**LAUREN FRENZEL SCHUCH**

# EXPRESSÃO DAS PROTEÍNAS CHK2, γH2AX E TP53 EM CARCINOMA DE CÉLULAS ESCAMOSAS DE BOCA DE INDIVÍDUOS FUMANTES E NÃO FUMANTES

**Faculdade de Odontologia**
**Universidade Federal de Minas Gerais**
**Belo Horizonte**
**2019**

**UNIVERSIDADE FEDERAL DE MINAS GERAIS**

**PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA**

# ATA DA DEFESA DE DISSERTAÇÃO DA ALUNA
# LAUREN FRENZEL SCHUCH

Aos 20 dias de novembro de 2019, às 14:00 horas, na sala 3403 da Faculdade de Odontologia da Universidade Federal de Minas Gerais, reuniu-se a Comissão Examinadora composta pelos professores Maria Cassia Ferreira de Aguiar (Orientadora) – FO/UFMG, Silvia Ferreira de Sousa – FO/UFMG, Manoela Domingues Martins - UFGRS e Vanessa de Fátima Bernardes – ICB/UFMG, para julgamento da dissertação de Mestrado, área de concentração em Estomatologia, intitulada: **Expressão das proteínas chk2, h2ax e p53 em carcinoma de células escamosas de boca de indivíduos fumantes e não fumantes.** A Presidente da Banca, abriu os trabalhos e apresentou a Comissão Examinadora. Após a exposição oral do trabalho pela aluna e arguição pelos membros da banca, a Comissão Examinadora considerou a dissertação:

⋈ Aprovada

( ) Reprovada

Finalizados os trabalhos, lavrou-se a presente ata que, lida e aprovada, vai assinada por mim e pelos demais membros da Comissão. Belo Horizonte, 20 dias de novembro de 2019.

Prof(a). Maria Cassia Ferreira de Aguiar

Prof(a). Silvia Ferreira de Sousa

Prof(a). Manoela Domingues Martins

Prof(a). Vanessa de Fátima Bernardes

Lauren Frenzel Schuch

# EXRESSÃO DAS PROTEÍNAS CHK2, γH2AX E TP53 EM CARCINOMA DE CÉLULAS ESCAMOSAS DE BOCA DE INDIVÍDUOS FUMANTES E NÃO FUMANTES

Dissertação apresentada ao Colegiado de Pós-Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do grau de Mestre em Odontologia - área de concentração em Estomatologia.

**Orientadora**: Profa. Dra. Maria Cássia Ferreira de Aguiar
**Coorientadora**: Profa. Dra. Vanessa de Fátima Bernardes

Belo Horizonte
2019

## Ficha Catalográfica

S384e 2019 T

Schuch, Lauren Frenzel.
Expressão das proteínas CHK2, H2AX e P53 em carcinoma de células escamosas de boca de indivíduos fumantes e não fumantes / Lauren Frenzel Schuch. -- 2019.

76 f. : il.

Orientadora: Maria Cássia Ferreira de Aguiar.
Coorientadora: Vanessa de Fátima Bernardes.

Dissertação (Mestrado) -- Universidade Federal de Minas Gerais, Faculdade de Odontologia.

1. Dano ao DNA. 2. Quinase do ponto de checagem 2. 3. Proteína supressora de tumor p53 . 4. Neoplasias bucais. 5. Tabaco. I. Aguiar, Maria Cássia Ferreira de. II. Bernardes, Vanessa de Fátima. III. Universidade Federal de Minas Gerais. Faculdade de Odontologia. IV. Título.

BLACK - D047

Elaborada por Sérgio Barbosa dos Santos – CRB6-3182

Dedico este trabalho aos meus pais, Beatriz Frenzel Schuch e Rony Schuch, pelo suporte e amor sem medida.

## AGRADECIMENTO

À minha orientadora, **Professora Maria Cássia Ferreira de Aguiar**, por toda orientação durante essa jornada. Pelos ensinamentos, pela liberdade e pelo carinho. Toda admiração e respeito pela pessoa e profissional que és.

À minha coorientadora**, Professora Vanessa de Fátima Bernardes**, por tanto ter me ensinado, de maneira doce e gentil.

Aos demais Professores da área de Estomatologia e Patologia Bucal da FAO-UFMG: **Felipe Fonseca**, **Patrícia Caldeira**, **Ricardo Gomez** e **Sílvia Ferreira**. Agradeço pelo aprendizado, pelas horas de convivência e pela atenção nesses dois anos. Em especial aos Professores **Ricardo Mesquita** e **Tarcília Silva**, pela acolhida e carinho.

Ao Professor **Mauro Abreu**, pelo auxílio precioso e sempre disposto na estatística deste trabalho.

Ao Professor **Lucas Abreu**, pela paciência em ensinar e pelo bom convívio. Às Professoras **Amália Moreno** e **Thaís Yumi** pela amizade.

Ao Colegiado de Pós-Graduação em Odontologia, no nome da **Professora Isabela Pordeus,** pelo apoio institucional. E à **Coordenação de Pessoal de Nível Superior (CAPES)** pelo apoio financeiro.

Às minhas professoras da FO-UFPel: **Adriana Etges**, **Ana Carolina Vasconcelos**, **Ana Paula Gomes** e **Sandra Tarquinio**. Obrigada por cultivarem em mim o amor pela Estomatologia e pela Patologia. Vocês sempre serão meus exemplos.

Aos técnicos do Laboratório de Patologia da FAO-UFMG: **Daniela**, **Domenico** e **Mara**, por sempre me tratarem com carinho e de forma prestativa.

Aos colegas de pós-graduação, por compartilharem de momentos e experiências. Obrigada pelas conversas, receptividade e parceria. Em especial, ao meu grande amigo **José Alcides**, com quem dividi os melhores e os piores momentos nesses dois anos – obrigada por sempre estar ao meu lado.

À **Renata Guimarães** e ao **Rafael Santos**, por me fornecerem as ferramentas necessárias para que eu pudesse seguir em frente. Sem eles, tudo seria mais sombrio e desanimador.

Aos meus tantos e bons amigos que deixei em Pelotas. Com vocês, tudo é mais lindo, divertido e leve. Vocês fazem falta nos meus dias. Obrigada por fazermos do nosso laço ainda mais forte.

Aos meus irmãos, cunhados e sobrinhos. Obrigada pelo incentivo e compreensão, e por sempre me esperarem com tanta festa quando retorno para casa. Em especial à minha irmã **Lisandra**, por ser meu porto seguro.

Aos meus pais, **Beatriz Frenzel Schuch** e **Rony Schuch**, pelo suporte, apoio e amor sem medidas. Com vocês, tudo vale a pena. Toda minha gratidão por viver essa vida com vocês.

A **Deus**, pela vida, pela escolha certa da minha caminhada e por nunca me deixar só.

“A vida te dará poucos presentes, acredita. Se queres uma vida, é preciso que a roubes.”

Lou Andreas-Salomé

## RESUMO

O câncer de boca corresponde a cerca de 4% das doenças neoplásicas, e o carcinoma de células escamosas representa o tipo mais frequente, englobando cerca de 90 a 95% dos casos. O cigarro é o principal fator etiológico para o câncer de boca, causando danos ao DNA e mutações que se não reparadas levam ao surgimento de lesões. O dano ao DNA associado ao cigarro tem sido estudado em diferentes tipos de câncer, mas ainda pouco explorado em relação ao câncer de boca. *Checkpoint kinase* 2 (CHK2) e P53 são proteínas que estão envolvidas no processo de checagem do ciclo celular, sendo responsáveis pelo reparo ao dano ao DNA. A proteína H2AX é uma histona nuclear que sofre fosforilação em resposta aos danos ao DNA, principalmente às quebras da dupla cadeia. O objetivo deste estudo foi avaliar a resposta a danos no DNA através da expressão de CHK2, γH2AX e TP53 entre fumantes e não fumantes com carcinoma de células escamosas de boca (CCEB). Além disso, foram analisadas associações entre imuno-expressão das proteínas estudadas, dados clínico-patológicos e classificação histopatológica. Foram incluídos 35 indivíduos (18 não fumantes e 17 fumantes) com CCEB de língua e soalho bucal. Reação imuno-histoquímica foi realizada para γH2AX para identificação de quebras de fita dupla, CHK2 e TP53 para avaliação da indução de parada do ciclo celular. Análises descritivas e estatísticas foram realizadas. A pesquisa foi composta por 22 homens (62,8%) e 13 mulheres (37,2%), com idade média de 63,9 anos. Entre os não fumantes, 50% apresentaram tumores bem diferenciados, enquanto que fumantes mostraram maior número em moderadamente diferenciados e pouco diferenciados (35,3% cada). No geral, 31 (88,6%) casos foram positivos para CHK2, 27 (77,2%) foram positivos para γH2AX e 23 (65,7%) foram positivos para TP53. Não foi observada associação entre essas proteínas com hábitos de fumar e não fumar ($p > 0,05$). Semelhanças entre os padrões imuno-histoquímicos de CHK2, γH2AX e TP53 em fumantes e não fumantes com CCEB foram observadas neste estudo, assim como entre os parâmetros clínico-patológicos. De forma geral, os resultados indicaram expressão positiva para essas proteínas no CCEB. Este estudo fornece informaçãoes sobre o dano ao DNA na carcinogênese oral.



**Palavras-chave**: Dano ao DNA. CHK2. Gama-H2AX. P53. Câncer oral. Tabaco.

## ABSTRACT

### Expression of CHK2, γH2AX and TP53 proteins in smokers and non-smokers oral squamous cell carcinoma

Oral cancer accounts for about 4% of neoplastic diseases, and squamous cell carcinoma is the most common type, accounting for about 90 to 95% of cases. Cigarette smoking is the main etiological factor for oral cancer, causing DNA damage and mutations that, if not repaired, lead to lesions. Cigarette-associated DNA damage has been studied in different cancers, but is still poorly explored in relation to oral cancer. Checkpoint kinase 2 (CHK2) and P53 are proteins that are involved in the cell cycle checking process and are responsible for repairing DNA damage. The H2AX protein is a nuclear histone that undergoes phosphorylation in response to DNA damage, especially double strand breaks. The aim of this study was to assess the DNA damage response through the expression of checkpoint kinase 2 (CHK2), γH2A histone family member X (γH2AX) and TP53 among smokers and non-smokers with oral squamous cell carcinoma (OSCC). In addition, associations amongst immunoexpression of studied proteins, clinicopathologic data and histopathological grading were analyzed. Thirty-five individuals (18 non-smokers and 17 smokers) with OSCC of the tongue and/or floor of the mouth were included. Immunohistochemistry was carried out for γH2AX for identification of double-strand breaks, CHK2 and P53 for evaluation of the induction of cell cycle arrest. Descriptive and statistical analyses were performed. The survey consisted of 22 males (62.8%) and 13 females (37.2%), with a mean age of 63.9 years. Fifty percent of non-smokers OSCC were well-differentiated tumors, whereas for smokers, OSCC were moderately differentiated and poorly differentiate tumors, equally (35.3% each). Overall, 31 (88.6%) cases were CHK2-positive, 27 (77.2%) were γH2AX-positive and 23 (65.7%) were TP53-positive. No association among these proteins with smoking and non-smoking habits was observed ($p>0.05$). Similarities in the CHK2, γH2AX and P53 immunohistochemical staining pattern were observed between smokers and non-smokers with OSCC in this survey, and the immunoexpression was not associated with clinicopathologic parameters. Overall, the results indicated consistent expression of these proteins in OSCC. This study provides information about the DNA damage in oral carcinogenesis.



***Keywords:*** DNA damage. Checkpoint Kinase 2. H2AX protein. Gamma-H2AX protein. Tumor suppressor protein P53. Oral cancer. Smoking.

## LISTA DE ABREVIATURA E SIGLAS

CCEB Carcinoma de Células Escamosas de Boca
CHK2 *Checkpoint Kinase* 2
FAO Faculdade de Odontologia
H2AX *Histona Family Member*
IHQ Imuno-histoquímica
INCA Instituto Nacional de Câncer
OMS Organização Mundial da Saúde
OSCC Oral Squamous Cell Carcinoma
SPSS *Statistical Package for the Social Sciences*
UFMG Universidade Federal de Minas Gerais

**SUMÁRIO**

## 1 CONSIDERAÇÕES INICIAIS

O carcinoma de células escamosas de boca (CCEB) possui uma incidência anual de 300.000 casos e está entre as dez malignidades mais comuns do mundo, correspondendo a 90% dos cânceres orais (ALI *et al.*, 2017; CUTILLI *et al.*, 2016; FERLAY *et al.*, 2015). Ainda, as estimativas indicam um aumento global significativo para 19,3 milhões de novos casos anuais até 2025 (D'SOUZA e SARANATH, 2015). Embora avanços recentes relacionados à terapia tenham sido relatados, a resposta à conduta terapêutica, altas taxas de recidivas e a permanência de baixa taxa de sobrevida em 5 anos para alguns pacientes ainda acarretam preocupação aos especialistas (CUTILLI *et al.*, 2016; LEE *et al.*, 2015).

O CCEB representa uma neoplasia maligna de origem epitelial caracterizado histologicamente por uma intensa proliferação de células epiteliais neoplásicas que invadem a lâmina própria subjacente formando ilhas ou cordões. Essas células são caracterizadas por apresentarem pleomorfismo celular e nuclear, hipercromatismo nuclear, nucléolos evidentes, figuras de mitoses atípicas e aumento da relação núcleo-citoplasma. Classificações histopatológicas para os CCEBs surgiram na tentativa de explicar o comportamento biológico discrepante dos tumores. Broders, em 1920, propôs uma gradação histopatológica baseada no grau de diferenciação celular. Contudo, muitos autores questionam o valor dessa classificação e o da proposta pela Organização Mundial de Saúde (OMS), destacando o papel de outras características histopatológicas no comportamento biológico do tumor e propondo novas gradações (ANNEROTH *et al.*, 1986; BARNES *et al.*, 2005; LINDEMANN *et al.*, 2018; WAGNER *et al.*, 2017).

O desenvolvimento de CCEB ocorre através de um processo multifatorial que requer o acúmulo de múltiplas alterações, influenciado tanto pela influência genética do paciente, quanto por fatores ambientais - tabaco, álcool, inflamação crônica e infecção viral (APPAH *et al.*, 2018; SCULLY e BAGAN, 2009; TSANTOULIS *et al.*, 2007). O cigarro continua a ser o principal fator etiológico do câncer de boca e o mecanismo de ação envolve danos diretos ao DNA. O tabaco pode causar alteração epigenética das células epiteliais orais, inibir múltiplas funções imunológicas sistêmicas do hospedeiro e, por meio de seus metabólitos tóxicos, causar estresse oxidativo nos tecidos para induzir o CCEB (JIANG *et al.*,

2019). Nos últimos anos, foi possível um entendimento maior dos componentes cancerígenos contidos nos produtos de tabaco, bem como seu papel na geração de tumores específicos (JETHWA e KHARIWALA, 2017).

Diversas alterações genéticas ocorrem tanto em células normais quanto em células displásicas, incluindo mutações pontuais, deleções, translocações, amplificações, metilações, instabilidade de microssatélites e perda de heterozigosidade (SINEVICI *et al.*, 2016). A superexpressão de genes que promovem crescimento, sobrevivência e disseminação de células pode levar ao desenvolvimento de câncer (SCULLY e BAGAN, 2009). No entanto, a identificação dessa superexpressão de oncogenes representa um desafio. Diante disso, uma demanda por biomarcadores clinicamente confiáveis levou a um aumento da produção científica na pesquisa de possíveis alvos em níveis genéticos, mRNA, proteicos e metabólicos, sendo a grande maioria intrinsecamente ligada ao desenvolvimento de células neoplásicas malignas (D'SOUZA e SARANATH, 2015; SINEVICI *et al.*, 2016).

O ciclo celular, regulado e monitorado por pontos de checagem, é caracterizado por uma sequência de eventos que ocorrem em uma célula resultando na sua replicação ou divisão (ABREU E HOWARD, 2015; ALI *et al.*, 2017). Esses pontos de verificação são importantes pois fornecem uma parada temporária controlada em um estágio específico do ciclo que permite que a célula corrija possíveis defeitos (TAO *et al.*, 2009). Danos ao DNA ocorrem de forma corriqueira, tanto por causas exógenas quanto endógenas, desencadeando respostas que combinam o reparo do DNA com a parada temporária do ciclo celular, evitando efeitos deletérios ou morte celular (DAVIDSON *et al.*, 2018).

A tirosina quinase CHK2 tem papel importante nesse metabolismo, uma vez que está envolvida na via de reparo ao dano do DNA, capaz de ativar o P53, modular a reparação e bloquear o ciclo celular (CARRASSA & DAMIA, 2011; GUFFANTI *et al.*, 2016). A primeira evidência de que a alteração genética em CHK2 pode predispor ao câncer foi relatada por Bell e colaboradores (1999), em mutações germinativas raras no gene em famílias com síndrome de Li-Fraumeni, caracterizada por múltiplos tumores em idade precoce, com predomínio de câncer de mama e sarcomas, e está frequentemente associada a mutações germinativas no gene P53. CHK1 e CHK2 regulam funções celulares fundamentais, como replicação do DNA e progressão do ciclo celular, reestruturação da cromatina e

apoptose, representando dois mensageiros críticos dos pontos de verificação da integridade do genoma, além do seu envolvimento na evolução do câncer humano (BARTEK & LUKAS, 2003). Na ausência de P53 funcional, a interrupção do ciclo celular e o reparo do DNA dependem da função do CHK1/2 para o reparo e replicação do DNA funcional (LINDEMANN *et al.*, 2018). Enquanto que a literatura defende que o CHK1 promove o reparo tumoral, sendo altamente encontrado numa variedade de tumores humanos, incluindo câncer de mama, cólon, fígado, gástrico e carcinoma nasofaríngeo, poucos estudos tem pesquisado sobre o desfecho do CHK2 em neoplasias de cabeça e pescoço (ZHANG & HUNTER , 2013).

A histona variante H2AX pertence à família histona H2A e, como outras variantes histonas, é altamente conservada e desempenha funções celulares críticas (WEYEMI *et al.*, 2018). O gene H2AX possui um papel essencial no reparo da quebra da fita dupla do DNA e na estabilidade do genoma, sendo considerado um gene supressor de tumor (MEADOR *et al.*, 2008; WEYEMI *et al.*, 2016). Em um estudo realizado por Osterman e colaboradores (2014), ao investigar a ligação entre o dano ao DNA e o câncer de pâncreas, ficou bem estabelecido que a H2AX desempenha um papel importante na disseminação do sinal ao dano, e que a H2AX fosforilada (γH2AX) é necessária para a estabilização de numerosos fatores de resposta ao dano nas lesões do DNA. Assim, observou-se ausência de expressão de γH2AX nas amostras de tecido pancreático normal, enquanto a expressão de γH2AX foi altamente positiva tanto no ducto quanto no tecido ao seu redor na amostra do tumor. Poucos estudos tem relatado CCEB e H2AX. Chou e colaboradores, em 2011, demonstraram a proteína em amostras de CCEB, que foi considerada um importante biomarcador de resposta ao dano ao DNA em estágio inicial da carcinogênese.

O gene supressor de tumor TP53, essencial na regulação da progressão do ciclo celular, diferenciação e reparo de DNA, é um gene bem documentado na literatura por estar associado não só ao carcinoma de boca, mas também a vários outros tipos de câncer (WHYTE *et al.*, 2002; SINEVICI, 2016). Cerca de 50% dos pacientes diagnosticados com CCEB possuem mutação no gene TP53 (SINEVICI, 2016). A ativação do TP53 é decorrência do estresse celular, o que inclui dano ao DNA, hipóxia e privação nucleotídea (LINDEMANN *et al.*, 2018). Tal proteína desempenha papel crítico para reprimir a invasão do câncer e a progressão

metastática (SINEVICI *et al.*, 2016; YANG *et al.*, 2015; ZEDAN *et al.*, 2015). Estudos na literatura mostraram que os CCEBs com superexpressão ou mutação de P53 proliferam mais rapidamente e possuem natureza mais agressiva, bem como um mau prognóstico (LEE *et al.*, 2015). Corroborando tal informação, Cutilli e colaboradores (2016), em um estudo avaliando a relação do P53 com o CCEB, observaram que os tumores com superexpressão de TP53 maior que 50% apresentaram uma taxa de sobrevida, em um período de 24 meses, de 47,4%, enquanto que tumores com expressão inferior a 50% tiveram uma taxa de sobrevida de 80,8%.

Embora a associação de TP53 esteja bem estabelecida quando relacionada ao CCEB, as proteínas CHK2 e H2AX ainda foram pouco exploradas no que se refere à expressão destas no câncer oral. A avaliação de possíveis danos que podem causar ao DNA é importante para a melhor compreensão da patogênese do CCEB associada ao tabaco, com impactos no estabelecimento de medidas preventivas e terapêuticas. Diante disso, o objetivo deste estudo foi avaliar a expressão imuno-histoquímica (IHQ) das proteínas CHK2, H2AX e P53, associadas ao processo de proteção e reparo ao DNA, comparando a expressão destas em carcinomas de indivíduos fumantes e não fumantes e verificando se há associação entre as mesmas, além de investigar a associação com características clínicas e histopatológicas da lesão.

## 1.1 Objetivos da pesquisa

### 1.1.1 Objetivos gerais

O objetivo deste trabalho foi avaliar a expressão imuno-histoquímica das proteínas CHK2, γH2AX e TP53 em carcinoma de células escamosas de boca de indivíduos fumantes e não fumantes.

### 1.1.2 Objetivos específicos

a) Investigar a expressão imuno-histoquímica das proteínas CHK2, γH2AX e TP53 em amostras de CCEB de indivíduos fumantes e não fumantes;

b) Verificar se existe associação entre a expressão destas proteínas;

c) Verificar por meio de análise estatística se algum fator clínico (idade, sexo, fumo e informações da lesão) ou histopatológico (grau de diferenciação) está associado à expressão destas proteínas.

## 2 METODOLOGIA EXPANDIDA

### 2.1 Aspectos éticos

O presente trabalho foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais (COEP/UFMG), sob o número CAAE: 03012618.1.0000.5149; parecer: 3.293.055 (Anexo A), obedecendo ao exigido pela legislação brasileira, conforme as resoluções CNS nº 466/12 e 340/04 do Conselho Nacional de Saúde, sobre Diretrizes e Normas Regulamentadoras de Pesquisas Envolvendo Seres Humanos.

### 2.2 Delineamento do estudo

Trata-se de um estudo transversal, do tipo retrospectivo, com população-alvo constituída por indivíduos com diagnóstico histopatológico de carcinoma de células escamosas de boca cujo material estava armazenado no arquivo do laboratório de Patologia Bucomaxilofacial da Faculdade de Odontologia da Universidade Federal de Minas Gerais.

### 2.3 Amostra

Inicialmente, foi conduzido um estudo piloto para determinação do cálculo amostral por meio de teste estatístico, através do programa OpenEpi (versão 3.01) (DEAN et al., 2013). No entanto, não foi possível a utilização da amostra estipulada e partiu-se para uma amostra de conveniência. O estudo foi composto apenas por casos de CCEB, através de um cálculo amostral, que tiverem laudo histopatológico pela FAO-UFMG. Os pacientes foram divididos em dois grupos: fumantes e não fumantes. Foram considerados não fumantes os indivíduos que nunca fumaram ou que fumaram até 0,5 maço/ano nos últimos 10 anos (PAULIN *et al.*, 2015). Como fumantes, aqueles que usam cigarro de forma contínua ou esporádica.

## 2.4 Coleta de dados

Foram coletados, da ficha de biópsia, os seguintes dados: sexo, idade, hábitos de fumo e álcool, sintomatologia, localização anatômica e tipo de amostra (incisional ou excisional). Ainda, foi feita uma análise histológica para determinar o grau do tumor, de acordo com a Organização Mundial da Saúde (OMS) 2017 (EL-NAGGAR *et al.*, 2017), em bem diferenciado, moderadamente diferenciado e mal diferenciado (QUADRO 1).

Quadro 1 - Sistema de gradação histológica para carcinoma de células escamosas de boca proposto pela OMS (2017)

| Parâmetros | Características |
|---|---|
| Bem diferenciado | Arquitetura tecidual semelhante ao padrão normal do epitélio escamoso |
| Moderadamente diferenciado | Certo grau de pleomorfismo nuclear e atividade mitótica;<br>Pouca ceratinização |
| Pouco diferenciado | Predomínio de células imaturas;<br>Numerosas mitoses típicas e atípicas;<br>Mínima ceratinização |

Fonte: EL-NAGGAR *et al.*, 2017.

## 2.5 Análise imuno-histoquímica

Os cortes histológicos apresentaram espessura de 4 µm e foram montados em lâminas de vidro StarFrost® (Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Alemanha). Foi realizada a avaliação IHQ das proteínas CHK2, H2AX e P53, e os anticorpos estão descritos na Tabela 1.

Tabela 1 - Anticorpos primários, fabricantes, soluções para recuperação antigênica, concentrações e sistema de detecção.

| **Anticorpo (Clone)** | **Fabricante** | **Recuperação antigênica** | **Concentração** | **Sistema de detecção** |
|---|---|---|---|---|
| Anti-Phospho-Chk2 (Thr68) | Cell Signaling Technology | *Trilogy* pH 7.5 *90ºC 20min* | 1:50 | Envision/HRP |
| Anti-H2AX (Ser139) | Cell Signaling Technology | *Trilogy* pH 7.5 *90ºC 20min* | 1:200 | Envision/HRP |

| | | | | |
|---|---|---|---|---|
| Anti-P53 (DO7) | DAKO | Ác. cítrico pH 6.0 *90ºC 20min* | 1:50 | Envision/HRP |

Fonte: elaborada pela autora, 2019.

2.5.1 Protocolo de IHQ para CHK2 e γH2AX

I. Desparafinização, hidratação e recuperação antigênica: reagente de "*trilogy"* (Rocklin, USA)*;*
II. Dois banhos "*trilogy*" em água destilada;
III. Cubas em panela no vapor até a temperatura do banho atingir 90ºC;
IV. Lavagem em 5 banhos de água destilada;
V. Etapa de bloqueio com metanol + $H_2O_2$ (10v);
VI. Lavagem em 5 banhos de água destilada;
VII. Lavagem em tampão TRIS- HCl 20 mM (3 banhos de 5 minutos);
VIII. Incubação do anticorpo primário diluído na solução diluente (DAKO; Glostrup Municipality, Denmark), em câmara úmida, *overnight* – 4ºC. Lavagem 3 vezes na solução de TRIS-HCl 20 mM;
IX. Incubação do anticorpo secundário *(DAKO ENVISION + DUAL LINK-SYSTEM HRP;* Glostrup Municipality, Denmark*)*;
X. Três lavagens na solução de TRIS-HCl 20 mM (5 minutos cada banho);
XI. Lavagem das lâminas em água destilada;
XII. Lavagem das lâminas em água corrente por 10 minutos (fluxo forte);
XIII. Incubação dos cortes com a hematoxilina de Mayer filtrada (3 minutos);
XIV. Passar as lâminas rapidamente pela solução de hidróxido de amônio 10%;
XV. Mergulhar as lâminas em água corrente (fluxo forte) por 5 minutos;
XVI. Desidratação em cadeia ascendente de etanol (70%, 90% e três vezes a de 100%), diafanização em três banhos de xilol, sendo as lâminas montadas ao final do procedimento com lamínulas de vidro e Tissue-Mount™ (TissueClear® based medium. Alphen aan den Rijn, Holanda).

2.5.1 Protocolo de IHQ para TP53

I. Bateria de hidratação em cadeia descendente de etanol e três banhos em xilol;
II. Dois banhos em Ácido Cítrico pH 6.0;
III. Cubas em panela no vapor até a temperatura do banho atingir 90ºC;
IV. Lavagem em 5 banhos de água destilada;
V. Etapa de bloqueio com metanol + $H_2O_2$ (10v);

VI. Lavagem em 5 banhos de água destilada;
VII. Lavagem em tampão TRIS- HCl 20 mM (3 banhos de 5 minutos);
VIII. Incubação do anticorpo primário diluído na solução diluente (DAKO; Glostrup Municipality, Denmark), em câmara úmida, *overnight* – 4ºC. Lavagem 3 vezes na solução de TRIS-HCl 20 mM;
IX. Incubação do anticorpo secundário *(DAKO ENVISION + DUAL LINK-SYSTEM HRP;* Glostrup Municipality, Denmark*)*;
X. Três lavagens na solução de TRIS-HCl 20 mM (5 minutos cada banho);
XI. Lavagem das lâminas em água destilada;
XII. Lavagem das lâminas em água corrente por 10 minutos (fluxo forte);
XIII. Incubação dos cortes com a hematoxilina de Mayer filtrada (3 minutos);
XIV. Passar as lâminas rapidamente pela solução de hidróxido de amônio 10%;
XV. Mergulhar as lâminas em água corrente (fluxo forte) por 5 minutos;
XVI. Desidratação em cadeia ascendente de etanol (70%, 90% e três vezes a de 100%), diafanização em três banhos de xilol, sendo as lâminas montadas ao final do procedimento com lamínulas de vidro e Tissue-Mount™ (TissueClear® based medium. Alphen aan den Rijn, Holanda).

## 2.6 Análise dos resultados

A análise dos dados foi realizada no software *Statistical Package for the Social Sciences* (SPSS), versão 23.0 (SPSS Inc., Chicago, IL, EUA). As variáveis desfecho foram avaliadas quanto à normalidade por meio dos testes de Shapiro-Wilk, nos grupos fumantes e não fumantes ($p<0,05$). Testes paramétricos ou não-paramétricos, quando indicados, foram desenvolvidos para comparar os valores das proteínas CHK2, γH2AX e TP53, entre grupos fumantes e não fumantes, considerando o nível de significância $p<0,05$.

# 3 ARTIGO

Os resultados foram escritos em língua inglesa na forma de artigo científico.

## 3.1 Artigo Científico

Artigo submetido ao periódico international: Archives of Oral Biology (Qualis A1 - Fator de impacto: 1.663; em 2019). Encontra-se aguardando resposta dos revisores.

**Immunoexpression of CHK2, H2AX and P53 is similar in oral squamous cell carcinoma samples from smoking and non-smoking patients**

**Running title:** Immunoexpression of CHK2, H2AX and P53

**Highlights**

- Similarities in CHK2, H2AX and P53 staining were observed in smokers and non-smokers with OSCC.
- There is a consistent expression of CHK2, H2AX and P53 proteins in OSCC.
- Pathway of DNA damage as an early and advanced event in oral carcinogenesis.

**Abbreviations**

CHK2: checkpoint kinase 2

DDR: DNA damage response

DSBs: double-strand breaks

H&E: hematoxylin and eosin

H2AX: H2A histone family member X

OSCC: oral squamous cell carcinoma

UFMG: Universidade Federal de Minas Gerais

**Abstract**

*Objective:* To assess the DNA damage response through the expression of checkpoint kinase 2 (CHK2), H2A histone family member X (H2AX) and P53 among smokers and non-smokers with oral squamous cell carcinoma (OSCC). In addition, associations between the immunoexpression of the studied proteins and clinicopathologic data and histopathological grading were analyzed.

*Design:* Thirty-five individuals (18 non-smokers and 17 smokers) with OSCC of the tongue and/or floor of the mouth were included. Immunohistochemistry for H2AX was carried out for the identification of double-strand breaks, CHK2 and P53 in order to evaluate the induction of the cell cycle. Descriptive and statistical analyses were performed.

*Results:* The sample consisted of 22 males (62.8%) and 13 females (37.2%), with a mean age of 63.9 ± 11.8 years. The OSCC of non-smokers were well-differentiated tumors in 50% of cases, and those of smokers were equally distributed into moderately differentiated and poorly differentiated tumors (35.3% each). Overall, 31 (88.6%) cases were CHK2-positive, 27 (77.1%) were H2AX-positive and 23 (65.7%) were P53-positive, with no difference between smokers and non-smokers ($p>0.05$). No association was found between proteins and clinicopathologic data ($p>0.05$).

*Conclusions:* Similarities in the CHK2, H2AX and P53 immunohistochemical staining pattern were observed between smokers and non-smokers with OSCC in this survey, and the immunoexpression was not associated with clinicopathologic parameters. Overall, the results indicated consistent expression of these proteins in OSCC. This study provides information about the pathway of DNA damage as an early and advanced event in oral carcinogenesis.

**Keywords:** DNA Damage, Checkpoint Kinase 2, H2AX protein, Gamma-H2AX protein, Tumor Suppressor Protein p53, Oral cancer.

## 1. Introduction

Oral squamous cell carcinoma (OSCC) is a global problem with an annual incidence of 300,000 cases (D'Cruz, Vaish, & Dhar, 2018). Genetic, epigenetic and environmental factors are involved in this multifactorial disease (Chi, Day, & Neville, 2015; Ali et al., 2017). Cigarette smoking is the main etiologic factor of oral cancer and its mechanism of action involves direct DNA damage (Jethwa & Khariwala, 2017). During carcinogenesis, some pathways may be altered, modifying tumor proliferation and apoptosis, and inducing cell transformation and clonal expansion of tumor cells (Monteiro et al., 2018). DNA damage is a recurrent phenomenon in metabolism that can be induced by exogenous and endogenous agents. When these factors accumulate, they can cause genomic instability, which eventually results in the carcinogenesis process (Nikitakis et al., 2018).

Cigarette smoke contains reactive oxidants which may cause macromolecular damage to exposed cells. Thus, the production of reactive oxygen species may damage DNA, occurring in the form of mutations, deletions, changes in sugar bases, cytosine halogenation or oxidation, and methylation (Murugan, Munirajan, & Tsuchida, 2012; De Oliveira, Da Silva, Mariz, Pereira, & De Oliveira, 2015). This fact suggests that the levels of specific proteins related to DNA damage such as checkpoint kinase 2 (CHK2), H2A histone family member X (H2AX) and P53 in smokers might serve as a measure of their cancer risk (Valinluck & Sowers, 2007).

The DNA damage response (DDR) represents the cell's ability to restore genomic changes caused by endogenous or exogenous mutagens (Nikitakis et al., 2018). H2AX phosphorylation is a key step in the DDR, playing a role in signaling and initiating the repair of double-strand breaks (DSBs) (Sedelnikova & Bonner, 2006; Bonner et al., 2008; Palla et al., 2017). DSBs must be repaired quickly and precisely to avoid cell death, chromosomal aberrations, mutations and, in certain cases, initiation of pathological effects

such as cancer. The rapid phosphorylation of H2AX represents an early cellular response to DSBs (Mah, El-Osta & Karagiannis, 2010).

Moreover, in response to DNA damage, a complex signaling network organizes cell-cycle checkpoints allowing cell cycle arrest and DNA repair, or activates senescence or cell death (Roos & Kaina, 2013). CHK2 is central to transducing the DNA damage signal and has been implicated in the mediation of both G1/S and G2/M cell-cycle arrest in a distinct pathway through P53 (Antoni, Sondha, Collins & Garrett, 2007; Zannini, Delia, & Buscemi, 2014). In addition, P53 is essential in regulating cell cycle progression, differentiation, DNA repair, and apoptosis. Almost 50% of individuals with oral cancer exhibit *P53* gene mutation and the high frequency of P53 mutations in human cancers highlights its role as a tumor suppressor (Sinevici & O'sullivan, 2016).

Nevertheless, the effect of smoking on the expression of CHK2 and H2AX in OSCC has not been well explored (De Oliveira et al., 2015; Nikitakis et al., 2018). In this study, we evaluated the immunoexpression of CHK2, H2AX and P53 proteins associated with the DNA protection and repair process among smokers and non-smokers with OSCC. Additionally, we analyzed the associations of proteins with clinicopathologic data and histopathological grading.

## 2. Materials and Methods

### *2.1 Study design and ethical approval*

This retrospective and cross-sectional study evaluated 35 paraffin-embedded tissue specimens of OSCC. The specimens were obtained from the archives of the Oral and Maxillofacial Pathology Laboratory of Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil. The study was approved by the Institutional Ethics Committee (No.

03012618.1.0000.5149) and the patient's identity remained anonymous according to the Declaration of Helsinki.

*2.2 Patients and samples*

Individuals with OSCC were analyzed according to sex, age, anatomical location (tongue and/or floor of the mouth), alcohol consumption (yes or no), and smoking habit. Never smokers with a ≤0.5 pack-years smoking history, as well as smokers with a >10 pack-years smoking history were considered (Paulin et al., 2015).

The histopathological diagnosis was confirmed by two independent oral and maxillofacial pathologists (V.F.B. and M.C.F.A.), who were blinded to the clinicodemographic data, by reviewing the sections stained with hematoxylin and eosin (H&E) retrieved from the files. Disagreements were jointly reviewed to reach a consensus.

All samples were graded as well differentiated, moderately differentiated or poorly differentiated according to the World Health Organization (El-Naggar, Chan, Grandis, Takata, & Slootweg, 2017). The exclusion criteria ruled out OSCC individuals submitted to radiotherapy, chemotherapy or other treatments before the surgical approach.

*2.3 Immunohistochemical staining*

For the immunohistochemical study, 4-µm thick sections were obtained from paraffin-embedded tissue blocks and mounted on polarized slides (StarFrost®, Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany). Monoclonal antibodies, including anti-phospho-CHK2 (clone Thr68; Rabbit; Cell Signaling Technology, Danvers, MA, US; 1:50), anti-H2AX (clone Ser19; Rabbit; Cell Signaling Technology, Danvers, MA, US; 1:200), and anti-P53 (clone DO7, Mouse; Dako, Carpinteria, CA, US; 1:50) were used. The antigen-retrieval step was performed using the TRILOGY™ Concentrate (Cell

Marque, Rocklin, CA, US; 1:100) at a temperature of 96°C in a digital water bath (DeLeo, Porto Alegre, RS, Brazil) for 30 minutes. Next, the sections were treated with the EnVision+ Dual Link System-HRP (Dako, Carpinteria, CA, US). 3.3'-Diaminobenzidine was used as the chromogen (Dako, Carpinteria, CA, US).

*2.4 Immunohistochemical assessment*

All cases were evaluated by one observer (L.F.S.) using an eyepiece grid coupled to a light microscope (Zeiss Axiostar, Ser. 48824, Oberkochen, Germany). Immunostaining was evaluated using a semiquantitative analysis of representative regions of each specimen. The slides were analyzed with a light microscope at a final magnification of 400×. Immunostaining was scored by counting the percentage of cells expressing the above-mentioned proteins in at last 10 to 15 different fields. Semi-quantitative analysis was performed based on the proportion of positive neoplastic cells relative to all neoplastic cells throughout the tissue section (Soares et al., 2017). For each brown stain, CHK2 expression was defined as nuclear, and cytoplasmic immunoreactivity (Lee et al., 2014), H2AX (Nikitakis et al., 2018) and P53 (Nagao et al., 2017) were defined as nuclear immunoreactivity. The immunoreactivity expression was considered negative (0%), normal (<50%) and overexpression (≥50%) (Lassus, Leminen, Lundin, Lehtovirta & Butzow, 2003; Karpathiou et al., 2006).

*2.6 Data analysis*

Statistical tests were carried out using by the Statistical Package for the Social Sciences (SPSS) software (IBM Corp., version 23.0, Armonk, USA). Outcome variables were assessed for normality using the Shapiro-Wilk test for the smoker and non-smoker groups ($p<0.05$). The Mann-Whitney test was used to compare the values of CHK2, H2AX,

and P53 proteins according to the clinical features (smoking habits, alcohol consumption, and anatomical location). The level of significance was set at $p<0.05$.

## 3. Results

### *3.1 Demographic and clinicopathological data*

The demographic and clinicopathological characteristics of the sample are depicted in Table 1. Thirty-five OSCC were included. The sample consisted of 22 males (62.8%) and 13 females (37.2%), with a male-to-female ratio of 1.7:1. The mean age of the sample as a whole was 63.9 years (range: 47 to 86 ± 11.8 years). Of the study subjects, 18 (51.4%) were non-smokers and 17 (48.6%) were smokers. Among the smokers, males (n=14, 82.3%) in the sixth decade of life (n=10, 58.9%) were most affected. Regarding non-smokers, females (n=10, 55.6%) in the eighth decade of life (n=6, 33.3%) were most affected. Alcohol consumption was reported by 70.6% (n=12) of smokers and 29.4% (n=5) of non-smokers.

As regards histological grading, 40% (n=14) were well-differentiated tumors, 31.4% (n=11) were moderately differentiated, and 28.6% (n=10) poorly differentiated. Fifty percent of non-smokers' OSCC were well-differentiated tumors, whereas for smokers, OSCC were moderately differentiated and poorly differentiated tumors in equal proportions (n=6, 35.3% each).

### *3.2 Immunoexpression of CHK2, H2AX and P53 positive cells*

Overall, most cases exhibited positivity (normal or overexpression) for CHK2, H2AX and P53. Thirty-one (88.6%) cases were CHK2-positive, 27 (77.2%) were H2AX-positive, and 23 (65.7%) were P53-positive. Figure 1 shows the distribution of immunostaining among non-smokers and smokers.

In smokers' OSCC samples, CHK2 staining ranged from 0 to 83.3% of positive cells (Figure 2A). One case was negative, six cases demonstrated normal positivity, and 10 showed overexpression. The expression in non-smokers' OSCC was closely similar (Figure 2B), with an expression index ranging from 0 to 91.2%. Three cases were negative, seven were normal, and eight showed overexpression.

The expression of H2AX was also quite similar in smokers (Figure 2C) and non-smokers (Figure 2D). However, disparities in mean number of positive cells were observed between smokers (0 to 92.9%) and non-smokers (0 to 53.5%). Two OSCC of smokers showed H2AX overexpression, 11 showed normal expression and four were negative. Four OSCC of non-smokers were negative, 11 were considered to have normal expression, and one showed overexpression.

The expression of P53 positive cells ranged from 0 to 91.9% for smokers and from 0 to 92.8% for non-smokers (Figure 2E and 2F). Six cases of each group were negative, and two cases of each group showed normal staining. Nine cases of smokers and 10 cases of non-smokers were considered to show overexpression.

No association was found between protein immunoexpression and the clinicopathological features of smokers and non-smokers ($p>0.05$). Also, no statistically significant difference was observed between groups (smokers vs. non-smokers) regarding CHK2 ($p=0.909$), H2AX ($p=0.807$) and P53 ($p=0.546$) proteins (Figure 3). No correlation was found among the proteins.

## 4. Discussion

Tobacco smoking involves a high risk for human malignancies, including oral cancer, because it contains multiple carcinogens that cause genetic instability (Beynon et al., 2018). Therefore, a worse prognosis would be expected for cancer patients who are

smokers. The present study investigated the expression of molecules related to the DNA damage response in smokers and non-smokers with OSCC. The selected markers participate in different stages of the DNA damage response, from identification of DSBs (H2AX) to induction of cell cycle arrest (CHK2 and P53). Herein, the immunoexpression of CHK2, H2AX and P53 was similar in smoking and non-smoking OSCC samples. Likewise, a previous study showed similar carcinogenic pathways and outcomes in oral premalignant lesions of smokers and non-smokers (de la Oliva et al., 2019).

The carcinogenic environment is represented by continuous genomic instability involving the activation of oncogenes and inactivation of tumor suppressor genes (Nikitakis et al., 2018; de la Oliva et al., 2019). As a consequence, the DNA damage response system suffers a breakdown. Moreover, epigenetic regulation, dietary factors, oral homeostasis and environmental pressures may be implicated in OSCC of both non-smokers and smokers (De Oliveira et al., 2015; Sarode, Sharma, Sarode, & Patil, 2019). Nevertheless, this similarity may explain the absence of difference between groups.

CHK2 is a key regulator kinase involved in the DNA-damage response-signaling pathway. Induced by DNA DSBs, ataxia telangiectasia mutated participates in the activation of CHK2, which is subsequently phosphorylated to block the cell cycle and to activate the transcription of repair genes (Squatrito et al., 2010; Matthews, Jones, & Collins, 2013; Tu et al., 2013). Studies have reported that CHK2 is linked to tumor progression in high-grade serous carcinoma and associated with an advanced TNM stage and poor prognosis in gastric malignant tumors (Lee et al., 2014; Davidson et al., 2018). In this study, although CHK2 expression was slightly lower in non-smokers than in smokers, protein immunoexpression did not differ between the two groups and no other association with clinicopathological features was demonstrated. The influence of tobacco on DNA repair and CHK2 action has been demonstrated in other studies (Tanaka et al., 2007; Zhao,

Albino, Jorgensen, Traganos & Darzynkiewicz, 2009; Li et al., 2013). An *in vitro* investigation showed an attenuation of CHK2 phosphorylation in lung epithelial cells treated with nicotine (Nishioka et al., 2011). In the present survey, the similarity between groups could be overridden by a large sample.

The tumor suppressor P53 has been reported to be another key target of CHK2 in response to DNA damage (Stolz, Ertych, & Bastians, 2011). P53 is reported to be mutated by over 80% in all cancers (Harjes, 2019). Loss of this protein prevents cell cycle arrest and apoptosis in the DNA damage scenario (Senturk & Manfredi, 2013). P53 changes are mainly caused by exogenous factors, particularly tobacco carcinogens in cases of OSCC (Hsieh et al., 2011). In the *P53* gene, the frequency of mutations is higher in lung tumors from smokers than in lung tumors from non-smokers Additionally, the occurrence of *P53* mutations in lung cancer samples from smokers has been observed to be dependent on lifetime cigarette consumption or duration of smoking (Takeshima et al., 1993). Regarding prostate tissue, smoking-related carcinogens can alter the expressions of some suppressor genes such as P53, suggesting that changes in these genes in prostate gland epithelia may possibly increase the risk for prostate carcinoma (Boran et al., 2017).

Herein, we observed an overexpression of P53 in 19/35 (54.3%) of cases, with no difference between smokers and non-smokers. Other studies have also found a similar expression of P53 in OSCC of smokers and non-smokers (Zaid, Azar-Maalouf, Barakat, & Chantiri 2018; de la Oliva et al., 2019). In one study, all specimens were P53 positive, independent of tobacco (Nikitakis et al., 2018). The authors stated that the main molecular alterations in oral premalignant lesions and those associated with the progression to OSCC are shared by non-smokers and smokers and appear in the early stages of carcinogenesis.

In recent years, the phosphorylated histone H2AX (γ-H2AX) marker has become a robust tool used to monitor DNA DSBs in cancer research, and has been suggested to play

a potential role in carcinogenesis and early cancer diagnosis (Sedelnikova & Bonner, 2006; Bonner et al., 2008; Palla et al., 2017). Accordingly, many studies have revealed a correlation between high γ-H2AX levels and worse prognosis and reduced disease-free survival in breast cancer (Wang, Zhang, Xia, Jiang, & Wang, 2019), ovarian cancer (Mei et al., 2015), colon carcinoma (Sedelnikova & Bonner, 2006), hepatocellular carcinoma (Xiao et al., 2015), and melanoma (Warters, Adamson, Pond, & Leachman, 2005). Interestingly, an association of H2AX positivity in OSCC with reduced overall survival time has been reported (Oliveira-Costa et al., 2014).

In the present study, no association was observed between clinicopathologic features of smokers and non-smokers and H2AX protein. However, a study has reported that the DNA DSB γ-H2AX marker exists in smoke-exposed placentae and the cessation of smoking reduces DSB DNA damage to the levels associated with non-smokers (Slatter et al., 2014). In addition, available literature data have demonstrated that exposure of A549 (human lung adenocarcinoma) cells to tobacco smoke or of NHBE (normal human bronchial epithelial) cells to smoke condensate induced γ-H2AX (Albino et al., 2004; Albino et al., 2009).

In our study, the majority of non-smokers (70.6%) did not have associated alcohol consumption and were women in the eighth and ninth decades of life. In fact, other OSCC series that included non-smokers also reported similar data (Koo, Barrowman, McCullough, Iseli, & Wiesenfeld, 2013; DeAngelis et al., 2018). Moreover, some studies that discussed changes in the genetic profile of OSCC when comparing smoker and non-smokers have been reported elsewhere with divergent results and the etiopathogenesis of OSCC in the non-smoking population remains unknown (DeAngelis et al., 2018).

While in some studies smoking history did not play a differential role in carcinogenesis (Katoh et al., 1998; Kietthubthew et al., 2001; Chaves et al., 2004), the vast

majority have reported that smokers are more subjected to genetic instability (Park et al., 2000; Prior et al., 2006; Sharma et al., 2006; Bau et al., 2017; Chen et al., 2007; Tsai et al., 2009). Although tobacco can cause epigenetic changes in oral epithelial cells, inhibit multiple systemic immune functions of the host, and induce oxidative stress in tissues through its toxic metabolites, leading to OSCC (Jiang, Wu, Wang & Huang, 2019), there is no specific mutation signature associated with smoking for OSCC (Chaves et al., 2004; Pickering et al., 2014; Jethwa & Khariwala, 2017; Jiang, Wu, Wang, & Huang, 2019; Tomar, 2019). The mutation signature from smoking appears to be site-specific and has been defined for lung tumors. Laryngeal squamous cell carcinoma also exhibits the strongest smoking signature among the head and neck squamous cell carcinoma sites, but there is no defined signature related to smoking for tumors at other sites (Brennan, Koenig, Gentles, Sunwoo, & Gevaerta, 2017).

Besides the limited sample, some other restrictions should be considered. The evaluation of tobacco smoking was based on self-reports documented in medical records. Thus, it was not possible to assess the amount of passive smoking of environmental tobacco and other carcinogens to which the patients may have been exposed. Furthermore, we did not consider patient diet, hormonal status, HPV infection or second-hand smoking. Thus, the effect of these factors on the immunoexpression of the studied proteins remains to be investigated.

## 5. Conclusions

The present study showed similarities in the CHK2, H2AX and P53 immunohistochemical staining pattern between smokers and non-smokers with OSCC. Overall, the results indicated consistent expression of these proteins in OSCC with marked nuclear and/or cytoplasmic labeling. It would be worth investigating if these characteristics

persist in non-smokers with poorly differentiated OSCC. This study provides insights on the pathway of DNA damage as an early and advanced event in oral carcinogenesis.

**Acknowledgements**

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES, Finance Code 001), Brazil. L.F.S. and J.A.A.A. are the recipients of fellowships. We gratefully acknowledge Prof. Carolina C. Gomes, Department of Pathology, Biological Sciences Institute, Universidade Federal de Minas Gerais, for donating the H2AX antibody. Mrs. E. Greene provided English editing of the manuscript.

**References**

Albino, A. P., Huang, X., Jorgensen, E., Yang, J., Gietl, D., Traganos, F., & Darzynkiewicz, Z. (2004). Induction of H2AX phosphorylation in pulmonary cells by tobacco smoke: a new assay for carcinogens. *Cell Cycle*, 3, 1062–1068.

Albino, A. P., Jorgensen, E. D., Rainey, P., Gillman, G., Clark, T. J., Gietl, D., Zhao, H., Traganos, F., & Darzynkiewicz, Z. (2009). gammaH2AX: A potential DNA damage response biomarker for assessing toxicological risk of tobacco products. *Mutation Research*, 678, 43–52. https://doi.org/10.1016/j.mrgentox.2009.06.009.

Ali, J., Sabiha, B., Jan, H. U., Haider, S. A., Khan, A. A., & Ali, S. S. (2017). Genetic etiology of oral cancer. *Oral Oncology*, 70, 23–28. https://doi.org/10.1016/j.oraloncology.2017.05.004.

Antoni, L., Sondha, N, Collins I., & Garrett, M. D. (2007). CHK2 kinase: cancer susceptibility and cancer therapy - two sides of the same coin? *Nature Reviews Cancer*, 7, 925–-936. https://doi.org/10.1038/nrc2251.

Bau, D. T., Tsai, M. H., Huang, C. Y., Lee, C. C., Tseng, H. C., Lo, Y. L., Tsai, Y., Tsai, F. (2007). Relationship between Polymorphisms of Nucleotide Excision Repair Genes and Oral Cancer Risk in Taiwan: Evidence for Modification of Smoking Habit. *Chinese Journal of Physiology*, 50, 294-300.

Beynon, R. A, Lang, S., Schimansky, S., Penfold, C. M., Waylen, A., Thomas, S. J., Pawlita, M., Waterboer, T., Martin, R. M., May, M., Ness, A. R. (2018). Tobacco smoking and alcohol drinking at diagnosis of head and neck cancer and all-cause mortality: Results from head and neck 5000, a prospective observational cohort of

people with head and neck cancer. *International Journal of Cancer*, 143, 1114-1127. http://doi.org/10.1002/ijc.31416.

Bonner, W. M., Redon, C. E., Dickey, J. S., Nakamura, A. J., Sedelnikova, O. A., Solier, S., & Pommier, Y. (2008). GammaH2AX and cancer. *Nature Reviews Cancer*, 8, 957–967. https://doi.org/10.1038/nrc2523https://doi.org/10.1038/nrc2523.

Boran, C. Kandirali, E. Yanik, S. Ahsen, H., Ulukaradağ, E., & Yilmaz, F. (2017). Does smoking change expression patterns of the tumor suppressor and DNA repair genes in the prostate gland? *Urologic Oncology: Seminars and Original Investigations*, 35, 533.e1-533.e8. https://doi.org/10.1016/j.urolonc.2017.03.001.

Brennan, K., Koenig, J. L., Gentles, A. J., Sunwoo, J. B., & Gevaert, O. (2017). Identification of an atypical etiological head and neck squamous carcinoma subtype featuring the CpG island methylator phenotype. *EBioMedicine*, 17, 223–236. https://doi.org/10.1016/j.ebiom.2017.02.025

Chaves, A., Cherubini, K., Herter, N., Furian, R., Santos, D., Squier, C., Domann, F. (2004). Characterzation of p53 gene mutations in a Brazilian population with oral squamous cell carcinomas. *International Journal of Oncology*, 24, 295-303.

Chen, H. H., Yu, C. H., Wang, J. T., Liu, B. Y., Wang, Y. P., Sun, A., Tsai, T. C., Chiang, C. P. (2007). Expression of human telomerase reverse transcriptase (hTERT) protein is significantly associated with the progression, recurrence and prognosis of oral squamous cell carcinoma in Taiwan. *Oral Oncology*, 43, 122-129. http://doi.org/10.1016/j.oraloncology.2006.01.011.

Chi, A. C., Day, T. A., & Neville, B. W. (2015). Oral cavity and oropharyngeal squamous cell carcinoma--an update. *CA: A Cancer Journal for Clinicians*, 65, 401–

421. https://doi.org/10.3322/caac.21293.

Davidson, B., Bjørnerem, M., Holth, A., Hellesylt, E., Hetland Falkenthal, T. E., & Flørenes, V. A. (2018). Expression, activation and clinical relevance of CHK1 and CHK2 in metastatic high-grade serous carcinoma. *Gynecologic Oncology*, 150, 136–142. https://doi.org/10.1016/j.ygyno.2018.05.007.

D'CruzD'Cruz, A. K., Vaish, R., & Dhar, H. (2018). Oral cancers: Current status. *Oral Oncology*, 87, 64–69. https://doi.org/10.1016/j.oraloncology.2018.10.013.

de la Oliva, J., Larque, A. B., Marti, C., Bodalo-Torruella, M., Nonell, L., Nadal, A., Castillo, P., Sieira, R., Ferrer, A., Garcia-Diez, E., & Alos, L. (2019). Oral premalignant lesions of smokers and non-smokers show similar carcinogenic pathways and outcomes. A clinicopathological and molecular comparative analysis. *Journal of Oral Pathology & Medicine*. https://doi.org/10.1111/jop.12864.

De Oliveira, S. R., Da Silva, I. C., Mariz, B. A., Pereira, A. M., & De Oliveira, N. F. (2015). DNA methylation analysis of cancer-related genes in oral epithelial cells of healthy smokers. *Archives of Oral Biology*, 60, 825–833. https://doi.org/10.1016/j.archoralbio.2015.02.022.

DeAngelis, A., Breik, O., Koo, K., Iseli, T., Nastri, A., Fua, T., Rischin, D., McCullough, M., & Wiesenfeld, D. (2018). Non-smoking, non-drinking elderly females, a 5 year follow-up of a clinically distinct cohort of oral squamous cell carcinoma patients. *Oral Oncology*, 86, 113–120. https://doi.org/10.1016/j.oraloncology.2018.09.004.

El-Naggar, A. K., Chan, J. K. C., Grandis, J. R., Takata, T., & Slootweg, P. J. (2017). *WHO Classification of Head and Neck Tumours*. (4th ed.). Lyon: IARC Press, (Chapter 8).

Harjes, U. (2019). Nothing to gain for p53. *Nature Reviews Cancer*. https://doi.org/10.1038/s41568-019-0202-0.

Hsieh, L. L., Wang, P. F., Chen, I. H., Liao, C. T., Wang, H. M., Chen, M. C., Chang J. T., & et al Cheng A. J. (2001). Characteristics of mutations in the p53 gene in oral squamous cell carcinoma associated with betel quid chewing and cigarette smoking in Taiwanese. *Carcinogenesis*, 22, 1497–1503. https://doi.org/10.1093/carcin/22.9.1497.

Jethwa, A. R., & Khariwala, S. S. (2017). Tobacco-related carcinogenesis in head and neck cancer. *Cancer and Metastasis Reviews*, 36, 411–423. https://doi.org/10.1007/s10555-017-9689-6.

Jiang, X., Wu, J., Wang, J., & Huang, R. (2019). Tobacco and oral squamous cell carcinoma: A review of carcinogenic pathways. *Tobacco Induces Diseases*, 17. https://doi.org/10.18332/tid/105844.

Karpathiou, G., Monaya, A., Forest, F., Froudarakis, M., Casteillo, F., Marc Dumollard, J., Prades, J. M., & Peoc'h, M. (2016). p16 and p53 expression status in head and neck squamous cell carcinoma: a correlation with histological, histoprognostic and clinical parameters. *Pathology*, 48, 341–348. https://doi.org/10.1016/j.pathol.2016.01.005.

Katoh, T., Kaneko, S., Boissy, S., Watson, M., Ikemura, K., Bell, D. (1998). A pilot study testing the association between N-acetyltransferases 1 and 2 and risk of oral squamous cell carcinoma in Japanese people. *Carcinogenesis,* 19, 1803-1807. http://doi.org/10.1093/carcin/19.10.1803.

Kietthubthew, S., Sriplung, H., Au, W. (2001). Genetic and environmental interactions on Oral Cancer in Sothern Thailand. *Environmental and molecular mutagenesis*, 37, 111-116. http://doi.org/10.1002/em.1018.

Koo, K., Barrowman, R., McCullough, M., Iseli, T., & Wiesenfeld, D. (2013). Non-smoking non-drinking elderly females: a clinically distinct subgroup of oral squamous cell carcinoma patients. *International Journal of Oral and Maxillofacial Surgery*, 42, 929–933. https://doi.org/10.1016/j.ijom.2013.04.010.

Lassus, H., Leminen, A., Lundin, J., Lehtovirta, P., & Butzow, R. (2003). Distinct subtypes of serous ovarian carcinoma identified by p53 determination. *Gynecologic Oncology*, 91, 504–512. https://doi.org/10.1016/j.ygyno.2003.08.034.

Lee, H. E., Han, N., Kim, M. A., Lee, H. S., Yang, H. K., Lee, B. L., & Kim, W. H. (2014). DNA damage response-related proteins in gastric cancer: ATM, Chk2 and p53 expression and their prognostic value. *Pathobiology*, 81, 25–35. https://doi.org/10.1159/000351072.

Li, B. Z., Chen, Z. L., Shi, S. S., Feng, X. L., Tan, X. G., Zhou, F., & He, J. (2013). Overexpression of Cdc25C predicts response to radiotherapy and survival in esophageal squamous cell carcinoma patients treated with radiotherapy followed by surgery. *Chinese Journal of Cancer*, 32, 403–4-09. https://doi.org/10.5732/cjc.012.10233.

Mah, L. J., El-Osta, A., & Karagiannis, T. C. (2010). γH2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia*, 24, 679–686. https://doi.org/10.1038/leu.2010.6.

Matthews, T. P., Jones, A. M., & Collins, I. (2013). Structure-based design, discovery and development of checkpoint kinase inhibitors as potential anticancer therapies.

*Expert Opinion on Drug Discovery*, 8, 621–640. https://doi.org/10.1517/17460441.2013.788496.

Mei, L., Hu, Q., Peng, J., Ruan, J., Zou, J., Huang, Q., Liu, S., & Wang, H. (2015). Phospho-histone H2AX is a diagnostic and prognostic marker for epithelial ovarian cancer. *International Journal of Clinical and Experimental Pathology*, 8, 5597–5602.

Monteiro, L. S., Diniz-Freitas, M., Warnakulasuriya, S., Garcia-Caballero, T., Forteza, J., & Fraga, M. (2018). An immunohistochemical score to predict the outcome for oral squamous cell carcinoma. *Journal of Oral Pathology & Medicine*, 47, 375–381. https://doi.org/10.1111/jop.12682.

Murugan, A. K., Munirajan, A. K., & Tsuchida, N. (2012). Ras oncogenes in oral cancer: the past 20 years. *Oral Oncology*, 48, 383–392. https://doi.org/10.1016/j.oraloncology.2011.12.006.

Nagao, T., Warnakulasuriya, S., Sakuma, H., Miyabe, S., Hasegawa, S., Machida, J., Suzuki, K., Fukano, H., Shimozato, K., & Hashimoto, S. (2017). p53 and ki67 as biomarkers in determining response to chemoprevention for oral leukoplakia. *Journal of Oral Pathology & Medicine*, 46, 346–352. https://doi.org/10.1111/jop.12498.

Nikitakis, N. G., Rassidakis, G. Z., Tasoulas, J., Gkouveris, I., Kamperos, G., Daskalopoulos, A., & Sklavounou, A. (2018). Alterations in the expression of DNA damage response-related molecules in potentially preneoplastic oral epithelial lesions. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, 125, 637–649. https://doi.org/10.1016/j.oooo.2018.03.006.

Nishioka, T., Yamamoto, D., Zhu, T., Guo, J., Kim, S. H., & Chen, C. Y. (2011). Nicotine Overrides DNA Damage-Induced G1/S Restriction in Lung Cells. *PLoS One*, 6, e18619. http://doi.org/10.1371/journal.pone.0018619.

Oliveira-Costa, J. P., Oliveira, L. R., Zanetti, R., Zanetti, J. S., da Silveira, G. G., Chavichiolli Buim, M. E., Zucoloto, S., Ribeiro-Silva, A., & Soares, F. A. (2014). BRCA1 and γH2AX as independent prognostic markers in oral squamous cell carcinoma. *Oncoscience*, 1, 383–391.

Palla, V. V., Karaolanis, G., Katafigiotis, I., Anastasiou, I., Patapis, P., Dimitroulis, D, & Perrea, D. (2017). gamma-H2AX: Can it be established as a classical cancer prognostic factor? *Tumor Biology*, 39, 1010428317695931. https://doi.org/10.1177/1010428317695931.

Park, J., Muscat, J., Kaur, T., Schantz, S., Stern, J., Richie, J., Lazarus, P. (2000). Comparison of GSTM polymorphisms and risk for oral cancer between African-American and Cacausians. *Pharmacogenetics*, 10, 123-131. http://doi.org/10.1097/00008571-200003000-00004.

Paulin, G. A., Ouriadov, A., Lessard, E., Sheikh, K., McCormack, D. G., & Parraga, G. (2015). Noninvasive quantification of alveolar morphometry in elderly never- and ex-smokers. *Physiological Reports*, 3, e12583. https://doi.org/10.14814/phy2.12583.

Pickering, C. R., Zhang, J., Neskey, D. M., Zhao, M., Jasser, S. A., Wang, J., Ward, A., Tsai, C. J., Ortega Alves, M. V., Zhou, J. H., Drummond, J., El-Naggar, A. K., Gibbs, R., Weinstein, J. N., Wheeler, D. A., Wang, J., Frederick, M. J., Myers, J. N. (2014). Squamous cell carcinoma of the oral tongue in young non-smokers is genomically similar to tumors in older smokers. *Clinical Cancer Research,* 20, 3842-8.

http://doi.org/10.1158/1078-0432.CCR-14-0565.

Prior, S., Griffiths, A., Baxter, J., Baxter, P., Hodder, S., Silvester, K., Lewis, P. (2006). Mitochondrial DNA mutations in oral squamous cell carcinoma. *Carcinogenesis*, 27, 945-950. http://doi.org/10.1093/carcin/bgi326

Roos, W. P., & Kaina, B. (2013). DNA damage-induced cell death: From specific DNA lesions to the DNA damage response and apoptosis. *Cancer Letters,* 332, 237–248. http://dx.doi.org/10.1016/j.canlet.2012.01.007.

Sarode, G. S., Sharma, N. K., Sarode, S. C., & Patil, S. (2019). Oral premalignant lesions of smokers and non-smokers show similar carcinogenic pathways and outcomes. *Journal of Oral Pathology & Medicine*, 48, 507. https://doi.org/10.1111/jop.12866.

Sedelnikova, O. A., & Bonner, W. M. (2006). GammaH2AX in cancer cells: a potential biomarker for cancer diagnostics, prediction and recurrence. *Cell Cycle*, 5, 2909–2913. https://doi.org/10.4161/cc.5.24.3569.

Senturk, E., & Manfredi, J. J. (2013). p53 and cell cycle effects after DNA damage. *Methods in Molecular Biology*, 962, 49–61. https://doi.org/10.1007/978-1-62703-236-0_4.

Sharma, A., Mishra, A., Das, B., Sardana, S., Sharma, J. (2006). Genetic polymorphism at GSTM1 and GSTT1 gene loci and susceptibility to oral cancer. *Neoplasma*, 53, 309-315.

Sinevici, N., & O'sullivan J. (2016). Oral cancer: Deregulated molecular events and their use as biomarkers. *Oral Oncology,* 61, 12–18. http://dx.doi.org/10.1016/j.oraloncology.2016.07.013.

Slatter, T. L., Park, L., Anderson, K., Lailai-Tasmania, V., Herbison, P., Clow, W., Royds, J. A., Devenish, C., & Hung, N. A. (2014). Smoking during pregnancy causes double-strand DNA break damage to the placenta. *Human Pathology*, 45, 17–26. https://doi.org/10.1016/j.humpath.2013.07.024.

Soares, A. B., Martinez, E. F., Ribeiro, P. F., Barreto, I. S., Aguiar, M. C., Furuse, C., Sperandio, M., Montalli, V. A., de Araújo, N. S., & de Araújo, V. C. (2017). Factors that may influence polymorphous low-grade adenocarcinoma growth. *Virchows Archiv*, 470, 437–443. https://doi.org/10.1007/s00428-017-2085-3.

Squatrito, M., Brennan, C. W., Helmy, K., Huse, J. T., Petrini, J. H., & Holland, E. C. (2010). Loss of ATM/Chk2/p53 pathway components accelerates tumor development and contributes to radiation resistance in gliomas. *Cancer Cell*, 18, 619–629. https://doi.org/10.1016/j.ccr.2010.10.034.

Stolz, A., Ertych, N., & Bastians, H. (2011). Tumor suppressor CHK2: regulator of DNA damage response and mediator of chromosomal stability. *Clinical Cancer Research*, 17, 401–405. https://doi.org/10.1158/1078-0432.CCR-10-1215.

Takeshima, Y., Seyama, T., Bennett, W. P., Akiyama, M., Tokuoka, S., Inai, K., Mabuchi, K., Land, C. E., & Harris, C. C. (1993). p53 Mutations in lung cancers from non-smoking atomic-bomb survivors., *Lancet*, 342, 1520–1521. https://doi.org/10.1016/s0140-6736(05)80087-x

Tanaka T., Huang, X., Jorgensen, E., Gietl, D., Traganos, F., Darzynkiewicz, Z., & Albino, A. P. (2007). ATM activation accompanies histone H2AX phosphorylation in A549 cells upon exposure to tobacco smoke. *BMC Cell Biology*, 26, 1–-11. https://doi.org/10.1186/1471-2121-8-26.

Tomar, S. L. (2019). The Transnational Tobacco Industry and Oral Health. *Community Dental Health*, 36, 163–168. https://doi.org/10.1922/CDH_SpecialIssueTomar06.

Tsai, M. H., Tseng, H. C., Liu, C. S., Chang, C. L., Tsai, C. W., Tsou, Y. A., Wang, R. F., Lin, C. C., Wang, H. C., Chiu, C. F., Bau, D. T. (2009). Interaction of Exo1 genotypes and smoking habit in oral cancer in Taiwan. *Oral oncology*, 45, 90-94. http://doi.org/10.1016/j.oraloncology.2009.03.011.

Tu, W. Z., Li, B., Huang, B., Wang, Y., Liu, X. D., Guan, H., Zhang, S. M., Tang, Y., Rang, W. Q., & Zhou, P. K. (2013). γH2AX foci formation in the absence of DNA damage: mitotic H2AX phosphorylation is mediated by the DNA-PKcs/CHK2 pathway. *FEBS Letters*, 587, 3437–3443. https://doi.org/10.1016/j.febslet.2013.08.028.

Valinluck, V., & Sowers, L. C. (2007). Inflammation-mediated cytosine damage: a mechanistic link between inflammation and the epigenetic alterations in human cancers. *Cancer Research*. 67, 5583–5586. https://doi.org/10.1158/0008-5472.CAN-07-0846.

Wang, B., Zhang, Z., Xia, S., Jiang, M., & Wang, Y. (2019). Expression of γ-H2AX and patient prognosis in breast cancer cohort. *Journal of Cellular Biochemistry*, 120, 12958–12965. https://doi.org/10.1002/jcb.28567.

Warters, R. L., Adamson, P. J., Pond, C. D., & Leachman, S. A. (2005). Melanoma cells express elevated levels of phosphorylated histone H2AX foci. *Journal of Investigative Dermatology*, 124, 807–817. https://doi.org/10.1111/j.0022-

202X.2005.23674.x.

Xiao, H., Tong, R., Ding, C., Lv, Z., Du, C., Peng, C., Cheng, S., Xie, H., Zhou, L., Wu, J., & Zheng, S. (2015). γ-H2AX promotes hepatocellular carcinoma angiogenesis via EGFR/HIF-1α/VEGF pathways under hypoxic condition. *Oncotarget*, 10, 2180–2192.

Zaid, K., Azar-Maalouf, E., Barakat, C., & Chantiri, M. (2018). p53 Overexpression in Oral Mucosa in Relation to Shisha Smoking in Syria and Lebanon. *Asian Pacific Journal of Cancer Prevention*, 19, 1879–1882. https://doi.org/10.22034/APJCP.2018.19.7.1879.

Zannini, L., Delia, D., & Buscemi, G. (2014). CHK2 kinase in the DNA damage response and beyond. *Journal of Molecular Cell Biology*, 6, 442–457. https://doi.org/10.1093/jmcb/mju045.

Zhao, H., Albino, A. P., Jorgensen, E., Traganos, F., & Darzynkiewicz, Z. (2009). DNA damage response induced by tobacco smoke in normal human bronchial epithelial and A549 pulmonary adenocarcinoma cells assessed by laser scanning cytometry. *Cytometry Part A*, 75, 840–84-7. https://doi.org/10.1002/cyto.a.20778.

**Figure legends**

**Figure 1.** Overall distribution of immunostaining in smokers and non-smokers.

**Figure 2.** CHK2 staining in a smoker **(A)** and non-smoker **(B)**. H2AX staining in a smoker **(C)** and non-smoker **(D)**. P53 staining in a smoker **(E)** and non-smoker **(F)**. Immunohistochemistry, 20×.

**Figure 3.** Immunoexpression of the proteins CHK2 (p=0.909), H2AX (p=0.807) and P53 (p=0.546) between smokers and non-smokers.

**Table 1**. Demographic data and clinicopathological characteristics of the sample

| Variable | n (%) | |
|---|---|---|
| | **Non-smokers, n=18 (51.4)** | **Smokers, n=17 (48.6)** |
| ***Sex, n=35*** | | |
| Male | 8 (44.4) | 14 (82.3) |
| Female | 10 (55.6) | 3 (17.7) |
| ***Age, n=35*** | | |
| 40–49 | 2 (11.1) | 1 (5.9) |
| 50–59 | 4 (22.2) | 10 (58.9) |
| 60–69 | 1 (5.6) | 6 (35.2) |
| 70–79 | 6 (33.3) | - |
| 80–89 | 5 (27.8) | - |
| ***Alcohol consumption, n=34*** | | |
| Yes | 5 (29.4) | 12 (70.6) |
| No | 12 (70.6) | 5 (29.4) |
| ***Symptoms, n=18*** | | |
| Yes | 7 (58.3) | 4 (66.7) |
| No | 5 (41.7) | 2 (33.3) |
| ***Anatomical location, n=35*** | | |
| Tongue | 14 (77.8) | 7 (41.2) |
| Floor of the mouth | 3 (16.7) | 8 (47.1) |
| Tongue + floor of the mouth | 1 (5.5) | 2 (11.7) |
| ***Type of sample, n=35*** | | |
| Incisional | 12 (66.7) | 8 (47.1) |
| Excisional | 6 (33.3) | 9 (52.9) |
| ***Histology/grade, n=35*** | | |
| Well-differentiated | 9 (50.0) | 5 (29.4) |
| Moderately differentiated | 4 (22.2) | 6 (35.3) |
| Poorly differentiated | 5 (27.8) | 6 (35.3) |

**Figure 1.** Overall distribution of immunostaining in smokers and non-smokers.

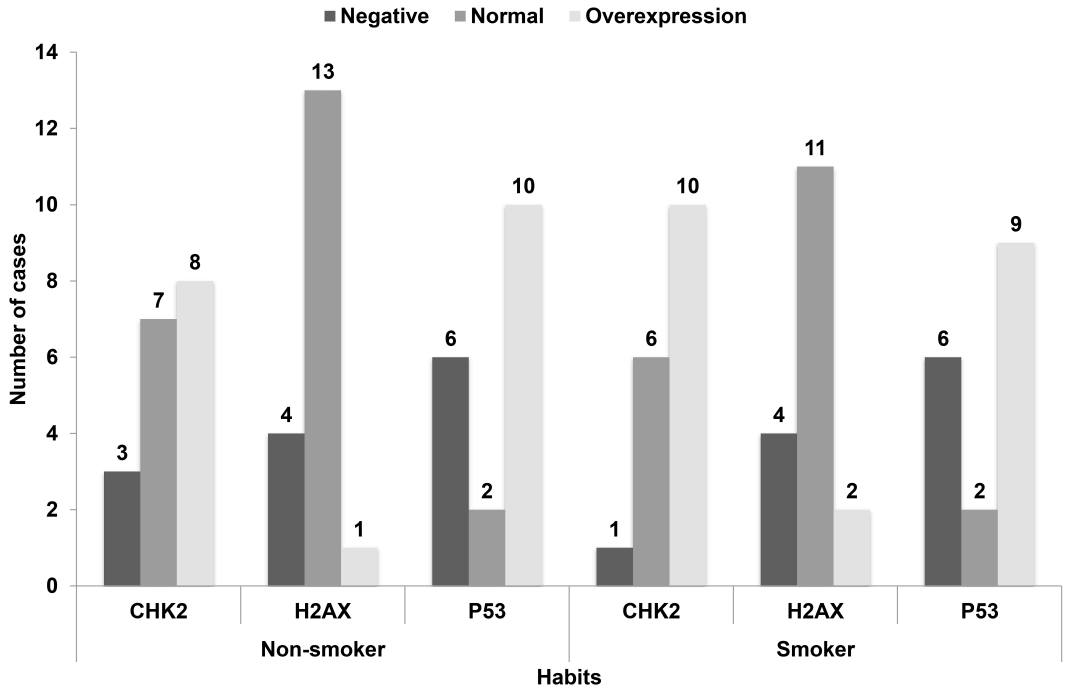


**Figure 2.** CHK2 staining in a smoker **(A)** and non-smoker **(B)**. H2AX staining in a smoker **(C)** and non-smoker **(D)**. P53 staining in a smoker **(E)** and non-smoker **(F)**. Immunohistochemistry, 20×.

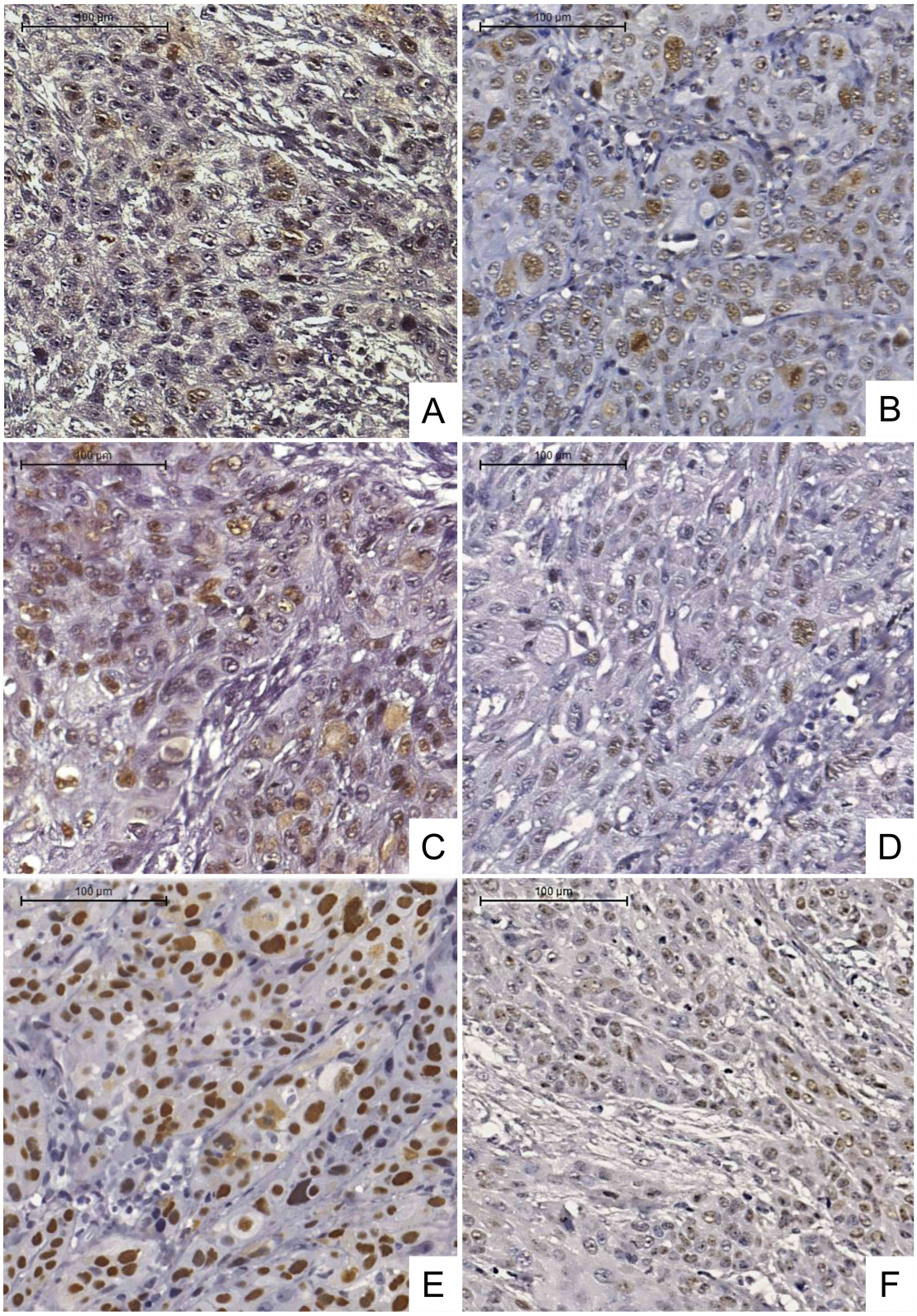
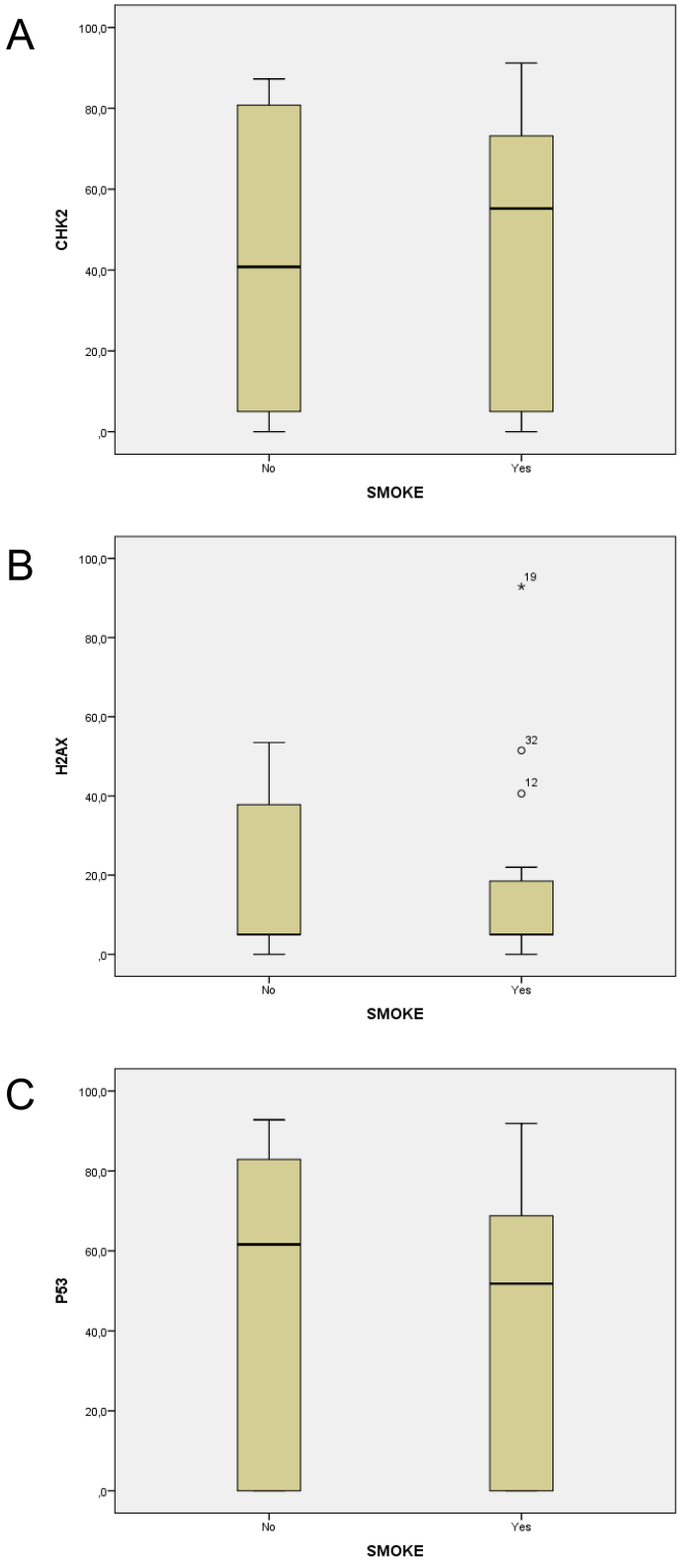


**Figure 3.** Immunoexpression of the proteins CHK2 (p=0.909), H2AX (p=0.807) and P53 (p=0.546) between smokers and non-smokers.



## 4 CONSIDERAÇÕES FINAIS

Este estudo fornece informações sobre algumas proteínas envolvidas no dano ao DNA, importantes no processo de carcinogênese oral. Observou-se semelhanças entre a expressão imuno-histoquímica de CHK2, γH2AX e TP53 em fumantes e não fumantes com CCEB. Os mecanismos de proteção ao DNA e as alterações relacionadas ao ciclo e checagem permaneceram as mesmas em pacientes fumantes e não fumantes. Porém, nos não fumantes, outras alterações genéticas que não foram abordadas neste trabalho influenciam no desenvolvimento do tumor, podendo levar a lesões mais agressivas. Esses achados não diminuem a importância dos programas de cessação do tabagismo e da ingestão de álcool para reduzir a incidência de câncer oral. No entanto, eles sugerem possíveis novos caminhos para a prevenção ou tratamento desta doença.

## REFERÊNCIAS


ABREU, V. A. M.; HOWARD, M. S. Tumor-supressor genes, cell cycle regulatory checkpoints and the skin. **North American Journal of Medical Sciences**, v. 7, n. 5, p. 176-188, 2015.

ALI, J.; SABIHA, B.; JAN, H. U.; HAIDER, S. A.; KHAN; A. A.; ALI, S. S. Genetic etiology of oral cancer. **Oral Oncology**, v. 70, p. 23-28, 2017.

ANNEROTH, G.; HANSEN, L. S.; SILVERMAN, S., JR. Malignancy grading in oral squamous cell carcinoma. I. Squamous cell carcinoma of the tongue and floor of mouth: histologic grading in the clinical evaluation. **Journal of Oral Pathology**, v.15, n.3, p.162-8, 1986.

APPAH, E. O.; BALLARD, B. R.; IZBAN, M. G.; JOLIN, C.; LAMMERS, P. E.; PARRISH, D. D.; MARSHALL, D. R. A rapidly growing human papillomavirus-positive oral tongue squamous cell carcinoma in a 21-year old female: A case report. **Oncology Letters**, v. 15, p. 7702-7706, 2018.

BARNES, L.; EVESON, J. W.; REICHART, P.; SIDRANSKY, D. Pathology and genetics of head and neck tumours**. World Health Organization classification of tumors**. IARC Press: Lyon, 2005.

BARTEK, J.; LUKAS, J. Chk1 and Chk2 kinases in checkpoint control and cancer. **Cancer Cell**, v.3, n.5, p.421-429, 2003.

BELL, D. W.; VARLEY, J. M.; SZYDLO, T. E.; KANG, D. H.; WAHRER, D. C.; SHANNON, K. E.; LUBRATOVICH, M.; VERSELIS, S. J.; ISSELBACHER, K. J.; FRAUMENI, J. F.; BIRCH, J. M.; LI, F. P.; GARBER, J. E.; HABER, D. A. Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. **Science**, v. 286, p. 2528–2531, 1999.

CARRASSA, L.; DAMIA, G. Unleashing Chk1 in cancer therapy. **Cell Cycle**, v. 10, n.13, p. 2121-2128, 2011.

CUTILLI, T.; LEOCATA, P.; DOLO, V.; ALTOBELLI, E. P53 as a prognostic marker associated with the risk of mortality for oral squamous cell carcinoma. **Oncology Letters**, v. 12, n. 2, p. 1046-1050, 2016.

DAVIDSON B.; BJØRNEREM, M.; HOLTH, A.; HELLESYLT, E.; HETLAND FALKENTHAL, T. E.; FLØRENES, V. A. Expression, activation and clinical relevance of CHK1 and CHK2 in metastatic high-grade serous carcinoma. **Gynecologic Oncology**, v. 150, p. 136–142, 2018.

D'CRUZ, A. K.; VAISH, R.; DHAR, H. (2018). Oral cancers: Current status. **Oral Oncology**, v. 87, p. 64–69, 2018.

DEAN, A.G.; SULLIVAN, K. M.; SOE, M. M. OpenEpi: Open Source Epidemiologic Statistics for Public Health, Versão. www.OpenEpi.com, atualizado 2013/04/06, acessado 2019/11/29.

D'SOUZA, W.; SARANATH, D. Clinical implications of epigenetic regulation in oral cancer. **Oral Oncology**, v.51, n.12, p. 1061-1068, 2015.

EL-NAGGAR, A. K. A. K.; CHAN, J. K. C.; GRANDIS, J. R.; TAKATA, T.; SLOOTWEG, P. J. (2017). **WHO Classification of Head and Neck Tumours. (4th ed.)**. Lyon: IARC Press, (Chapter 8).

FERLAY, J. et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. **Int J Cancer**, v. 136, n. 5, p. e359-386, 2015.

GUFFANTI, F.; FRUSCIO, R.; RULLI, E.; DAMIA, G. The impact of DNA damage response gene polymorphisms on therapeutic outcomes in late stage ovarian cancer. **Scientific Reports**, v. 1, n. 6, p. 38142.

JETHWA, A. R.; KHARIWALA, S. S. Tobacco-related carcinogenesis in head and neck cancer. **Cancer Metastasis Review**, 2017.

JIANG, X.; WU, J.; WANG, J.; HUANG, R. Tobacco and oral squamous cell carcinoma: A review of carcinogenic pathways. **Tobacco Induces Diseases**, v. 17, 2019

LEE, H. J. et al. Positive expression of NANOG, mutant P53, and CD44 is directly associated with clinicopathological features and poor prognosis of oral squamous cell carcinoma. **BMC Oral Health**, v. 15, n. 1, p. 153, 2015.

LINDEMANN, A., TAKAHASHI, H.; PATEL, A. A.; OSMAN, A. A.; MYERS, J. N. Targeting the DNA Damage Response in OSCC with TP53 Mutations. **Journal of Dental Research**, v. 97, n. 6, p. 635-644, 2018.

MEADOR, J. A; ZHAO, M.; SU, Y.; NARAYAN, G.; GEARD, C. R.; BALAJEE, A. S. Histone H2AX is a critical factor for cellular protection against DNA alkylating agents. **Oncogenese**, v. 27, p.5662-5671, 2008.

OSTERMAN, M.; KATHAWA, D.; LIU, D.; GUO, H.; ZHANG, C.; LI, M.; YU, X.; LI, F. Elevated DNA damage response in pancreatic cancer. **Histochemistry and Cell Biology**, v. 142, n. 6, p. 713-20, 2014.

PAULIN, G. A.; OURIADOV, A.; LESSARD, E.; SHEIKH, K.; MCCORMACK, D. G.; PARRAGA, G. Noninvasive quantification of alveolar morphometry in elderly never- and ex-smokers. **Physiological Reports**, v. 3, n. 10, 2015.

SCULLY, C.; BAGAN, J. V. Oral squamous cell carcinoma: overview of current understanding of aetiopathogenesis and clinical implications. **Oral Diseases**, v. 15, p. 388-399, 2009.

SINEVICI, N.; O'SULLIVAN, J. Oral cancer: Deregulated molecular events and their use as biomarkers. **Oral Oncology**, v. 61, p. 12-18, 2016.

TAO, Y.; LETEUR, C.; YANG, C.; ZHANG, P.; CASTEDO, M.; PIERRÉ, A.; GOLSTEYN, R. M.; BOURHIS, J.; KROEMER, G.; DEUTSCH, E. Radiosensitization by Chir-124, a selective CHK1 inhibitor: effects of P53 and cell cycle checkpoints. **Cell Cycle**, v. 8, n. 8, p. 1196-1205, 2009.

TSANTOULIS, P. K.; KASTRINAKIS, N. G.; TOURVAS, A. D.; LASKARIS, G.; GORGOULIS, V. G. Advances in the biology of oral cancer. **Oral Oncology**, v. 43, n. 6, p. 523-534, 2007.

WAGNER, V. P. et al. Bryne's grading system predicts poor disease-specific survival of oral squamous cell carcinoma: a comparative study among different histologic grading systems. **Oral Surg Oral Med Oral Pathol Oral Radiol,** v. 123, n. 6, p. 688-696, 2017.

WEYEMI, U.; REDON, C. E.; CHOUDHURI, R.; AZIZ, T.; MAEDA, D.; BOUFRAQECH, M.; PAREKH, P. R.; SETHI, T. K.; KASOJI, M.; ABRAMS, N.; MERCHANT, A.; RAJAPAKSE, V. N.; BONNER, W. M. The histone variant H2A.X is a regulator of the epithelial-mesenchymal transition. **Nature Communications**, v. 7, 2016.

WHYTE, D. A.; BROTON, C. E.; SHILLITOE, E. J. The unexplained survival of cells in oral cancer: whats is the role of P53? **Journal of Pathology and Medicine**, v. 31, p. 125-133, 2002.

YANG, L.; WANG, Y.; GUO, L.; WANG, L.; CHEN, W.; SHI, B. The Expression and Correlation of iNOS and P53 in Oral Squamous Cell Carcinoma. **Biomed Research International**, 2015.

ZHANG, Y.; HUNTER, T. Roles of Chk1 in cell biology and cancer therapy. International Journal of Cancer, 2013.

ZEDAN, W.; MOURAD. M. I.; EL-AZIZ, S. M.; SALAMAA, N. M.; SHALABY, A. A. Cytogenetic significance of chromosome 17 aberrations and P53 gene mutations as prognostic markers in oral squamous cell carcinoma. **Diagnostic Pathology**, 2015.

# ANEXO A – Aprovação do comitê de ética em pesquisa

UNIVERSIDADE FEDERAL DE MINAS GERAIS

## PARECER CONSUBSTANCIADO DO CEP

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** PADRÃO DE CONSUMO DE TABACO EM PACIENTES COM CÂNCER DA CAVIDADE ORAL: ESTUDO DAS ASSOCIAÇÕES CLINICOPATOLÓGICAS E IMUNOISTOQUÍMICAS

**Pesquisador:** Maria Cássia Ferreira de Aguiar

**Área Temática:**

**Versão:** 2

**CAAE:** 03012618.1.0000.5149

**Instituição Proponente:** PRO REITORIA DE PESQUISA

**Patrocinador Principal:** Financiamento Próprio

**DADOS DO PARECER**

**Número do Parecer:** 3.293.055

**Apresentação do Projeto:**

A incidência dos casos de câncer da cavidade oral, lábios e orofaringe, representados principalmente pela variante carcinoma de células escamosas (CCE), apesar das variações entre os países, na maioria deles ainda apresenta trajetória ascendente e preocupante. O presente estudo é do tipo prospectivo caso-controle dos casos de câncer da cavidade oral atendidos na Clínica de Patologia, Cirurgia e Radiologia da Faculdade de Odontologia da Universidade Federal de Minas Gerais, que será desenvolvido conjuntamente com um estudo transversal, do tipo retrospectivo, com população-alvo constituída por indivíduos com diagnóstico histopatológico de carcinoma de células escamosas de boca que tiveram laudo gerado pelo Laboratório de Patologia Bucomaxilofacial da Faculdade de Odontologia da Universidade Federal de Minas Gerais.

**Objetivo da Pesquisa:**

O objetivo principal é avaliar prospectivamente o padrão de consumo de tabaco, incluindo o índice de exposição cumulativa ao alcatrão (IEC), em pacientes com CCEB, e verificar sua relação com características clínicas e histopatológicas da doença, bem como investigar a expressão de algumas proteínas do ciclo celular em amostras de CCEB já coletadas, de pacientes fumantes e não fumantes.

**Endereço:** Av. Presidente Antônio Carlos,6627 2° Ad SI 2005
**Bairro:** Unidade Administrativa II **CEP:** 31.270-901
**UF:** MG **Município:** BELO HORIZONTE
**Telefone:** (31)3409-4592 **E-mail:** coep@prpq.ufmg.br

UNIVERSIDADE FEDERAL DE MINAS GERAIS



**Avaliação dos Riscos e Benefícios:**

De acordo com o pesquisador, a pesquisa em suas duas vertente oferece riscos mínimos aos indivíduos, uma vez que será realizada por meio da aplicação de questionários, após consentimento dos pacientes; e por meio da avaliação imunoistoquímica de material biológico previamente coletado. Em relação à aplicação dos questionários, os participantes poderão se sentir cansados ou constrangidos com alguma pergunta. Os pesquisadores irão minimizar este risco cuidando para que a entrevista seja feita de forma reservada, breve e deixando claro que o sigilo quanto a identidade dos participantes será mantido. Os mesmos cuidados quanto à preservação da identidade serão tomados em relação ao uso do material de arquivo. Ressalta-se ainda, que os pacientes não terão seu tratamento e acompanhamento prejudicados.

Como benefícios, é descrito conhecer melhor o padrão de consumo de tabaco entre indivíduos com e sem CCEB, bem como avaliar a relação do uso de tabaco com características clínicas, patológicas e imunoistoquímicas dessa neoplasia. Sendo assim, espera-se gerar resultados que subsidiem políticas públicas de combate ao tabagismo e alertem a população sobre a importância do consumo de tabaco no desenvolvimento do câncer de boca.

**Comentários e Considerações sobre a Pesquisa:**

Constam no processo: Projeto original; Formulário de Informações Básicas; Carta Resposta ao Comitê de Ética; TCLE modificado de acordo com as solicitações do Comitê de Ética; Termo de Compromisso de Utilização de Dados; Declaração de Compromisso do Pesquisador; Parecer Departamental e Orçamento.

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

A pesquisadora atendeu todas as solicitações do Comitê de Ética, enviando carta resposta, TCLE com alterações e TCUD.

**Recomendações:**

Não há recomendações.

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

S.M.J. julgo aprovado o projeto de pesquisa.

**Considerações Finais a critério do CEP:**

Tendo em vista a legislação vigente (Resolução CNS 466/12), o CEP-UFMG recomenda aos Pesquisadores: comunicar toda e qualquer alteração do projeto e do termo de consentimento via emenda na Plataforma Brasil, informar imediatamente qualquer evento adverso ocorrido durante o

**Endereço:** Av. Presidente Antônio Carlos,6627 2° Ad SI 2005
**Bairro:** Unidade Administrativa II **CEP:** 31.270-901
**UF:** MG **Município:** BELO HORIZONTE
**Telefone:** (31)3409-4592 **E-mail:** coep@prpq.ufmg.br

UNIVERSIDADE FEDERAL DE MINAS GERAIS



desenvolvimento da pesquisa (via documental encaminhada em papel), apresentar na forma de notificação relatórios parciais do andamento do mesmo a cada 06 (seis) meses e ao término da pesquisa encaminhar a este Comitê um sumário dos resultados do projeto (relatório final).

**Este parecer foi elaborado baseado nos documentos abaixo relacionados:**

| Tipo Documento | Arquivo | Postagem | Autor | Situação |
|---|---|---|---|---|
| Informações Básicas do Projeto | PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1229178.pdf | 22/03/2019 13:02:31 | | Aceito |
| Outros | TCUD.pdf | 22/03/2019 13:00:56 | Karine Duarte da Silva | Aceito |
| Projeto Detalhado / Brochura Investigador | Projeto_corrigido.pdf | 22/03/2019 12:59:11 | Karine Duarte da Silva | Aceito |
| Outros | Carta_resposta_COEP.pdf | 22/03/2019 12:58:56 | Karine Duarte da Silva | Aceito |
| Declaração de Manuseio Material Biológico / Biorepositório / Biobanco | Autorizacao_novo2.pdf | 22/03/2019 12:58:21 | Karine Duarte da Silva | Aceito |
| TCLE / Termos de Assentimento / Justificativa de Ausência | TCLE_novo2.pdf | 22/03/2019 11:27:42 | Karine Duarte da Silva | Aceito |
| TCLE / Termos de Assentimento / Justificativa de Ausência | dispensa.pdf | 08/10/2018 08:30:44 | Karine Duarte da Silva | Aceito |
| Outros | Parecer_departamento.pdf | 08/10/2018 08:29:56 | Karine Duarte da Silva | Aceito |
| Orçamento | orcamento.pdf | 08/10/2018 08:29:00 | Karine Duarte da Silva | Aceito |
| Declaração de Pesquisadores | Declaracao.pdf | 08/10/2018 08:27:51 | Karine Duarte da Silva | Aceito |
| Cronograma | Cronograma.pdf | 08/10/2018 08:27:32 | Karine Duarte da Silva | Aceito |
| Folha de Rosto | Folha_de_rosto.pdf | 08/10/2018 08:26:59 | Karine Duarte da Silva | Aceito |

**Situação do Parecer:**
Aprovado

**Endereço:** Av. Presidente Antônio Carlos,6627 2° Ad SI 2005
**Bairro:** Unidade Administrativa II **CEP:** 31.270-901
**UF:** MG **Município:** BELO HORIZONTE
**Telefone:** (31)3409-4592 **E-mail:** coep@prpq.ufmg.br

UNIVERSIDADE FEDERAL DE MINAS GERAIS



**Necessita Apreciação da CONEP:**
Não

BELO HORIZONTE, 29 de Abril de 2019

**Assinado por:**
**Eliane Cristina de Freitas Rocha**
**(Coordenador(a))**

**Endereço:** Av. Presidente Antônio Carlos,6627 2° Ad SI 2005
**Bairro:** Unidade Administrativa II **CEP:** 31.270-901
**UF:** MG **Município:** BELO HORIZONTE
**Telefone:** (31)3409-4592 **E-mail:** coep@prpq.ufmg.br

# ANEXO B – Informações da revista

Revista: Archives of Oral Biology – A1

Editors-in-Chief:
Professor G B Proctor, London, UK
Professor S W Cadden, Dundee, Scotland

Archives of Oral Biology is an international journal which aims to publish papers of the highest scientific quality reporting new knowledge from the orofacial region including:

• developmental biology

• cell and molecular biology

• molecular genetics

• immunology

• pathogenesis

• microbiology

• biology of dental caries and periodontal disease

• forensic dentistry

• neuroscience

• salivary biology

• mastication and swallowing

• comparative anatomy

• paeleodontology

Archives of Oral Biology will also publish expert reviews and articles concerned with advancement in relevant methodologies. The journal will consider clinical papers only where they make a significant contribution to the understanding of a disease process.

These guidelines generally follow the Uniform Requirements for Manuscripts Submitted to Biomedical Journals

**Types of Contribution**

Original papers and review articles are welcomed. There will be no differentiation on the basis of length into full or short communications. Editorial commentaries will also be considered but only by invitation. All submissions will be refereed.

***Page charges***

This journal has no page charges.

**Submission checklist**

You should use this list to carry out a final check of your submission before you send it to the journal for review. Please check all relevant sections in this Guide for Authors for more details.

**Ensure that the following items are present:**

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address

All necessary files have been uploaded:

*Manuscript*:

• Include Keywords

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print *Graphical Abstracts* (where applicable)

*Highlights* (where applicable)

*Supplemental files* (where applicable)

Further considerations

• Manuscript has been 'spell checked' and 'grammar checked'

• All references mentioned in the Reference List are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Internet)

• Relevant declarations of interest have been made

• Declarations of authors' contributions have been made if there are four or more authors

• Journal policies detailed in this guide have been reviewed

• Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Center

**Ethics in publishing**

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

**Studies in humans and animals**

If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The manuscript should be in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity) as per those recommendations. The terms sex and gender should be used correctly.

Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed. The sex of animals must

be indicated, and where appropriate, the influence (or association) of sex on the results of the study.

**Declaration of interest**

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places: 1. A summary declaration of interest statement in the title page file (if double-blind) or the manuscript file (if single-blind). If there are no interests to declare then please state this: 'Declarations of interest: none'. This summary statement will be ultimately published if the article is accepted. 2. Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information match.

**Submission declaration and verification**

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see 'Multiple, redundant or concurrent publication' section of our ethics policy for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically, without the written consent of the copyright-holder. To verify originality, your article is likely to be checked by the originality detection service CrossCheck.

***Preprints***

Please note that preprints can be shared anywhere at any time, in line with Elsevier's sharing policy. Sharing your preprints e.g. on a preprint server will not count as prior publication (see 'Multiple, redundant or concurrent publication' for more information).

**Use of inclusive language**

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of race, sex, culture or any other characteristic, and should use inclusive language throughout. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (e.g. 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess').

**Contributors**

If there are four or more authors, then each is required to declare his or her individual contribution to the article: all authors must have materially participated in the research and article preparation, so roles for all authors should be described. The statement that "All authors have read and approved the final article" should be true and included in the disclosure.

**Authorship**

All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

***Article transfer servisse***

This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. Please note that your article will be reviewed again by the new jornal.

**Copyright**

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement'. An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases.

For gold open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (more information). Permitted third party reuse of gold open access articles is determined by the author's choice of user license.

***Author rights***

As an author you (or your employer or institution) have certain rights to reuse your work.

***Elsevier supports responsible sharing***

Find out how you can share your research published in Elsevier journals.

**Role of the funding source**

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

***Funding body agreements and policies***

Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some funding bodies will reimburse the author for the gold open access publication fee. Details of existing agreements are available online.

After acceptance, open access papers will be published under a noncommercial license. For authors requiring a commercial CC BY license, you can apply after your manuscript is accepted for publication.

**Open access**

This journal offers authors a choice in publishing their research:

***Subscription***

• Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs.
• No open access publication fee payable by authors.
• The Author is entitled to post the accepted manuscript in their institution's repository and make this public after an embargo period (known as green Open Access). The published journal article cannot be shared publicly, for example on ResearchGate or Academia.edu, to ensure the sustainability of peer-reviewed research in journal publications. The embargo period for this journal can be found below.

***Gold open access***

• Articles are freely available to both subscribers and the wider public with permitted reuse.

• A gold open access publication fee is payable by authors or on their behalf, e.g. by their research funder or institution.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For gold open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

***Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)***

For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

The gold open access publication fee for this journal is **USD 2700**, excluding taxes. Learn more about Elsevier's pricing policy: https://www.elsevier.com/openaccesspricing.

***Green open access***

Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our open access page for further information. Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form.

This journal has an embargo period of 12 months.

***Elsevier Researcher Academy***

Researcher Academy is a free e-learning platform designed to support early and mid-career researchers throughout their research journey. The "Learn" environment at Researcher Academy offers several interactive modules, webinars, downloadable guides and resources to guide you through the process of writing for research and going through peer review. Feel free to use these free resources to improve your submission and navigate the publication process with ease.

***Language (usage and editing services)***

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate

possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's Author Services.

**Submission**

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

**Peer review**

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final.

***Use of word processing software***

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To minimize unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

**Article structure**

***Manuscript Structure***

Follow this order when typing manuscripts: Title, Authors, Affiliations, Abstract, Keywords, Main text (Introduction, Materials & Methods, Results, Discussion for an original paper), Acknowledgments, Appendix, References, Figure Captions and then Tables. Do not import the Figures or Tables into

your text. The corresponding author should be identified with an asterisk and footnote. All other footnotes (except for table footnotes) should be identified with superscript Arabic numbers.

***Introduction***

This should be a succinct statement of the problem investigated within the context of a brief review of the relevant literature. Literature directly relevant to any inferences or argument presented in the Discussion should in general be reserved for that section. The introduction may conclude with the reason for doing the work but should not state what was done nor the findings.

***Materials and Methods***

Enough detail must be given here so that another worker can repeat the procedures exactly. Where the materials and methods were exactly as in a previous paper, it is not necessary to repeat all the details but sufficient information must be given for the reader to comprehend what was done without having to consult the earlier work.

Authors are requested to make plain that the conditions of animal and human experimentation are as outlined in the "Ethics" and "Studies on Animals" sections above

***Results or Findings***

These should be given clearly and concisely. Care should be taken to avoid drawing inferences that belong to the Discussion. Data may be presented in various forms such as histograms or tables but, in view of pressure on space, presentation of the same data in more than one form is unacceptable.

***Discussion***

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is occasionally appropriate. Avoid extensive citations and discussion of published literature. ***Conclusions***
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion section.

**Essential title page information**

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-

case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

As titles frequently stand alone in indexes, bibliographic journals etc., and indexing of papers is, to an increasing extent, becoming computerized from key words in the titles, it is important that titles should be as concise and informative as possible. Thus the animal species to which the observations refer should always be given and it is desirable to indicate the type of method on which the observations are based, e.g. chemical, bacteriological, electron-microscopic, histochemical, etc. A "running title" of not more than 40 letters and spaces must also be supplied. A keyword index must be supplied for each paper.

**Highlights**

Highlights are mandatory for this journal as they help increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any).

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

**Structured abstract**

The paper should be prefaced by an abstract aimed at giving the entire paper in miniature. Abstracts should be no longer than 250 words and should be structured as per the guidelines published in the Journal of the American Medical Association (JAMA 1995; 273: 27-34). In brief, the abstract should be divided into the following sections: (1) Objective; (2) Design - if clinical, to include setting, selection of patients, details on the intervention, outcome measures, etc.; if laboratory research, to include details on methods; (3) Results; (4) Conclusions.

**Keywords**

Immediately after the abstract, provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

**Abbreviations**

As Archives of Oral Biology is a journal with a multidisciplinary readership, abbreviations, except those universally understood such as mm, g, min. u.v., w/v and those listed below, should be avoided if possible. Examples of abbreviations which may be used without definition are: ADP, AMP, ATP, DEAE-cellulose, DNA, RNA, EDTA, EMG, tris.
Other abbreviations used to improve legibility should be listed as a footnote on the title page as well as being defined in both the abstract and the main text on first usage. Chemical symbols may be used for elements, groups and simple compounds, but excessive use should be avoided. Abbreviations other than the above should not be used in titles and even these should be avoided if possible.

***Acknowledgements***

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.) but who did not meet all the criteria for authorship (see Authorship section above).

***Formatting of funding sources***

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Bacterial nomenclature**

Organisms should be referred to by their scientific names according to the binomial system. When first mentioned the name should be spelt in full and in italics. Afterwards the genus should be abbreviated to its initial letter, e.g. 'S. aureus' not 'Staph. aureus'. If abbreviation is likely to cause confusion or render the intended meaning unclear, the names of microbes should be spelt in full. Only those names which were included in the Approved List of Bacterial Names, Int J Syst Bacteriol 1980; 30: 225-420 and those which have been validly published in the Int J Syst Bacteriol since 1 January 1980 have standing in nomenclature. If there is good reason to use a name that does not have standing in nomenclature, the names should be enclosed in quotation marks and an appropriate statement concerning the nomenclatural status of the name should be made in the text (for an example see Int J Syst Bacteriol 1980; 30: 547-556). When the genus alone is used as a noun or adjective, use lower case Roman not italic, e.g.'organisms were staphylococci' and 'streptococcal infection'. If the genus is specifically referred to use italics e.g. 'organisms of the genus Staphylococcus'. For genus in plural, use lower case roman e.g. 'salmonellae'; plurals may be anglicized e.g.'salmonellas'. For trivial names, use lower case Roman e.g. 'meningococcus'

**Artwork**

***Image manipulation***

Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

***Electronic artwork***

*General points*

• Make sure you use uniform lettering and sizing of your original artwork.

• Embed the used fonts if the application provides that option.

• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.

• Number the illustrations according to their sequence in the text

• Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately.

• Size the illustrations close to the desired dimensions of the published version.

• Submit each illustration as a separate file.

**You are urged to visit this site; some excerpts from the detailed information are given here.**

*Formats*

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format. Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts. TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi. TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi. TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

**Please do not:**

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

***Illustration services***

Elsevier's Author Services offers Illustration Services to authors preparing to submit a manuscript but concerned about the quality of the images accompanying their article. Elsevier's expert illustrators can produce scientific, technical and medical-style images, as well as a full range of charts, tables and graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

**Tables**

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in

accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

***Data references***

This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

***Reference management software***

Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley. Using citation plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes from different reference management software.

***Reference style***

*Text:* Citations in the text should follow the referencing style used by the American Psychological Association. You are referred to the Publication Manual of the American Psychological Association, Sixth Edition, ISBN 978-1-4338-0561-5, copies of which may be ordered online or APA Order Dept., P.O.B. 2710, Hyattsville, MD 20784, USA or APA, 3 Henrietta Street, London, WC3E 8LU, UK.

*List:* references should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

*Examples:*

Reference to a journal publication:

Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2010). The art of writing a scientific article. *Journal of Scientific Communications, 163*, 51–59. https://doi.org/10.1016/j.Sc.2010.00372.

Reference to a journal publication with an article number:

Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2018). The art of writing a scientific article. *Heliyon*, *19*, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.

Reference to a book:

Strunk, W., Jr., & White, E. B. (2000). *The elements of style.* (4th ed.). New York: Longman, (Chapter 4).

Reference to a chapter in an edited book:

Mettam, G. R., & Adams, L. B. (2009). How to prepare an electronic version of your article. In B. S. Jones, & R. Z. Smith (Eds.), *Introduction to the electronic age* (pp. 281–304). New York: E-Publishing Inc.

Reference to a website:

Cancer Research UK. Cancer statistics reports for the UK. (2003). http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ Accessed 13 March 2003.

Reference to a dataset:

[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T. (2015). *Mortality data for Japanese oak wilt disease and surrounding forest compositions*. Mendeley Data, v1. https://doi.org/10.17632/xwj98nb39r.1.

Reference to a conference paper or poster presentation:

Engle, E.K., Cash, T.F., & Jarry, J.L. (2009, November). The Body Image Behaviours Inventory-3: Development and validation of the Body Image Compulsive Actions and Body Image Avoidance Scales. Poster session presentation at the meeting of the Association for Behavioural and Cognitive Therapies, New York, NY.

**Data visualization**

Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research. Follow the instructions here to find out about available data visualization options and how to include them with your article.

***Research data***

This journal encourages and enables you to share data that supports your research publication where appropriate, and enables you to interlink the data with your published articles. Research data refers to the results of observations or experimentation that validate research findings. To facilitate reproducibility and data reuse, this journal also encourages you to share your software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. If you are sharing data in one of these ways, you are encouraged to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation. For more information on depositing, sharing and using research data and other relevant research materials, visit the research data page. ***Data linking***
If you have made your research data available in a data repository, you can link your article directly to the dataset. Elsevier collaborates with a number of repositories to link articles on ScienceDirect with relevant repositories, giving readers access to underlying data that gives them a better understanding of the research described.

There are different ways to link your datasets to your article.

When available, you can directly link your dataset to your article by providing the relevant information in the submission system. For more information, visit the database linking page . For supported data repositories a repository banner will automatically appear next to your published article on ScienceDirect.

In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). ***Mendeley Data***
This journal supports Mendeley Data, enabling you to deposit any research data (including raw and processed data, video, code, software, algorithms, protocols, and methods) associated with your manuscript in a free-to-use, open access repository. During the submission process, after uploading your manuscript, you will have the opportunity to upload your relevant datasets directly to *Mendeley Data*. The datasets will be listed and directly accessible to readers next to your published article online.

For more information, visit the Mendeley Data for journals page. ***Data statement***
To foster transparency, we encourage you to state the availability of your data in your submission. This may be a requirement of your funding body or institution. If your data is unavailable to access or unsuitable to post, you will have the opportunity to indicate why during the submission process, for example by stating that the research data is confidential. The statement will appear with your published article on ScienceDirect. For more information, visit the Data Statement page.

**Online proof correction**

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type

your corrections, eliminating the potential introduction of errors.
If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.
We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

**Offprints**

The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Author Services. Corresponding authors who have published their article gold open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link.

**Statistical analysis**

Authors should ensure that the presentation and statistical testing of data are appropriate and should seek the advice of a statistician if necessary. A number of common errors should be avoided, e.g.: -

• Use of parametric tests when non-parametric tests are required

• Inconsistencies between summary statistics and statistical tests such as giving means and standard deviations for data which were analysed with non-parametric tests.

• Multiple comparisons undertaken with multiple t tests or non-parametric equivalents rather than with analysis of variance (ANOVA) or non-parametric equivalents.

• Post hoc tests being used following an ANOVA which has yielded a non-significant result.

• Incomplete names for tests (e.g. stating "Student's t test" without qualifying it by stating "single sample", "paired" or "independent sample")

• n values being given in a way which obscures how many independent samples there were (e.g. stating simply n=50 when 10 samples/measurements were obtained from each of 5 animals/human subjects).

• Stating that P=0.000 (a figure which is generated by some computer packages). The correct statement (in this case) is $P<0.0005$.