group, moderate in the ED group and severe in the OSCC group. There was statistical difference in the median count of inflammatory cells between OSCC and NNE groups (p=0.000) and between ED and NNE groups (p=0.000) (Figure 2h). Furthermore, in the ED group, cases with severe dysplasia had more inflammatory cells than mild and moderate dysplasia (p= 0.000 and p= 0.027, respectively) (Figure 3h); and "high-risk" lesions more than "low-risk" (p= 0.000) (Figure 5a; c Table 3;).

*Correlation between markers*

Spearman’s correlation test revealed a positive correlation between the mean count of inflammatory cells and the expression of VIM (p= 0.000) (Figure 5b, d). Moreover, the expression of MMP-9 in the epithelium correlated with the expression of TIMP-1 in the epithelium and the connective tissue (p= 0.000 and p=0.006, respectively).

**Table 3:** Expression of MMP-9, TIMP-1, TIMP-2 and VIM, and the intensity of inflammation in the epithelium and the connective tissue according to grade of dysplasia (Binary)

| | Epithelium | | | Connective Tissue | | |
|---|---|---|---|---|---|---|
| | LR | HR | P | LR | HR | P |
| MMP-9 | 14.4± 12.1 | 12.8± 10.8 | 0.227 | 6.2± 8.1 | 6.6± 7.9 | 1.000 |
| TIMP-1 | 7.0± 9.9 | 5.9± 7.0 | 0.760 | 2.5± 3.1 | 1.8± 2.2 | 0.256 |
| TIMP-2 | 3.5± 8.3 | 4.2± 8.8 | 1.000 | 0.9± 1.8 | 1.6± 3.5 | 1.000 |
| VIM | 7.7± 13.7 | 9.9± 16.6 | 1.000 | - | - | |
| Inflammation | - | - | | 38.4± 29.2 | 72.7± 29.5 | 0.000 |

MMP, Matrix metalloproteinase; TIMP, Tissue inhibitor of matrix metalloproteinase; VIM, Vimentin; HR, High Risk; LR, Low risk;
Values are expressed as the means± standard deviation (%).
Mann-Whitney statistical test ($P < 0.05$)

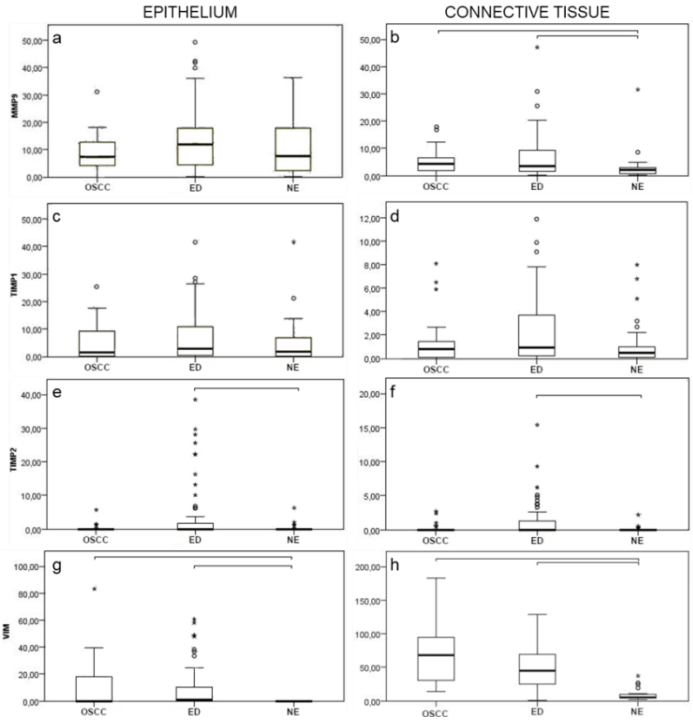


**Figure 2:** OSCC, Oral squamous cell carcinoma; ED, Epithelial dysplasia; NNE, Non-neoplastic epithelium; Distribution of (a,b) matrix metalloproteinase (MMP-9), (c, d) tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), (e, f) tissue inhibitor of matrix metalloproteinase 2 (TIMP-2), (g) vimentin (VIM) and (h) inflammatory infiltrate in the epithelium and connective tissue of OSCC, ED and NNE. Connecting lines indicate statistical difference ($p<0.05$). Kruskal-Wallis statistical test with Dunn-Bonferroni post-Roc test for multiple comparisons.

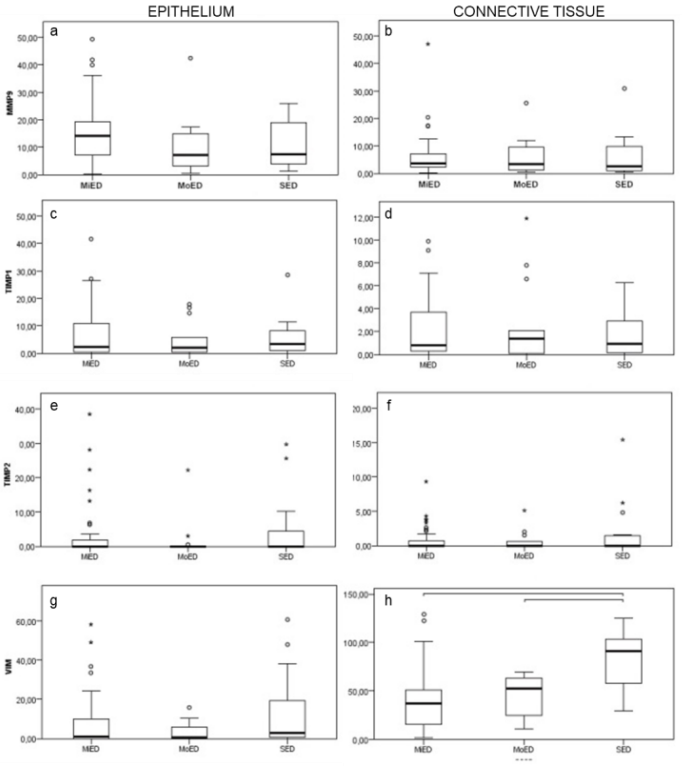


**Figure 3:** MED, Mild epithelial dysplasia; MoED, Moderate epithelial dysplasia; SED, Severe epithelial dysplasia; Distribution of (a, b) matrix metalloproteinase (MMP-9), (c, d) tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), (e, f) tissue inhibitor of matrix metalloproteinase 2 (TIMP-2), (g) vimentin (VIM) and (h) inflammatory infiltrate in the epithelium and connective tissue of epithelial dysplasia, according to the degree of dysplasia (WHO system). Connecting lines indicate statistical difference ($p<0.05$). Kruskal-Wallis statistical test with Dunn-Bonferroni post-Roc test for multiple comparisons.

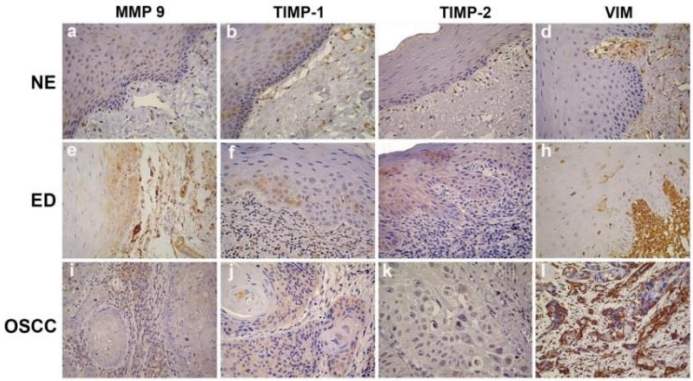


**Figure 4:** Photomicrograph of matrix metalloproteinase (MMP-9), tissue inhibitor of matrix metalloproteinase (TIMP-1 and TIMP-2) and vimentin (VIM) expressions in non-neoplastic epithelium (NNE), epithelial dysplasia (ED) and oral squamous cell carcinoma (OSCC) samples. (a) Expression of MMP-9 in NNE; (b) expression of TIMP-1 in NNE; (c) expression of TIMP-2 in NNE; (d) expression of VIM in NNE; (e) expression of MMP-9 in ED; (f) expression of TIMP-1 in ED; (g) expression of TIMP-2 in ED; (h) expression of VIM in ED; (i) expression of MMP-9 in OSCC; (j) expression of TIMP-1 in OSCC; (k) expression of TIMP-2 in OSCC; (l) expression of VIM in OSCC (400x).

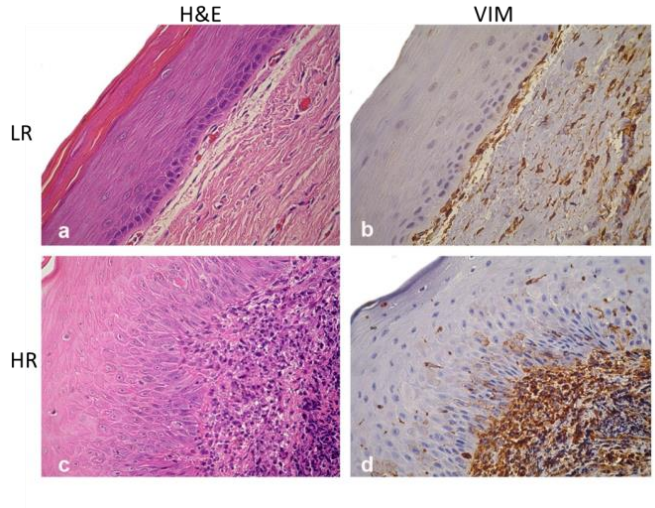


**Figure 5**: Relationship between vimentin expression and inflammatory process: a) Low-Risk Epithelial Dysplasia with mild inflammation (haematoxylin and eosin [H&E] 400×); b) Immunohistochemical expression of Vimentin in the same (Envision 400×); c) High-Risk Epithelial Dysplasia with intense inflammation (H&E 400×); d) Immunohistochemical expression of Vimentin in the same case (Envision 400×).

## 4.5 DISCUSSION

Over the past decades several studies have been published regarding the influence of matrix metalloproteinases (MMPs) on the aggressiveness and prognostic of oral squamous cell carcinoma (OSCC) (Lee et al., 2009; N. N. Lin et al., 2017; Miranda Galvis et al., 2018; Pramanik et al., 2018). In oral potentially malignant disorders (OPMD), MMPs are involved in the breakdown of the basement membrane, which favors the invasion of the underlying connective tissue by the epithelial malignant cells. Thus, MMPs are directly involved in the

process of invasion since the first stages of carcinogenesis (Yadav et al., 2014).

In this study, the expression of MMP-9 was evaluated in the epithelium and the underlying connective tissue of non-neoplastic epithelium (NNE), epithelial dysplasia (ED) and OSCC. The expression of MMP-9 was higher in ED and OSCC, both in the epithelium and the connective tissue, when compared to NNE. However, although without a statistical difference, the expression of this MMP was higher in ED when compared to OSCC. Bianco *et al* (2015) reported similar results. They found a higher expression of MMP-9 in the epithelium of actinic cheilitis (AC) when compared to lip squamous cell carcinoma (LSCC) (Bianco et al., 2015). On the other hand, Bindhu *et al* (2006) reported an increased expression of MMP-9 with the progression from normal to dysplastic and from dysplastic to invasive carcinoma, both in the epithelium and stroma (Bindhu, Ramadas, Sebastian, & Pillai, 2006). Other studies that investigated the expression of MMP-9 also reported an increase with the progression to OSCC (Chandolia, Basu, & Kumar, 2016; de Carvalho Fraga et al., 2014; He et al., 2016; Tortorici et al., 2008). Contrarily, Tamamura *et al* (2013) in their sample found almost negative expression of MMP-9 in normal tissue and ED, whereas in OSCC the expression varied from mild to moderate (Tamamura et al., 2013). In our sample, the higher expression of MMP-9 in ED and OSCC confirms that this enzyme is required during carcinogenesis. MMP-9 location in the epithelium and the connective tissue in these lesions suggest that in fact, both neoplastic and stromal cells can produce this protein.

The activity of MMPs is regulated by their inhibitors called tissue inhibitor of metalloproteinases (TIMP). There are four types of TIMPs, designated as TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Generally, they can bind to any type of MMPs,

but there are some differences in affinity (Verstappen & Von den Hoff, 2006). At present work, the expression of TIMP-1 and TIMP-2 was assessed and the overall expression was low in all groups, which agrees with other reports (Shrestha, Bajracharya, Byatnal, Kamath, & Radhakrishnan, 2017). The expression of TIMP-2 was higher in the ED group and increased with the severity of dysplasia. Although the differences did not achieve statistical significance, they could suggest that TIMP-2 is required during the progression to cancer. Bajracharya *et al* (2014) demonstrate an increase in TIMP-2 expression with the severity of dysplasia in leukoplakias and a positive correlation between TIMP-2/MMP-2 expressions. MMP-2 is also a gelatinase, as MMP-9, which degrades collagen type IV from the basement membrane. MMP-2 is released as an inactive form, called pro-MMP-2, and to become active needs to suffer hydrolysis from an enzyme complex of which TIMP-2 is part of. Thus, TIMP-2 expression is needed during carcinogenesis to activate MMP2, which together with MMP-9, will participate in the breakdown of the basement membrane, enabling invasion (Bajracharya, Shrestha, Kamath, Menon, & Radhakrishnan, 2014).

The expression of MMP-9 correlated positively with TIMP-1, which is different from the result reported by Nanda *et al* (2009). In their study, the correlation of these markers was negative. However, in their sample the expression of MMP-9 was found to be very intense, what can be explained by the low expression of TIMP-1, which is the main inhibitor of MMP-9 (Nanda et al., 2014). In our study, the mean expression of MMP-9 wasn't very high and this could be because of the inhibitory effect of TIMP-1.

Vimentin (VIM) is a protein of the intermediate filament family expressed by mesenchymal cells (Chaw et al., 2012; Sawant et al., 2014). In the epithelium, it has been used

as marker for epithelial-mesenchymal transition (EMT) and when expressed in neoplastic cells is associated with a migratory phenotype and metastasis (Paccione et al., 2008; Sawant et al., 2014; Scanlon, Van Tubergen, Inglehart, & D'Silva, 2013; Zhou, Tao, Xu, Gao, & Tang, 2015). There haven't been many studies investigating VIM expression in oral ED. In this study, VIM expression was absent in NNE and this was expected since this is a mesenchymal marker not expressed by normal epithelial cells (Sawant et al., 2014). The expression of VIM was detected in 33% of ED, which is similar to the results of Sawant *et al* (2014)(44%). On the other hand, the percentage of OSCC with positive immunoreactivity for VIM was 37%, which is lesser than the reported by these same authors (77%), what could be due to the larger sample used by them. Moreover, our results revealed a higher immunoreactivity of VIM in severe and "high-risk" lesions, but this wasn't statistically significant. Other studies reported a statistically significant increase in VIM expression with the progression of dysplasia (Sawant et al., 2014). The expression of VIM in OPMD lesions is necessary because to progress to cancer, epithelial cells need to migrate into the underlying connective tissue.

Furthermore, our results revealed that VIM expression wasn't detected in all cases of ED and there were some cases graded as mild dysplasia and classified as "low-risk" lesions that presented with more than 30% of positive cells. Although the presence of moderate/severe dysplasia is more predictive of malignant transformation, in a few cases cancer can arise from lower grades (Schepman, Van der Meij, Smeele, & Van der Waal, 1998). Vimentin could be a predictive factor for malignant transformation, but in order to confirm this, further studies need to be conducted

The tumor microenvironment is characterized by a cross-talk between neoplastic cells and stroma. Neoplastic cells stimulate inflammatory cells to produce cytokines, chemokines, growth factors, proteinases and prostaglandins, which in turn induce cell proliferation and survival, and tissue angiogenesis, resulting in tumor progression (Feller, Altini, & Lemmer, 2013). In the present study, there was an increase in the intensity of inflammation with the progression from NNE to ED and from ED to OSCC. Moreover, ED with severe dysplasia had significantly more inflammatory cells in the underlying connective tissue than mild and moderate dysplasia, as did "high-risk" lesions. These findings are in agreement with the report of Mashhadiabbas *et al* (2017), in which the intensity of inflammatory infiltrate increased with the progression of dysplasia towards OSCC (Mashhadiabbas & Fayazi-Boroujeni, 2017). On the other hand, Piva *et al* (2011) reported a moderate inflammatory infiltrate in ED and found a higher expression of CD8 in ED than in OSCC, suggesting a possible protective role of inflammation against malignization. However, the authors also found a correlation between the intensity of inflammation and the expression of TNF-α and NF-κB in cases of ED (Piva et al., 2013; Piva et al., 2011). According to them, it could be speculated that the inflammatory response at the beginning of the carcinogenesis would first have a protective effect, with the presence of CD8 lymphocytes, which has a tumor suppressive effect by targeting tumor cells. As the inflammatory response endures, the profile of cytokines would change, with the expression of TNF-α and NF-κB, two pro-inflammatory mediators associated with amplification of the inflammatory response and stromal remodeling, which favors the process of transformation (Balkwill & Mantovani, 2001; Pikarsky et al., 2004; Piva et al., 2013). Furthermore, it was suggested that the persistence of the inflammation would also result in a reduced response of malignant cells to transforming growth factor-β

(TGF-β). TGF-β act as a tumor suppressor in the early stages of tumorigenesis; however, as the tumor progress and cells lose they response to its inhibitory effect, malignant cells may respond by entering in epithelial-mesenchymal transition (EMT) (Pickup, Novitskiy, & Moses, 2013).

In the present study, the expression of VIM was positively correlated with the intensity of inflammatory infiltrate. Thus, it could be suggested that inflammation is a coadjutant in the malignant transformation process of ED by triggering EMT. Moreover, there wasn't a correlation between MMP-9expression and the intensity of inflammatory infiltrate. These results could suggest that the regulation of MMP-9 is not associated with the quantity of inflammation, but rather with the quality of it. Thus, the type of inflammatory cells along with the profile of cytokines and growth factors secreted would determine the expression of MMPs. In this matter, the expression of TGF-β1 could possibly be the link between inflammation, MMP-9 expression and EMT, since there are evidences that this growth factor is associated both with the up-regulation of MMP-9 at protein and gene level in SCC (Hawinkels et al., 2014; Qiao, Johnson, & Gao, 2010; Sun et al., 2008; Takayama et al., 2009) and the triggering of EMT in epithelial cells (Qiao et al., 2010). Taken together these results, it all points out to the malignant transformation of OPMD being a multistep process in which inflammation would play a pivotal role, through the regulation of MMP expression and EMT triggering.

## 4.6 CONCLUSION

In summary, our data confirmed the hypothesis that severe intensity of inflammatory infiltrate is associated with the progression of OPMD to OSCC. Furthermore, vimentin

expression correlated with the intensity of inflammatory infiltrate. On the other hand, MMP-9, TIMP-1, and TIMP-2 didn't show correlation with the severity of epithelial dysplasia or with the intensity of inflammatory infiltrate.

**Acknowledgments**

The authors would like to thank Helison Bertoli Alves Dias for his contribution to the statistical analysis

This work was supported by the Brazilian National Council for Scientific and Technological Development (CNPq, 406744/20165).

Buba Embaló (grant number 1642898) was supported by CAPES (Coordination for the Improvement of Higher Education Personnel), Ministry of Education, Brazil and Andressa Fernanda Paza Miguel (grant number 131412/2017-5) was supported by CNPq (Brazilian National Council for Scientific and Technological Development)..

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# 5 CONCLUSÕES

A intensidade de infiltrado inflamatório presente na lâmina própria de lesões potencialmente malignas intra-bucais teve relação com a intensidade de displasia presente no epitélio das mesmas. A correlação positiva entre intensidade do infiltrado inflamatório e a expressão de Vimentina, sugere uma possível relação entre inflamação e transição-epitélio mesenquimal (EMT). Diante disso, seria possível inferir que a inflamação estaria envolvida na progressão das displasias epiteliais para carcinoma epidermoide bucal ao contribuir na invasão do tecido conjuntivo adjacente, por meio da ativação de EMT.

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# ANEXO I

## PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

**Título da Pesquisa:** O papel do estroma no desenvolvimento e progressão do câncer de boca

**Pesquisador:** Elena Riet Correa Rivero

Área Temática:

**Versão:** 1

**CAAE:** 42976715.3.0000.0121

**Instituição Proponente:** Departamento de Patologia

**Patrocinador Principal:** Financiamento Próprio

DADOS DO PARECER

**Número do Parecer:** 1.005.587

**Data da Relatoria:** 30/03/2015

**Apresentação do Projeto:**

Trata-se de projeto vinculado à linha de pesquisa "Etiologia, Diagnóstico, Prevenção e Terapias aplicadas à Odontologia", do Programa de Pós-graduação em Odontologia da UFSC. A professora coordenadora faz parte do grupo de Pesquisa em Diagnóstico Bucal da UFSC. O projeto desdobrar-se-á em uma tese de doutorado e um Trabalho de Conclusão de Curso. Como amostra positiva de neoplasia invasiva serão incluídos casos de carcinoma epidermoide de boca (CEB) e como amostra de tecido não neoplásico serão incluídos casos

de HFI (hiperplasia fibrosa inflamatória). A seleção dos casos será feita com base no diagnóstico histopatológico e na análise das lâminas coradas em H&E. Com base na casuística desse Serviço de Diagnóstico espera-se no final ao menos 25 casos de DEBM; 25 casos de DEBM, 20 casos de carcinoma epidermoides de boca e 20 casos de HFI.

**Objetivo da Pesquisa**:

Objetivo Primário:

- O objetivo principal deste projeto é contribuir com o entendimento sobre o processo de invasão do CEB (carcinoma epidermoide de boca), por meio do estudo das interações parênquima/estroma nos mecanismos de crescimento e invasão tumoral.

Objetivo Secundário:

1- Promover um levantamento dos laudos histopatológicos de lesões diagnosticadas como displasias epiteliais, CEB e hiperplasia fibrosa inflamatória (HFI), presentes nos arquivos do Laboratório de Patologia Bucal (LPB) da Universidade Federal de Santa Catarina (UFSC);

2- Proceder a avaliação histológica dos casos selecionados e Classificar as displasias epiteliais segundo o sistema binário, em displasias de alto risco de malignização (DEAM) e baixo risco de malignização (DEBM);

3- Investigar a presença de fibroblastos senescentes, por meio de marcadores de senescência celular (p16 e beta

galactosidase), assim como por meio de marcadores de FAC (podoplanina), na lâmina própria de DEBM, DEAM e HFI, assim como no estroma de CEB.

4- Investigar a expressão de caveolina-1, osteopontina e MMP-2 na lâmina própria de DEBM, DEAM e HFI, e no CEB.

5- Estabelecer o índice de proliferação epitelial, por meio da marcação do antígeno Ki-67, em DEBM, DEAM, HFI e CEB;

6- Comparar a expressão das proteínas em estudo nos casos de DEBM, DEAM, HFI e CEB;

7- Comparar a expressão das proteínas em estudo nos casos de displasias epiteliais que evoluíram para carcinoma epidermoide;

8- Fazer a correlação das proteínas em estudo nos casos de DEBM, DEAM, HFI e CEB.

9- Correlacionar os achados deste estudo com os já existentes na literatura.

**Avaliação dos Riscos e Benefícios:**

Em relação aos riscos da pesquisa, os pesquisadores esclarecem que "Durante a pesquisa será apenas utilizado o material resultante de biópsia da lesão, previamente realizada, o qual encontra-se armazenado nos arquivos do LPB, sem causar qualquer tipo de desconforto aos pacientes. Como haverá acesso aos dados presentes nas fichas de

biopsia e laudos histopatológicos, há um risco de perda de sigilo dessas informações, mas os pesquisadores garantem que tomarão todos os cuidados para evitar que isso ocorra".

No que se refere aos benefícios do estudo, observa-se que "envolvem a produção de conhecimento científico podendo servir de base para outros estudos, e possivelmente tentar ajudar os próximos pacientes que tenham a mesma doença no futuro, facilitando o seu diagnóstico".

**Comentários e Considerações sobre a Pesquisa:**

Sem comentários adicionais.

Continuação do Parecer: 1.005.587

**Considerações sobre os Termos de apresentação obrigatória:**

Todos os documentos necessários ao processo estão disponíveis na Plataforma Brasil e de acordo com a legislação vigente: folha de rosto; projeto de pesquisa; informações detalhadas sobre o projeto, incluindo cronograma e orçamento; e termo de consentimento livre e esclarecido (TCLE) a ser apresentado aos participantes da pesquisa.

**Recomendações:**

Não há.

**Conclusões ou Pendências e Lista de Inadequações:**

De acordo com o exposto nesse parecer, o projeto de pesquisa "O papel do estroma no desenvolvimento e progressão do câncer de boca" deve ser considerado APROVADO.

**Situação do Parecer:**

Aprovado
Necessita Apreciação da CONEP:
Não
Considerações Finais a critério do CEP:

FLORIANOPOLIS, 30 de Março de 2015

Assinado por:
**Washington Portela de Souza**
**(Coordenador)**

# ANEXO II

## Author Guidelines

**The median processing time from submission to first decision for manuscripts submitted to *Oral Diseases* in the prior 12 months is 22 days.**

**Content of Author Guidelines**: 1. General, 2. Ethical Guidelines, 3. Manuscript Submission Procedure, 4. Manuscript Types Accepted, 5. Manuscript Format and Structure, 6. After Acceptance.

**Relevant Documents**: Online Open Order Form, Colour Work Agreement Form, Standard Release Form for photographic consent

**Useful Websites**: Submission Site, Articles Published in *Oral Diseases*, Author Services, Wiley-Blackwell's Ethical Guidelines, Guidelines for Figures

### 1. GENERAL

The editors encourage submissions of original articles, review articles, reports of meetings, book reviews and correspondence in the form of letters to the editor. *Oral Diseases* does not accept case reports.

Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in *Oral Diseases*. Authors are encouraged to visit Wiley-Blackwell Author Services for further information on the preparation and submission of articles and figures.

### Avoiding allegations of plagiarism

The journal to which you are submitting your manuscript employs text matching software (iThenticate) to ensure against plagiarism. By submitting your manuscript to this journal you accept that your manuscript may be screened for plagiarism against previously published work. Authors should consider whether their manuscript may raise concerns via iThenticate,

which will signal whether a paper is likely in any way to be plagiarized in a formal sense. iThenticate will also, however, signal whether a paper may be plagiarized by repeating work of the submitting authors and thus be regarded as duplicate or redundant publication. Experience shows that, on occasion, large sections of submitted manuscripts can be close to verbatim in word choice from that seen in other papers from the authors' group. This has nothing to do with simple repetition of names/affiliations, but does involve common (not necessarily "standard") phrases that are more appropriately referenced instead of repeating. Alternatively, they can be rephrased differently. Previously published results, including numerical information and figures or images, should be labeled to make it clear where they were previously reported. Papers that present new analyses of results that have already been published (for example, subgroup analyses) should identify the primary data source, and include a full reference to the related primary publications. *Oral Diseases* will review and publish accepted mansucripts that report data included in conference proceedings in abstract form. In such cases, authors must be clear to readers that part of all of the manuscript's data have already been published in abstract form by so indicating using a footnote to the title that states the confrence proceedings in which the relevant abstract was published. For full guidance on text matching and plagiarism, please refer to Section 3 ('Research Integrity') of Wiley's Ethics Guidelines at https://authorservices.wiley.com/ethics-guidelines/index.html.

## 2. ETHICAL GUIDELINES

*Oral Diseases* adheres to the ethical guidelines given below for publication and research.

### 2.1. Authorship and Acknowledgements

**Authorship**: *Oral Diseases* adheres to the International Standards for Authors published by the Committee on Publication Ethics (COPE). All authors named on a paper should agree to be named on the paper, and all authors so named should agree to the submission of the paper to *Oral*

*Diseases* and approve the submitted and accepted versions of the publication. Any change to the author list should be approved by all authors, including any author who has been removed from the list.

*Oral Diseases* also adheres to the definition of authorship set up by The International Committee of Medical Journal Editors (ICMJE). According to the ICMJE authorship criteria should be based on 1) substantial contributions to conception and design of, or acquisition of data or analysis and interpretation of data, 2) drafting the article or revising it critically for important intellectual content and 3) final approval of the version to be published. Authors should meet conditions 1, 2 and 3.

It is a requirement that the corresponding author submit a short description of each individual's contribution to the research and its publication. Upon submission of a manuscript all co-authors should also be registered with a correct e-mail addresses. If any of the e-mail addresses supplied are incorrect, the corresponding author will be contacted by the Journal Administrator.

**Acknowledgements**: Authors must acknowledge individuals who do not qualify as authors but who contributed to the research. Authors must acknowledge any assistance that they have received (e.g. provision of writing assistance, literature searching, data analysis, administrative support, supply of materials). If/how this assistance was funded should be described and included with other funding information. “Acknowledgements” should be brief and should not include thanks to anonymous referees and editors. Where people are acknowledged, a covering letter demonstrating their consent must be provided.

### 2.2. Ethical Approvals

**Human Subjects**: Experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical AssociationDeclaration of Helsinki (version 2002) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be

accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included.

**Photographs of People**: Oral Diseases follows current HIPAA guidelines for the protection of patient/subject privacy. If an individual pictured in a digital image or photograph can be identified, his or her permission is required to publish the image. The corresponding author must either submit a letter signed by the patient authorizing Oral Diseases to publish the image/photo, or complete the 'Standard Release Form for photographic consent' available at the top of this page or by clicking the "instructions and Forms" link on the ScholarOne Manuscripts submission site. The approval must be received by the Editorial Office prior to final acceptance of the manuscript for publication. Otherwise, the image/photo must be altered such that the individual cannot be identified (black bars over eyes, tattoos, scars, etc.). Oral Diseases will not publish patient photographs that will in any way allow the patient to be identified, unless the patient has given their express consent.

Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

**Animal Study**: When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

### 2.3 Clinical Trials

Clinical Trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist and flowchart should also be included

in the submission material. Clinical trials can be registered in any free, public clinical trials registry such as http://www.clinicaltrials.gov or http://isrctn.org/. A list of further registries is available at http://www.who.int/ictrp/network/primary/en/. As stated in an editorial published in *Oral Diseases*(12:217-218), 2006), all manuscripts reporting results from a clinical trial must indicate that the trial was fully registered at a readily accessible website. The clinical trial registration number and name of the trial register will be published with the paper.

### 2.4 DNA Sequences and Crystallographic Structure Determinations

Papers reporting protein or DNA sequences and crystallographic structure determinations will not be accepted without a Genbank or Brookhaven accession number, respectively. Other supporting data sets must be made available on the publication date from the authors directly.

### 2.5 Conflict of Interest and Source of Funding

All sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential grant holders should be listed. Authors are also required to disclose any possible conflict of interest. These include financial (for example patent, ownership, stock ownership, consultancies, speaker's fee). Information on sources of funding and any potential conflict of interest should be disclosed at submission under the heading "Acknowledgements".

### 2.6 Appeal of Decision

The decision on a paper is final and cannot be appealed.

### 2.7 Permissions

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**3. MANUSCRIPT SUBMISSION PROCEDURE**

*Oral Diseases* only accepts online submission of manuscripts. Manuscripts should be submitted at the online submission site: http://mc.manuscriptcentral.com/odi. Complete instructions for submitting a manuscript are available at the site upon creating an account. Assistance for submitting papers can be sought with the editorial assistant Lisa Walton at: odiedoffice@wiley.com

Upon successful submission, the journal administrator will check that all parts of the submission have been completed

correctly. If any necessary part is missing or if the manuscript does not fulfil the requirements as specified below, the corresponding author will be asked either to adjust the submission according to specified instructions or to submit their paper to another journal.

### 3.1. Getting Started

Launch your web browser (supported browsers include Internet Explorer 5.5 or higher, Safari 1.2.4, or Firefox 1.0.4 or higher) and go to the journal's online Submission Site: http://mc.manuscriptcentral.com/odi

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After you have logged into your 'Corresponding Author Centre', submit your manuscript by clicking the submission link under 'Author Resources'.

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### 3.3. Manuscript Files Accepted

Manuscripts should be uploaded as Word (.doc/.docx) or Rich Text Format (.rft) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and PDF on upload and will be used for the review process. The text file must contain the entire manuscript

including title page, abstract, text, references, acknowledgements, tables, and figure legends, but no embedded figures. In the text file, please reference figures as for instance 'Figure 1', 'Figure 2' etc to match the tag name you choose for individual figure files uploaded. Manuscripts should be formatted as described in the Author Guidelines below.

### 3.4. Blinded Review

All manuscripts submitted to *Oral Diseases* will be reviewed by two experts in the field. *Oral Diseases*uses single blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper.

### 3.5. Suggest a Reviewer

*Oral Diseases* attempts to keep the review process as short as possible to enable rapid publication of new scientific data. In order to facilitate this process, you must suggest the names and current e-mail addresses of from 2-4 potential reviewers whom you consider capable of reviewing your manuscript in an unbiased way.

### 3.6. Suspension of Submission Mid-way in the Submission Process

You may suspend a submission at any phase before clicking the 'Submit' button and save it to submit later. The manuscript can then be located under 'Unsubmitted Manuscripts' and you can click on 'Continue Submission' to continue your submission when you choose to.

### 3.7. E-mail Confirmation of Submission

After submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation e-mail after 24 hours, please check your e-mail address carefully in the system. If the e-mail address is correct please contact your IT department. The error may be caused by some sort of spam filtering on your e-mail server. Also, the e-mails should be received if the IT department adds our e-mail server (uranus.scholarone.com) to their whitelist.

### 3.8. Manuscript Status

The average time from submission to first decision for manuscripts submitted to *Oral Diseases* is 20 days. You can access ScholarOne Manuscripts (formerly known as Manuscript Central) any time to check your 'Author Centre' for the status of your manuscript. The Journal will inform you by e-mail once a decision has been made.

### 3.9. Submission of Revised Manuscripts

To upload a revised manuscript, locate your manuscript under 'Manuscripts with Decisions' and click on 'Submit a Revision'. Please remember to delete any old files uploaded when you upload your revised manuscript.

## 4. MANUSCRIPT TYPES ACCEPTED

**Original Research Articles**: Manuscripts reporting laboratory investigations, well-designed and controlled clinical research, and analytical epidemiology are invited. Studies related to aetiology, pathogenesis, diagnosis, prevention and treatment are all of interest, but all papers must be based on rigorous hypothesis-driven research. Areas of interest include autoimmune, endocrine, genetic, infectious, metabolic and mucosal diseases; cancer and pre-cancerous conditions; chemosensory, developmental, geriatric and motor disorders, pain and wound healing. Randomised trials must adhere to the CONSORT guidelines, and a CONSORT checklist and flowchart must be submitted with such papers. Please also refer to the notes under section 2.3 above. Observational studies must adhere to the STROBE guidelines, and a STROBE checklist must be submitted with such papers. Diagnostic accuracy studies must adhere to the STARD guidelines, and a STARD checklist must be submitted with such papers.

**Review Papers**: *Oral Diseases* commissions review papers and also welcomes uninvited reviews. Systematic reviews with or without meta-analyses must adhere to the PRISMA guidelines, and aPRISMA checklist and flowchart must be submitted with such papers.

**Letters to the Editors**: Letters, if of broad interest, are encouraged. They may deal with material in papers published

in *Oral Diseases* or they may raise new issues, but should have important implications. Only one letter may be submitted by any single author or group of authors on any one published paper.

**Case Reports**: *Oral Diseases* does not accept case reports and instead recommends that authors submit to *Clinical Case Reports* an open access journal published by Wiley.

**Meeting Reports**: Will be considered by the editors for publication only if they are of wide and significant interest.

**Invited Concise Reviews**: These may be submitted by invitation of the Senior Editors only, and consist of around 2500-2750 words, with a maximum of one table or image and 25 references.

**Invited Medical Reviews**: These may be submitted by invitation of the Senior Editors only, and consist of around 2500-2750 words, with a maximum of one table or image and 25 references.

**Invited Commentaries**: These may be submitted by invitation of the Senior Editors only.

**Invited Editorials**: These may be submitted by invitation of the Senior Editors only.

**Invited Book Reviews**: These may be submitted by invitation of the Senior Editors only.

## 5. MANUSCRIPT FORMAT AND STRUCTURE

### 5.1. Page Charge

Articles exceeding 6 published pages, including title page, abstract, references, table/figure legends and tables and figures, are subject to a charge of GBP70 per additional page. As a guide, one published page amounts approximately to 850 words, or two to four small tables/figures. Additional supplementary material (including text and figures), which does not fit within the page limits, can be published online only as supporting information.

### 5.2. Format

**Language**: Authors should write their manuscripts in British English using an easily readable style. Authors whose native language is not English should have a native English speaker read and correct their manuscript. Spelling and phraseology should conform to standard British usage and should be consistent throughout the paper. A list of independent suppliers of editing services can be found at http://authorservices.wiley.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

**Presentation**: Authors should pay special attention to the presentation of their findings so that they may be communicated clearly. The background and hypotheses underlying the study as well as its main conclusions should be clearly explained. Titles and abstracts especially should be written in language that will be readily intelligible to any scientist.

**Technical jargon**: should be avoided as much as possible and clearly explained where its use is unavoidable.

**Abbreviations**: Oral Diseases adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. Non-standard abbreviations must be used three or more times and written out completely in the text when first used.

**5.3. Structure:** All papers submitted to *Oral Diseases* should include:

- Title Page
- Structured Abstract (reviews need not include a structured abstract)
- Main text
- References
- (Figures)
- (Figure Legends)
- (Tables)

**Title Page**: should be part of the manuscript uploaded for review and include:

- A title of no more than 100 characters including spaces
- A running title of no more than 50 characters
- 3-6 keywords
- Complete names and institutions for each author
- Corresponding author's name, address, email address and fax number
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**Abstract:** is limited to 200 words in length and should contain no abbreviations. The abstract should be included in the manuscript document uploaded for review as well as separately where specified in the submission process. The abstract should convey the essential purpose and message of the paper in an abbreviated form set out under:

- Objective(s),
- Subject(s) (or Materials) and Methods,
- Results,
- Conclusions(s).

**The Main Text of Original Research Articles** should be organised as follows

**Introduction**: should be focused, outlining the historical or logical origins of the study and not summarize the results; exhaustive literature reviews are inappropriate. It should close with the explicit statement of the specific aims of the investigation.

**Materials and Methods** must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced. As a condition of publication, authors are required to make materials and methods used freely available to academic researchers for their own use. This includes antibodies and the constructs used to make transgenic animals, although not the animals

themselves. Other supporting data sets must be made available on the publication date from the authors directly.

***(i) Clinical trials***: As noted above, these should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist should also be included in the submission material. Clinical trials can be registered in any of the following free, public clinical trials registries: www.clinicaltrials.gov, http://clinicaltrials.ifpma.org/clinicaltrials/, http://isrctn.org/.As stated in an editorial published in *Oral Diseases* (12:217-218), 2006), all manuscripts reporting results from a clinical trial must indicate that the trial was fully registered at a readily accessible website. The clinical trial registration number and name of the trial register will be published with the paper.

***(ii)Experimental subjects***: As noted above, experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2002) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included.Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used. When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

***(iii) Suppliers*:** Suppliers of materials should be named and their location (town, state/county, country) included.

**Results**: should present the observations with minimal reference to earlier literature or to possible interpretations.

**Discussion:** may usually start with a brief summary of the major findings, but repetition of parts of the abstract or of the results sections should be avoided. The section should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.

**Acknowledgements**: Should be used to provide information on sources of funding for the research, any potential conflict of interest and to acknowledge contributors to the study that do not qualify as authors. All sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential grant holders should be listed. Acknowledgements should be brief and should not include thanks to anonymous referees and editors. Where people are acknowledged, a covering letter demonstrating their consent must be provided.

### 5.4. References

References should be prepared according to the *Publication Manual of the American Psychological Association* (6th edition). This means in-text citations should follow the author-date method whereby the author's last name and the year of publication for the source should appear in the text, for example, (Jones, 1998). For references with three to five authors, all authors should be listed only on the first occurrence of the in-text citation, and in subsequent in-text occurrences only the first author should be listed followed by '*et al.*'. The complete reference list should appear alphabetically by name at the end of the paper.

A sample of the most common entries in reference lists appears below. Please note that a DOI should be provided for all references where available. For more information about APA referencing style, please refer to the APA website. Please note

that for journal articles, issue numbers are not included unless each issue in the volume begins with page one.

**Journal article**

Example of reference with 2 to 7 authors

Beers, S. R., & De Bellis, M. D. (2002). Neuropsychological function in children with maltreatment-related posttraumatic stress disorder. *The American Journal of Psychiatry, 159*, 483–486. doi: 10.1176/appi.ajp.159.3.483

Ramus, F., Rosen, S., Dakin, S. C., Day, B. L., Castellote, J. M., White, S., & Frith, U. (2003). Theories of developmental dyslexia: Insights from a multiple case study of dyslexic adults. *Brain, 126*(4), 841–865. doi: 10.1093/brain/awg076

Example of reference with more than 7 authors

Rutter, M., Caspi, A., Fergusson, D., Horwood, L. J., Goodman, R., Maughan, B., … Carroll, J. (2004). Sex differences in developmental reading disability: New findings from 4 epidemiological studies. *Journal of the American Medical Association, 291*(16), 2007–2012. doi: 10.1001/jama.291.16.2007

**Book edition**

Bradley-Johnson, S. (1994). *Psychoeducational assessment of students who are visually impaired or blind: Infancy through high school* (2nd ed.). Austin, TX: Pro-ed.

**5.5. Tables, Figures and Figure Legends**

**Figures**: All figures and artwork must be provided in electronic format. Please save vector graphics (e.g. line artwork) in Encapsulated Postscript Format (EPS) and bitmap files (e.g. half-tones) or clinical or in vitro pictures in Tagged Image Format (TIFF).

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Unnecessary figures and parts (panels) of figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Figures should not contain more than one panel unless the parts are logically connected.

Figures divided into parts should be labelled with a lower-case, boldface, roman letter, a, b, and so on, in the same type size as used elsewhere in the figure. Lettering in figures should be in lower-case type, with the first letter capitalized. Units should have a single space between the number and unit, and follow SI nomenclature common to a particular field. Unusual units and abbreviations should be spelled out in full or defined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In general visual cues (on the figures themselves) are preferred to verbal explanations in the legend (e.g. broken line, open red triangles etc).

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## 6. AFTER ACCEPTANCE

Upon acceptance of a paper for publication, the manuscript will be forwarded to the Production Editor who is responsible for the production of the journal.

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