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ARIELI CARINI MICHELS

POLIMORFISMOS NO GENE *NANOG* ESTÃO ASSOCIADOS COM LEUCOPLASIA BUCAL: ESTUDO CASO-CONTROLE

Curitiba
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Orientadora: Profa. Dra. Aline Cristina Batista Rodrigues Johann.
Coorientador: Prof. Dr. Cleber Machado de Souza.

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Curitiba, 10 de fevereiro de 2020.

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Título: POLIMORFISMOS NO GENE *NANOG* ESTÃO ASSOCIADOS COM LEUCOPLASIA BUCAL: ESTUDO CASO-CONTROLE

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Resumo

Objetivo: Investigar a associação dos polimorfismos no gene *NANOG* com a leucoplasia bucal. **Materiais e métodos**: Pesquisa de caso-controle. A amostra foi constituída por 68 casos de leucoplasia bucal e 21 de mucosa bucal normal (controle), submetidos a genotipagem dos polimorfismos tagSNPs: rs877716 e rs10845877 no gene *NANOG*, por meio da Reação em Cadeia da Polimerase (PCR) em tempo real. Os testes estatísticos utilizados foram Qui–quadrado de Pearson e Exato de Fisher, com significância de 5%. **Resultados**: Para o modelo genético dominante para o alelo G do rs877716, os genótipos AG+GG revelaram frequência aumentada no grupo leucoplasia bucal quando comparado ao controle (75,4 e 50% respectivamente) (p=0,031). Este genótipo apresentou 3,063 vezes chances de leucoplasia bucal comparado com o AA. No modelo genético alélico, para o rs10845877, o alelo C apresentou-se mais frequente em leucoplasia quando comparado ao controle (25 e 7,5% respectivamente) (p=0,01). Nos demais modelos genéticos não houve associação. **Conclusão:** Os polimorfismos no gene *NANOG* estão associados com a leucoplasia bucal.

Palavras-chave: Polimorfismo Genético. *NANOG*. Células tronco embrionárias. Leucoplasia, oral.

Introdução

O carcinoma de células escamosas de boca é uma neoplasia maligna epitelial que está entre os dez cânceres mais comuns no mundo (Rivera, 2015), apresentando incidência global de 300 mil casos, com mais de 145 mil mortes em 2012 (Ferlay et al, 2015). No Brasil, ocorreram 5.898 mortes por câncer de boca em 2015, com uma incidência de diagnósticos da doença, para 2018-2019, estimada em 14.700 casos para cada ano do biênio (Instituto Nacional De Câncer José Alencar Gomes Da Silva, 2017). Além disso, o tratamento apresenta altos custos e o paciente apresenta diferentes condições associadas, como dor, inanição e agravos na função da fala (Rivera, 2015), que remetem a reflexões importantes no âmbito da ciência e Saúde Pública em todo o mundo.

O carcinoma de células escamosas de boca pode ter origem nas desordens bucais potencialmente malignas, e a leucoplasia bucal é a mais frequente destas desordens (Awadallah et al, 2018), sendo “um termo clínico usado para descrever placas brancas de risco questionável, uma vez que outras condições específicas e outras desordens bucais potencialmente malignas tenham sido descartadas” (El-Naggar; Adel, 2017). As leucoplasias bucais podem ser causadas pelo uso de tabaco, álcool e *betel quid*, resultando em evolução sequencial da desordem. Tem sido reportado uma taxa de transformação maligna de leucoplasia de 3,5% (0,13% e 34,0%), sendo que a idade mais avançada, sexo feminino, lesão clinicamente não homogênea e grau de displasia foram considerados fatores de risco para a transformação (Warnakulasuriya; Ariyawardana, 2016). Fatores ambientais e genéticos podem aumentar o risco do desenvolvimento da doença, considerando que a progressão para a transformação maligna, envolve diferentes eventos celulares e genéticos complexos que levam a um acúmulo e progressão de mutações moleculares (Awadallah et al, 2018).

O padrão ouro para determinar risco de transformação maligna da leucoplasia bucal é a análise histológica da gradação da displasia epitelial. Esta, entretanto, apresenta-se subjetiva diante da variabilidade entre os examinadores (Warnakulasuriya et al, 2008), sendo necessárias as investigações de biomarcadores biológicos para determinação de risco. Portanto, a compreensão da suscetibilidade genética à leucoplasia bucal poderia permitir uma análise do

risco de câncer de boca por meio de estágios precoces da doença (Shridhar et al, 2016).

Evidências indicam que a iniciação, progressão, recorrência e metástase de câncer estão relacionados com o comportamento de uma pequena população de células-tronco tumorais (Reers et al, 2014). Estas células apresentam um aumento da plasticidade epigenética e maior variabilidade de expressão de genes (Capp, 2019). O gene Nanog homeobox (*NANOG)*, codifica um fator de transcrição responsável pela auto-renovação e pluripotência de células tronco embrionárias, e ainda pode bloquear a diferenciação dessas células (Jeter et al, 2015). Sob o ponto de vista imuno-histoquímico, a imunoexpressão de Nanog já foi associada ao carcinoma de células escamosas de boca, sendo mais expresso quando há menor grau de diferenciação histológica (Lee et al, 2015, Zhang et al, 2012) e prognóstico ruim (Zhang et al, 2012; Watanabe et al, 2013; Qiao et al, 2014). Com relação a leucoplasia bucal, em um estudo prévio, observou-se uma maior imunoexpressão do fator de transcrição Nanog quando comparado com a mucosa bucal normal (Kitahara; Johann, 2019).

Sabe-se, entretanto, que variações genéticas, chamadas de polimorfismos, dentro da normalidade, podem alterar o fenótipo por meio de diferentes mecanismos: alteração da sequência da proteína, na regulação do gene, do processamento do $_m$RNA e da síntese de proteínas (Wang; Sadée, 2006), e assim se associarem a um aumento do risco de determinada doença. Mas não há estudos que investiguem a associação das variações genéticas no gene *NANOG* em leucoplasia bucal.

Portanto, o objetivo do presente estudo foi investigar a associação dos polimorfismos no gene *NANOG* com leucoplasia bucal.

Material e Métodos

Foi realizado um estudo retrospectivo, observacional, de caso-controle. A metodologia foi realizada no Laboratório de Multiusuário da Pontifícia Universidade Católica do Paraná. Obteve-se aprovação do Comitê de Ética em Pesquisa Local, sob parecer número 2.971.307.

3.1 Amostra

Foram incluídos 68 casos de leucoplasia bucal, diagnosticados clinicamente e confirmados por meio de diagnóstico histopatológico, obtidos no banco secundário de dados dos laboratórios de Patologia bucal da Pontifícia Universidade Católica do Paraná, Universidade Federal de Minas Gerais e Universidade Federal de Santa Catarina e 21 controles de mucosa bucal normal do rebordo alveolar obtidas por indicação de remoção, por meio de cunha distal para acesso em exodontias de terceiro molar incluso, na Universidade Positivo.

Todas as lâminas das amostras foram submetidas a confirmação de diagnóstico segundo os critérios da OMS (El-Naggar; Adel, 2017) por dois patologistas experientes e calibrados. Para os casos de leucoplasia bucal, foram excluídas amostras em que havia diagnóstico de hiperceratose sem atipia epitelial. Para caso e controle, foram excluídas as amostras com presença de inflamação tecidual e blocos de parafina ausentes.

Com relação a idade, no grupo caso, 20 (30,8%) indivíduos tinham menos de 48 anos e 45 (69,2%) indivíduos apresentavam idade igual ou superior a 48 anos. O grupo controle foi constituído 17 (100%) indivíduos com idade inferior a 48 anos, sendo que não foram identificadas idades em 3 fichas no grupo caso e em 4 fichas do grupo controle. Quanto ao gênero, 37 (54,4%) indivíduos eram do sexo feminino e 31 (45,6%) do sexo masculino no grupo caso e 10 (47,6%) e 11 (52,4%), respectivamente no grupo controle.

3.2 Análise Genética

A extração de DNA foi realizada com o QIAamp DNA minikit® de acordo com as normas do fabricante. Utilizou-se Buffer ATL (Tissue Lysis Buffer), proteinase K, Buffer AL, etanol, QIAamp DNA minikit® e Buffer AW1 (Wash Buffer 1) e Buffer AW2 (Wash Buffer 2) e Buffer AE (Simonato et al, 2007).

Foram selecionados Polimorfismo de Nucleotídeo Único (SNP), do tipo *tag*, que apresenta alto equilíbrio de ligação com demais SNPs, possibilitando a cobertura total do gene em relação a variabilidade genética, sem a necessidade de estudo individual dos SNPs. Os *tag*SNPs no gene *NANOG* foram escolhidos com base no International HapMap Project (http://www.hapmap.org), segundo os

parâmetros: frequência alélica mínima de 5% e Desequilíbrio de Ligação de 80% na população europeia (CEU) proporcionando total cobertura ao gene (tabela 1).

Tabela 1. Seleção de *tag*SNPs no gene *NANOG* para o estudo.

*tag*SNP	Troca de Base	Frequência Alélica Mínima	Localização	FAM
rs877716	A/G	0,46	íntron	G 0,31-0,69
rs10845877	C/T	0,15	íntron	C 0,14-0,86

O DNA purificado dos pacientes foi amplificado pela técnica de Reação em Cadeia da Polimerase (PCR) em Tempo Real (Applied Biosystems 7500 Real Time PCR System; Applied Biosystems, Foster City, CA, USA) utilizando a tecnologia TaqMan® Genotyping Master Mix (Applied Biosystems) para a amplificação dos polimorfismos (Simonato et al, 2007).

3.3 Análise Estatística

Por meio Haploview 4.2 obteve-se o equilíbrio de Hardy-Weinberg e o cálculo de Desequilíbrio de Ligação entre os *tag*SNPs. As análises estatísticas foram realizadas por meio do software SPSS, versão 25.0 para Windows (Inc. Chicago. Illions, USA). Para a análise no modelo genético aditivo, utilizou-se a regressão logística binária. Os modelos alélico, dominante e recessivo, foram analisados pelos testes Qui-quadrado de Pearson ou teste exato de Fisher Odds Ratio (OR). O nível de significância adotado foi de 5% ($p<0,05$).

Resultados

A distribuição de genótipos do gene *NANOG* apresentou-se em equilíbrio de Hardy-Weinberg no grupo controle. O cálculo do Desequilíbrio de Ligação entre os *tag*SNPs rs10845877 e rs877716 para a população do presente estudo, indicaram baixo Desequilíbrio de Ligação (DL) (r^2=15%).

Quanto aos modelos genéticos, observou-se que, no modelo dominante para o alelo G; AG+GG vs AA, do rs877716, no gene *NANOG*, os genótipos AG+GG estão com uma frequência aumentada no grupo leucoplasia bucal com

diferença significativa entre os grupos. Indivíduos com o genótipo AG+GG apresentam 3,063 maiores chances de leucoplasia bucal comparado com o genótipo AA (Tabela 3). Além disso, apresentou valor *borderline* no modelo genético aditivo entre caso e controle (p=0,086). Nos demais modelos (alélico e recessivo) não houve associação (Tabelas 2 e 3).

O *tag*SNPs rs10845877, no modelo alélico, foi associado à leucoplasia bucal (p=0,01) e a presença do alelo C parece ser fator de risco para Leucoplasia Bucal (Tabela 2).

Tabela 2 – Análise genotípica e alélica dos *tag*SNPs no gene *NANOG* para o modelo aditivo para os grupos Leucoplasia e Controle.

Tag SNPs DbSNP ID[a]	Grupo	Homozigoto 1/1 N (%)	Heterozigoto 1/2 N (%)	Homozigoto 2/2 N (%)	Valor p*	Alelo N (%)		Valor p*
rs877716		AA	AG	GG		A	G	
	Leucoplasia	16 (24,6)	34 (52,3)	15 (23,1)	0,086	66 (50,8)	64(49,2)	0,11
	Controle	10 (50)	6 (30)	4 (20)		26 (65)	14 (35)	
rs10845877		TT	CT	CC		T	C	
	Leucoplasia	40 (62,5)	16 (25)	8 (12,5)	0,116	96 (75)	32 (25)	**0,01**
	Controle	17 (85)	3 (15)	0 (0)		37 (92,5)	3 (7,5)	

[a] Identificador de SNP baseado no dbSNP do NCBI. *Qui-quadrado.

Tabela 3 – Análise da associação genotípica dos *tag*SNPs do gene *NANOG* para os modelos dominante e recessivo para os grupos Leucoplasia e Controle.

*Tag*SNPS dbSNP ID[a]	Grupo	Genótipo n (%)	Genótipo N (%)	Valor de p	OR (IC 95%)
rs877716 DOM G		AG+GG	AA		
	Leucoplasia	49 (75,4)	16 (24,6)	**0,031***	3.063 (1.080 – 8.686)
	Controle	10 (50)	10 (50)		
		CT+CC	TT		
rs10845877 DOM C	Leucoplasia	64,4 (37,5)	40 (62,5)	0,50**	
	Controle	3 (15)	17 (85)		
rs877716 REC G		GG	AA+AG		
	Leucoplasia	15 (23,1)	50 (76,9)	0,520**	
	Controle	4 (20)	16 (80)		
rs10845877 REC C		CC	CT+TT		
	Leucoplasia	8 (12,5)	56 (87,5)	0,102**	
	Controle	0 (0,0)	20 (100)		

[a] Identificador de SNP baseado no dbSNP do NCBI. *Teste Qui-quadrado de Pearson **Teste Exato de Fisher. OR: Odds Ratio; IC: Intervalo de Confiança.

Discussão

A leucoplasia bucal é uma desordem bucal com potencial maligno (El-Naggar; Adel, 2017). A teoria das células tronco tumorais, indica que um grupo específico de células poderia iniciar e progredir o tumor: células tronco. O *NANOG* é um gene importante na manutenção de pluripotencialidade e auto-renovação de células tronco embrionárias (Jeter et al, 2015), e já se identificou sua expressão por meio de imuno-histoquímica em carcinoma de células escamosas de boca (Lee et al, 2015, Zhang et al,2012; Watanabe et al,2013; Qiao et al, 2014) e leucoplasia bucal (Kitahara; Johann, 2019). Entretanto pela primeira vez investigou-se a associação das variações genéticas (rs877716 e rs10845877) no *NANOG* com a leucoplasia bucal e encontrou-se associação entre eles. O polimorfismo rs877716 no modelo genético aditivo revelou valores *borderline* quando os grupos leucoplasia e controle foram comparados, e no modelo dominante, indivíduos com o genótipo AG+GG apresentaram risco elevado para leucoplasia bucal. O segundo polimorfismo estudado, rs10845877, foi associado com leucoplasia bucal no modelo alélico e parece que indivíduos com alelo C tem maior risco para leucoplasia bucal.

Estudos anteriores, analisados em uma revisão de literatura, com intuito de identificar associações de variações genéticas à desordens bucais potencialmente malignas, dentre elas a leucoplasia bucal, indicaram SNPs nos genes *GSRM1* (4 estudos), *CCND1* (2 estudos), *MMP3* (2 estudos), *TNFα* (2 estudos), *XPD* (2 estudos) e *Gemin3* (2 estudos) como marcadores sugestivos de suscetibilidade às desordens bucais potencialmente malignas, em diferentes populações ao redor do mundo (asiáticos, caucasianos, brasileiros) e *p53* (2 estudos) para população indiana. Entretanto, outros estudos não indicaram associação entre as desordens bucais potencialmente malignas e os SNPs em *GSTM1* (10 estudos), *XPD* (10 estudos) e *p53* (10 estudos). Além disso, o tamanho da amostra era inferior a 200 indivíduos na maioria dos estudos, o que poderia dificultar a detecção de associações baixas e moderadas de risco às lesões; as frequências dos alelos e dos genótipos de risco foram relatadas de forma não padronizada entre os estudos e as pesquisas precisam de validação para diferentes populações, dessa forma, os autores da revisão indicam que não é possível forte inferência para nenhum

SNP identificado até o momento em nenhuma população (Shridhar et al, 2016). Estudos posteriores, em população indiana, identificaram associação de SNPs em *XPD* e *XPG* com desordens bucais potencialmente malignas (Nigam et al, 2019); e SNPs em *GSTM1, GSTT1* com risco de leucoplasia bucal (Rao et al, 2017), mas sem investigação de polimorfismos no *NANOG* com leucoplasia bucal.

Na ausência de estudos anteriores que investiguem a associação de *tag*SNPs no *NANOG* em leucoplasia bucal e em carcinoma de células escamosas de boca, estes já foram investigados em diferentes tipos de câncer. Na próstata, ao analisar o polimorfismo rs1105786 no *NANOG*, baseado em população "Gujarati Indians in Houston", não se observou associação com a doença, nem com características clínico-patológicas (Nong et al, 2018). Em câncer de mama, em uma população "Gujarati Indians in Houston", também não foi possível observar diferenças estatísticas nas frequências entre grupo caso e controle, nos diferentes modelos genéticos (aditivo, dominante e recessivo), nem associação com parâmetros clinico-patológicos com o rs11055786, no *NANOG* (Tulsyan et al, 2014). Da mesma forma, não foram observadas associações entre o rs11055786 no *NANOG* e o câncer de vesícula biliar (Yadav et al, 2016).

Entretanto a associação da expressão imuno-histoquímica do fator de transcrição expresso pelo gene *NANOG* com carcinoma de células escamosas de boca (Lee et al, 2015, Zhang et al,2012; Watanabe et al,2013; Qiao et al, 2014), bem como com leucoplasia bucal (Kitahara; Johann, 2019) nos motivou a investigação da associação das variabilidades genéticas desse gene com a leucoplasia bucal, e diferentemente dos estudos anteriores com diferentes tipos de câncer, encontramos associação de ambos os *tag*SNPs (cobertura total do gene): rs877716 no modelo genético dominante e borderline no modelo aditivo e rs10845877, no modelo alélico, sugerindo este gene como marcador de suscetibilidade à leucoplasia bucal.

Devido ao seu caráter retrospectivo, com coleta de dados em base de dados secundários, o estudo apresenta limitações relacionadas ao pareamento e tamanho da amostra e avaliação das interferências de fatores ambientais. Dentro das limitações deste estudo, os resultados evidenciam associação de ambos os polimorfismos genéticos no *NANOG* com a leucoplasia bucal, para a população

estudada. Contudo, sugere-se estudos futuros para validação em outras populações, associações com fatores ambientais bem como o uso de outras metodologias, como a epigenética, a fim de identificar um possível papel desses polimorfimos na metilação do DNA, interferência no RNA ou modificações das histonas (Angarica, Del Sol, 2017).

Este estudo poderá contribuir para elaboração de futuras ferramentas de diagnóstico precoce, bem como de terapêuticas alvo e, assim, diminuir os altos índices de morbimortalidade por carcinoma de células escamosas, bem como os altos custos de tratamento da doença para os sistemas públicos de saúde.

Conclusão

Baseado nos resultados obtidos, este estudo fornece evidências de associação entre *NANOG* e leucoplasia bucal.

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Title Page

Title: POLYMORPHISMS IN THE NANOG GENE ARE ASSOCIATED WITH ORAL LEUKOPLASIA: CASE-CONTROL STUDY

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Abstract

Objective: To investigate the association of polymorphisms in the *NANOG* gene with oral leukoplakia. Materials and methods: Case-control research. The sample consisted of 68 cases of oral leukoplakia and 21 of normal oral mucosa (control), submitted to genotyping of the tagSNPs polymorphisms: rs877716 and rs10845877 in the *NANOG* gene, through the Polymerase Chain Reaction (PCR) in real time. The statistical tests used were Chi-square and Fisher's Exact, with a significance of 5%. Results: For the dominant genetic model for the G allele of rs877716, the AG + GG genotypes showed an increased frequency in the oral leukoplakia group when compared to the control group (75.4 and 50% respectively) ($p = 0.031$). This genotype had 3.063 times chances of oral leukoplakia compared to AA. In the allelic genetic model, for rs10845877, the C allele was more frequent in leukoplakia when compared to the control (25 and 7.5% respectively) ($p = 0.01$). In the other genetic models there was no association. Conclusion: Polymorphisms in the NANOG gene are associated with oral leukoplakia.

Keywords: Polymorphism, Genetic. *NANOG*. Embryonic Stem Cells. Leukoplakia, Oral.

Introduction

Oral squamous cell carcinoma is an epithelial malignancy among the ten most common cancers in the world (Rivera, 2015), with a global incidence of 300 thousand cases and more than 145 thousand deaths in 2012 (Ferlay et al, 2015). In Brazil, there were 5,898 deaths from oral cancer in 2015 and an incidence of diagnoses of the disease for 2018-2019, estimated at 14,700 cases for each year of the biennium (Instituto Nacional De Câncer José Alencar Gomes Da Silva, 2017). In addition, the treatment has high costs and the patient has different associated conditions, such as pain, starvation and disorders in the function of speech (Rivera, 2015), that lead to important reflections in the field of science and public health around the world.

Oral squamous cell carcinoma can originate from potentially malignant oral disorders, and oral leukoplakia is the most frequent of these disorders (Awadallah et al, 2018), being “a clinical term used to describe white plaques of questionable risk, once that other specific conditions and other potentially malignant oral disorders have been ruled out ”(El-Naggar; Adel, 2017). Oral leukoplakias can be caused by the use of tobacco, alcohol and betel quid, resulting in the sequential evolution of the disorder. A rate of leukoplakia malignant transformation of 3.5% (0.13% and 34.0%) has been reported, with older age, female gender, clinically inhomogeneous lesion and dysplasia’s degree being considered risk factors (Warnakulasuriya; Ariyawardana, 2016). Environmental and genetic factors can increase the risk of developing the disease, considering that the progression to malignant transformation involves different complex cellular and genetic events that lead to an accumulation and progression of molecular mutations (Awadallah et al, 2018).

The gold standard for determining the risk of malignant transformation of oral leukoplakia is the histological analysis of the gradation dysplasia epithelial. However this is subjective because of variability among examiners (Warnakulasuriya et al, 2008), thats requiring the investigation of biological biomarkers to determine risk. Therefore, understanding the genetic susceptibility to oral leukoplakia could be allow an analysis of oral câncer risk through early stages of the disease (Shridhar et al, 2016).

Evidence indicates that cancer initiation, progression, recurrence and metastasis are related to the behavior of a small population of tumor stem cells (Reers et al, 2014). These cells show an increase in epigenetic plasticity and greater variability in gene expression (Capp, 2019). The Nanog homeobox (NANOG) gene encodes a transcription factor responsible for the self-renewal and pluripotency of embryonic stem cells, and can also block the differentiation of these cells (Jeter et al, 2015). From an immunohistochemical, Nanog has already been associated with oral squamous cell carcinoma, and it is more expressed when there is histological differentiation less degree (Lee et al, 2015, Zhang et al, 2012) and poor prognosis (Zhang et al, 2012; Watanabe et al, 2013; Qiao et al, 2014). About oral leukoplakia, in a previous study, a greater immunoexpression of the transcription factor Nanog was observed when compared to the normal oral mucosa (Kitahara; Johann, 2019).

However, it is known that genetic variations, the polymorphisms, within normality, can alter the phenotype through different mechanisms: alteration of the protein sequence, in the regulation of the gene, in the processing of mRNA and in protein synthesis (Wang ; Sadée, 2006), and thus are associated with an increased risk of a particular disease. But there are no studies that investigate the association of genetic variations in the NANOG gene in oral leukoplakia.

The aim of the present study was to investigate the association of polymorphisms in the NANOG gene with oral leukoplakia.

Material and Methods

A retrospective, observational, case-control study was carried out. Approval was obtained from the Local Research Ethics Committee (2.971.307).

3.1 Sample

The sample comprised 68 cases of oral leukoplakia, clinically diagnosed and confirmed by means of histopathological diagnosis, obtained from the secondary database of the oral pathology laboratories of the Pontifical Catholic University of Paraná, Federal University of Minas Gerais and Federal University of Santa Catarina and 21 controls of normal buccal mucosa of the alveolar ridge obtained by

indication of removal, by means of a distal wedge for access to extractions of the third molar included, at Positivo University.

All slides of the samples were submitted to diagnostic confirmation according to WHO criteria (El-Naggar; Adel, 2017) by two experienced and calibrated pathologists. Samples of oral leukoplakias in which there was a diagnosis of hyperkeratosis without epithelial atypia were excluded. For case and control, samples with tissue inflammation and missing paraffin blocks were excluded.

Regarding age, in the case group, 20 (30.8%) individuals were younger than 48 years old and 45 (69.2%) individuals were 48 years old or older. The control group consisted of 17 (100%) individuals under the age of 48 years, and ages were not identified in 3 records in the case group and in 4 records in the control group. As for gender, 37 (54.4%) individuals were female and 31 (45.6%) male in the case group and 10 (47.6%) and 11 (52.4%), respectively in the group control.

3.2 Genetic Analysis

DNA extraction was performed with the QIAamp DNA minikit® according to the manufacturer's standards. Buffer ATL (Tissue Lysis Buffer), proteinase K, Buffer AL, ethanol, QIAamp DNA minikit® and Buffer AW1 (Wash Buffer 1) and Buffer AW2 (Wash Buffer 2) and Buffer AE (Simonato et al, 2007) were used.

*Single Nucleotide Polymorphism (*SNP), of the tag type, which has a high link balance with other SNPs, allowing full coverage of the gene in relation to genetic variability, without the need for individual study of the SNPs. The tagSNPs in the NANOG gene were chosen based on the International HapMap Project (http://www.hapmap.org), according to the parameters: minimum allelic frequency of 5% and Link Imbalance of 80% in the European Population providing total gene coverage (table 1).

Table 1. Selection of tagSNPs in the NANOG gene for the study.

*tag*SNP	Base Exchange	Minimum Allele Frequency	Location	FAM
rs877716	A/G	0,46	intron	G 0,31-0,69
rs10845877	C/T	0,15	intron	C 0,14-0,86

The patients' purified DNA was amplified by the Polymerase Chain Reaction (PCR) technique in Real Time (Applied Biosystems 7500 Real Time PCR System; Applied Biosystems, Foster City, CA, USA) using the technology TaqMan® Genotyping Master Mix (Applied Biosystems) for the polymorphisms amplification (Simonato et al, 2007).

3.2 Statistical Analysis

Using Haploview 4.2, the Hardy-Weinberg balance and the Link Imbalance calculation between the tagSNPs were obtained. Statistical analyzes were performed using SPSS software, version 25.0 for Windows (Inc. Chicago. Illions, USA). For the analysis in the additive genetic model, chi-square was used. The allelic, dominant and recessive models were analyzed using Pearson's chi-square test or Fisher Odds Ratio (OR) exact test. The level of significance adopted was 5% ($p < 0.05$).

Results

NANOG gene genotypes distribution was in Hardy-Weinberg equilibrium in the control group. The calculation of the Link Imbalance between the rs10845877 and rs877716 tagSNPs for the population of the present study, indicated a low Link Imbalance (DL) ($r2 = 15\%$).

As for the genetic models, it was observed that, in the dominant model for the G allele; AG + GG vs AA, from rs877716, in the NANOG gene, the AG + GG genotypes are with an increased frequency in the oral leukoplakia group with a significant difference between the groups. Individuals with the AG + GG genotype have 3.063 higher chances of oral leukoplakia compared to the AA genotype (Table

3). In addition, it showed *borderline* value in the additive genetic model between case and control (p = 0.086). In the other models (allelic and recessive) there was no association (Tables 2 and 3).

The rs10845877 tagSNPs, in the allelic model, was associated with oral leukoplakia (p = 0.01) and the presence of the C allele seems to be a risk factor for oral leukoplakia (Table 2).

Table 2 - Genotypic and allelic analysis of the tagSNPs in the NANOG gene for the additive model for the Leukoplakia and Control groups.

Tag SNPs DbSNP ID[a]	Group	Homozygous 1/1 N (%)	Heterozygote 1/2 N (%)	Homozygous 2/2 N (%)	Value p*	Allele N (%)		Value p*
rs877716		AA	AG	GG		A	G	
	Leukoplakia	16 (24,6)	34 (52,3)	15 (23,1)	0,086	66 (50,8)	64(49,2)	0,11
	Control	10 (50)	6 (30)	4 (20)		26 (65)	14 (35)	
rs10845877		TT	CT	CC		T	C	
	Leukoplakia	40 (62,5)	16 (25)	8 (12,5)	0,116	96 (75)	32 (25)	**0,01**
	Control	17 (85)	3 (15)	0 (0)		37 (92,5)	3 (7,5)	

[a] SNP identifier based on NCBI's dbSNP. * Chi-square.

Table 3 - Analysis of the genotypic association of the NANOG gene tagSNPs for the dominant and recessive models for the Leukoplakia and Control groups.

*Tag*SNPS dbSNP ID[a]	Group	Genotype n (%)	Genotype N (%)	Value p	OR (CI 95%)
rs877716 DOM G		AG+GG	AA		
	Leukoplakia	49 (75,4)	16 (24,6)	**0,031***	3.063 (1.080 – 8.686)
	Control	10 (50)	10 (50)		
		CT+CC	TT		
rs10845877 DOM C	Leukoplakia	64,4 (37,5)	40 (62,5)	0,50**	
	Control	3 (15)	17 (85)		
rs877716 REC G		GG	AA+AG		
	Leukoplakia	15 (23,1)	50 (76,9)	0,520**	
	Control	4 (20)	16 (80)		
rs10845877 REC C		CC	CT+TT		
	Leukoplakia	8 (12,5)	56 (87,5)	0,102**	
	Control	0 (0,0)	20 (100)		

[a] SNP identifier based on NCBI's dbSNP. * Pearson's Chi-square test ** Fisher's exact test. OR: Odds Ratio; CI: Confidence Interval.

Discussion

Oral leukoplakia is an oral disorder with malignant potential (El-Naggar; Adel, 2017). The stem cell cancer theory indicates that a specific group of cells could initiate and progress the tumor: stem cells. NANOG is an important gene in the maintenance of pluripotentiality and self-renewal of embryonic stem cells (Jeter et

al, 2015), and its expression has already been identified through immunohistochemistry in oral squamous cell carcinoma (Lee et al, 2015, Zhang et al, 2012; Watanabe et al, 2013; Qiao et al, 2014) and oral leukoplakia (Kitahara; Johann, 2019). However, for the first time, the association of genetic variations (rs877716 and rs10845877) in NANOG with oral leukoplakia was investigated and an association was found between them. The rs877716 polymorphism in the additive genetic model revealed borderline values when the leukoplakia and control groups were compared, and in the dominant model, individuals with the AG + GG genotype were at high risk for oral leukoplakia. The second polymorphism studied, rs10845877, was associated with oral leukoplakia in the allelic model and it appears that individuals with C allele are at higher risk for oral leukoplakia.

Previous studies, analyzed in a literature review, in order to identify associations of genetic variations with potentially malignant oral disorders, among them oral leukoplakia, indicated SNPs in the GSRM1 (4 studies), CCND1 (2 studies), MMP3 genes (2 studies), TNFα (2 studies), XPD (2 studies) and Gemin3 (2 studies) as markers suggestive of susceptibility to potentially malignant oral disorders, in different populations around the world (Asians, Caucasians, Brazilians) and p53 (2 studies) for Indian population. However, other studies have not indicated an association between potentially malignant oral disorders and SNPs in GSTM1 (10 studies), XPD (10 studies) and p53 (10 studies). In addition, the sample size was less than 200 individuals in most studies, which could make it difficult to detect low and moderate associations of risk to injuries; the frequencies of alleles and risk genotypes have been reported in a non-standardized way between studies and research needs validation for different populations, thus, the authors of the review indicate that no strong inference is possible for any SNP identified so far in no population (Shridhar et al, 2016). Subsequent studies in an Indian population identified an association of SNPs in XPD and XPG with potentially malignant oral disorders (Nigam et al, 2019); and SNPs in GSTM1, GSTT1 at risk of oral leukoplakia (Rao et al, 2017), but without investigation of polymorphisms in NANOG with oral leukoplakia.

In the absence of previous studies investigating the association of tagSNPs in NANOG in oral leukoplakia and oral squamous cell carcinoma, these have

already been investigated in different types of cancer. In the prostate, when analyzing the rs1105786 polymorphism in the NANOG, based on the “Gujarati Indians in Houston” population, there was no association with the disease, nor with clinical-pathological characteristics (Nong et al, 2018). In breast cancer, in a population “Gujarati Indians in Houston”, it was also not possible to observe statistical differences in the frequencies between case and control groups, in the different genetic models (additive, dominant and recessive), nor association with clinical-pathological parameters with o rs11055786, in the NANOG (Tulsyan et al, 2014). Likewise, no associations were observed between rs11055786 in NANOG and gallbladder cancer (Yadav et al, 2016).

However, the association of immunohistochemical expression of the transcription factor expressed by the NANOG gene with oral squamous cell carcinoma (Lee et al, 2015, Zhang et al, 2012; Watanabe et al, 2013; Qiao et al, 2014), as well as with oral leukoplakia (Kitahara; Johann, 2019) motivated us to investigate the association of the genetic variability of this gene with oral leukoplakia, and unlike previous studies with different types of cancer, we found an association of both tagSNPs (full coverage of the gene) : rs877716 in the dominant and borderline genetic model in the additive model and rs10845877 in the allelic model, suggesting this gene as a marker of susceptibility to oral leukoplakia.

Due to its retrospective character, with data collection in secondary databases, the study has limitations related to the pairing and sample size and evaluation of the interference of environmental factors. Within the limitations of this study, the results show an association of both genetic polymorphisms in the NANOG with oral leukoplakia, for the population studied. However, future studies are suggested for validation in other populations, associations with environmental factors as well as the use of other methodologies, such as epigenetics, in order to identify a possible role of these polymorphisms in DNA methylation, RNA interference or histone modifications (Angarica, Del Sol, 2017).

This study may contribute to the development of future tools for early diagnosis, as well as target therapies and, thus, reduce the high rates of morbidity and mortality from squamous cell carcinoma, as well as the high costs of treating the disease for public health systems.

Conclusion

Based on the results obtained, this study provides evidence of an association between NANOG and oral leukoplakia.

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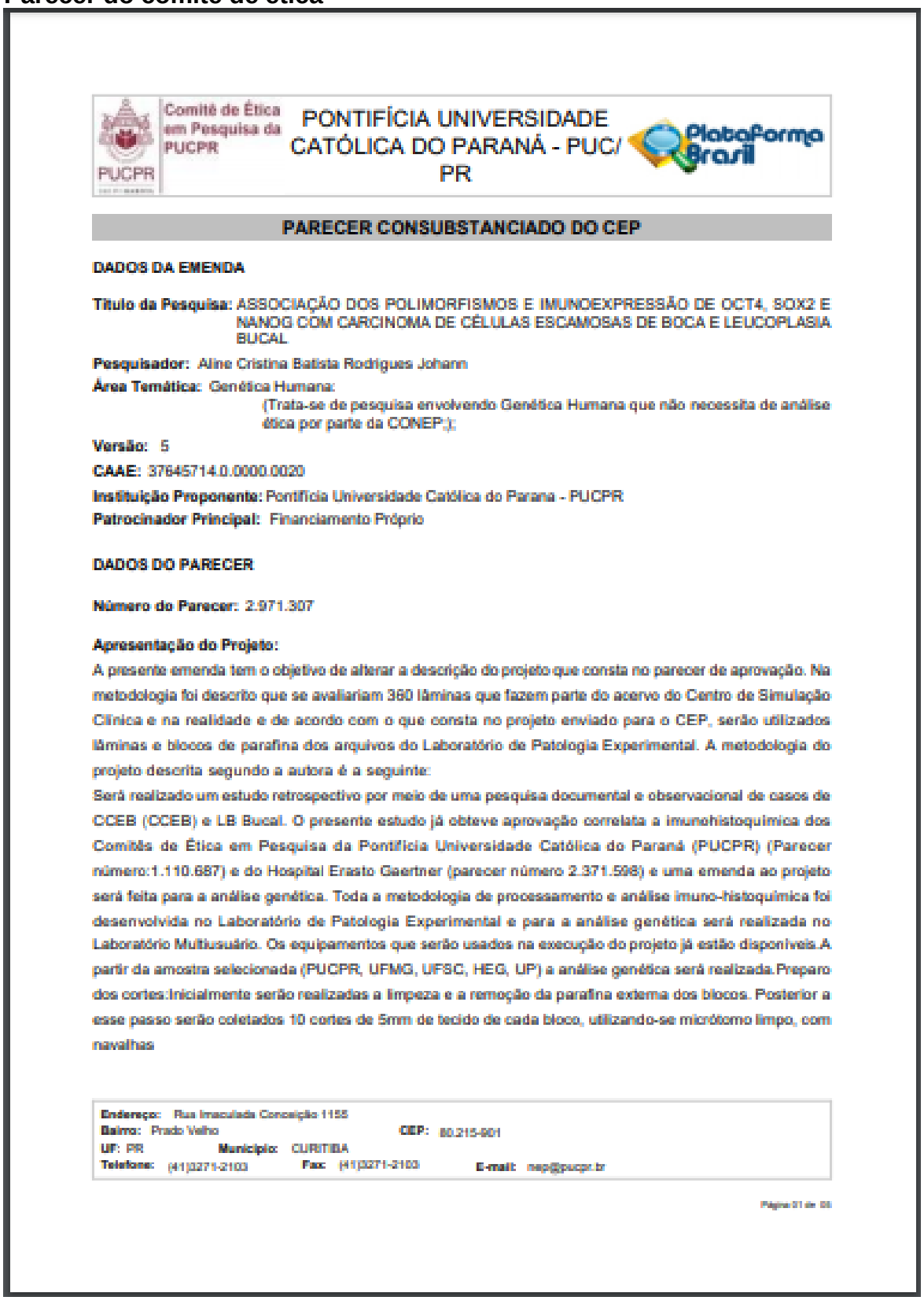
ANEXOS

Parecer do comitê de ética



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PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: ASSOCIAÇÃO DOS POLIMORFISMOS E IMUNOEXPRESSÃO DE OCT4, SOX2 E NANOG COM CARCINOMA DE CÉLULAS ESCAMOSAS DE BOCA E LEUCOPLASIA BUCAL

Pesquisador: Aline Cristina Batista Rodrigues Johann

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 5

CAAE: 37645714.0.0000.0020

Instituição Proponente: Pontifícia Universidade Católica do Parana - PUCPR

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.971.307

Apresentação do Projeto:

A presente emenda tem o objetivo de alterar a descrição do projeto que consta no parecer de aprovação. Na metodologia foi descrito que se avaliariam 360 lâminas que fazem parte do acervo do Centro de Simulação Clínica e na realidade e de acordo com o que consta no projeto enviado para o CEP, serão utilizados lâminas e blocos de parafina dos arquivos do Laboratório de Patologia Experimental. A metodologia do projeto descrita segundo a autora é a seguinte:

Será realizado um estudo retrospectivo por meio de uma pesquisa documental e observacional de casos de CCEB (CCEB) e LB Bucal. O presente estudo já obteve aprovação correlata a imunohistoquímica dos Comitês de Ética em Pesquisa da Pontifícia Universidade Católica do Paraná (PUCPR) (Parecer número:1.110.687) e do Hospital Erasto Gaertner (parecer número 2.371.598) e uma emenda ao projeto será feita para a análise genética. Toda a metodologia de processamento e análise imuno-histoquímica foi desenvolvida no Laboratório de Patologia Experimental e para a análise genética será realizada no Laboratório Multiusuário. Os equipamentos que serão usados na execução do projeto já estão disponíveis.A partir da amostra selecionada (PUCPR, UFMG, UFSC, HEG, UP) a análise genética será realizada.Preparo dos cortes:Inicialmente serão realizadas a limpeza e a remoção da parafina externa dos blocos. Posterior a esse passo serão coletados 10 cortes de 5mm de tecido de cada bloco, utilizando-se micrótomo limpo, com navalhas

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descartáveis. Após o corte, as fatias serão acondicionadas em tubos plásticos de 1,5ml identificados previamente e mantidas à temperatura ambiente até o momento da extração. Os procedimentos laboratoriais serão realizados por um único técnico de laboratório.Antes da extração do DNA, os cortes passarão pelo processo de desparafinização, sendo que cada método requereu procedimentos distintos.Para purificação com o kit da Qiagen (QIAamp DNA minikit®) será seguido os seguintes procedimentos: será adicionado 1.200l de xilol as amostras, agitando-se por 15 segundos (1). Em seguida, os tubos serão centrifugados a 14.000rpm (rotações por minuto) durante cinco minutos (2). O sobrenadante será desprezado (3) e 1.200l de etanol será adicionado ao sedimento formado (4). Os tubos serão agitados por 15 segundos (5) e centrifugados a 14.000rpm durante cinco minutos (6). Esse procedimento será repetido e, ao final, os tubos com as tampas abertas serão colocados em centrífuga à vácuo a 37°C durante 15 minutos, objetivando a evaporação do etanol remanescente (7). Extração manualO protocolo para extração de DNA a partir de material parafinado com o sistema comercial QIAamp DNA minikit® será realizado de acordo com as normas do fabricante. Em cada tubo será adicionado 180l de Buffer ATL (Tissue Lysis Buffer), além de 20l de proteinase K. Após homogeneização, as amostras serão incubadas em banho-maria a 55°C, durante três horas, e agitadas gentilmente a cada hora. Após esse período, deverá ser adicionado a cada tubo 200l de Buffer AL (fornecido pelo fabricante), sendo os mesmos previamente aquecidos a 70°C durante 10 minutos para a inativação da proteinase residual. Em seguida, será adicionado 200l de etanol seguido de agitação durante 15 segundos e centrifugando brevemente. A solução resultante será transferida para o dispositivo da coluna QIAamp DNA minikit® e que deverá ser centrifugada a 8.000 rpm por um minuto. O dispositivo com a coluna será removido do tubo e recolocado em um tubo limpo. Posteriormente serão adicionados 500l de Buffer AW1 (Wash Buffer 1) ao dispositivo com a coluna, que será centrifugado a 8.000 rpm por um minuto. O procedimento de lavagem será repetido com 500l de Buffer AW2 (Wash Buffer 2), seguido de centrifugação a 14.000 rpm durante três minutos. O DNA extraído da coluna será eluído pela adição de 100l de Buffer AE (fornecido pelo fabricante). Adicionando primeiramente 50l Buffer AE, aguardando-se um minuto e centrifugando-se a 8.000 rpm durante um minuto. Em seguida, será adicionado o restante (50l) de Buffer AE. Aguardar por cinco minutos e centrifugar a 8.000 rpm por um minuto. Após as amostras serem transferidas do dispositivo para tubo de recuperação, as mesmas serão armazenadas a -20°C. Análises de polimorfismos genéticos - Genotipagem por PCR em tempo real: Os polimorfismos alvo (tag SNPs) nos genes dos biomarcadores de lesão e resposta tecidual propostos foram escol.

Objetivo da Pesquisa:

A presente emenda tem o objetivo de alterar a descrição do projeto que consta no parecer de aprovação. Na metodologia foi descrito que se avaliariam 360 lâminas que fazem parte do acervo do Centro de Simulação Clínica e na realidade e de acordo com o que consta no projeto enviado para o CEP, serão utilizados lâminas e blocos de parafina dos arquivos do Laboratório de Patologia Experimental.

Avaliação dos Riscos e Benefícios:

Os riscos e benefícios apresentados estão adequados e de acordo com a Resolução 466/2012.

Comentários e Considerações sobre a Pesquisa:

Os objetivos e a metodologia apresentados estão adequados e de acordo com a Resolução 466/2012.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos de apresentação obrigatória foram anexados e estão adequados e em acordo com a Resolução 466/2012.

Recomendações:

Sem recomendações.

Conclusões ou Pendências e Lista de Inadequações:

Emenda aprovada.

Considerações Finais a critério do CEP:

Lembramos aos senhores pesquisadores que, no cumprimento da Resolução 466/2012, o Comitê de Ética em Pesquisa (CEP) deverá receber relatórios anuais sobre o andamento do estudo, bem como a qualquer tempo e a critério do pesquisador nos casos de relevância, além do envio dos relatos de eventos adversos, para conhecimento deste Comitê. Salientamos ainda, a necessidade de relatório completo ao final do estudo. Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP/PUCPR de forma clara e sucinta, identificando a parte do protocolo a ser modificado e as suas justificativas. Se a pesquisa, ou parte dela for realizada em outras instituições, cabe ao pesquisador não iniciá-la antes de receber a autorização formal para a sua realização. O documento que autoriza o início da pesquisa deve ser carimbado e assinado pelo responsável da instituição e deve ser mantido em poder do pesquisador responsável, podendo ser requerido por este CEP em qualquer tempo.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1231281_E4.pdf	02/10/2018 16:49:33		Aceito
Outros	ProjetoEmenda.docx	02/10/2018 16:39:53	Aline Cristina Batista Rodrigues Johann	Aceito
Declaração de Instituição e Infraestrutura	Autorizacao_Hospital_Erasto_Gaertner.pdf	04/04/2017 10:34:48	Aline Cristina Batista Rodrigues Johann	Aceito
Declaração de Instituição e Infraestrutura	Autorização UFSC.pdf	29/05/2015 11:21:57		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	tcle.doc	28/05/2015 15:31:57		Aceito
Outros	Carta solicitação de correção do parecer-signed.pdf	24/03/2015 16:04:11		Aceito
Outros	TCUD 1.pdf	21/10/2014 09:24:50		Aceito
Folha de Rosto	Folha de rosto.pdf	21/10/2014 09:24:28		Aceito
Projeto Detalhado / Brochura Investigador	Projeto edital universal.doc	10/10/2014 18:21:33		Aceito
Outros	declaração custos-signed.pdf	10/10/2014 18:20:22		Aceito
Declaração de Instituição e Infraestrutura	Autorização UFMG.pdf	10/10/2014 17:50:56		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CURITIBA, 19 de Outubro de 2018

Assinado por:
NAIM AKEL FILHO
(Coordenador(a))

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

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3.2. Submitting Your Manuscript

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

- Enter data and answer questions as appropriate. You may copy and paste directly from your manuscript and you may upload your pre-prepared cover letter. If you are submitting for a thematic or special issue, please specify clearly in your cover letter.
- Click the 'Next' button on each screen to save your work and advance to the next screen.
- You are required to register all of your co-authors with a functioning e-mail address. If the e-mail address is incorrect, you will be contacted by the journal administrator.
- You are required to upload your files: Click on the 'Browse' button and locate the file on your computer. Select the designation of each file in the drop down next to the Browse button. When you have selected all files you wish to upload, click the 'Upload Files' button.
- Review your submission (in HTML and PDF format) before completing your submission by sending it to the Journal. Click the 'Submit' button when you are finished reviewing.

Data protection: By submitting a manuscript to or reviewing for this publication, your name, email address, and affiliation, and other contact details the publication might require, will be used for the regular operations of the publication, including, when necessary, sharing with the publisher (Wiley) and partners for production and publication. The publication and the publisher recognize the importance of protecting the personal information collected from users in the operation of these services and have practices in place to ensure that steps are taken to maintain the security, integrity, and privacy of the personal data collected and processed. You can learn more at https://authorservices.wiley.com/statements/data-protection-policy.html.

3.3. Manuscript Files Accepted

Manuscripts should be uploaded as Word (.doc/.docx) or Rich Text Format (.rft) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and PDF on upload and will be used for the review process. The text file must contain the entire manuscript including title page, abstract, text, references, acknowledgements, tables, and figure legends, but no embedded figures. In the text file, please reference figures as for instance 'Figure 1', 'Figure 2' etc to match the tag name you choose for individual figure files uploaded. Manuscripts should be formatted as described in the Author Guidelines below.

3.4. Blinded Review

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

3.5. Suggest a Reviewer

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

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

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

3.7. E-mail Confirmation of Submission

After submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation e-mail after 24 hours, please check your e-mail address carefully in the system. If the e-mail address is correct please contact your IT department. The error may

be caused by some sort of spam filtering on your e-mail server. Also, the e-mails should be received if the IT department adds our e-mail server (uranus.scholarone.com) to their whitelist.

3.8. Manuscript Status

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

3.9. Submission of Revised Manuscripts

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

4. MANUSCRIPT TYPES ACCEPTED

Original Research Articles: Manuscripts reporting laboratory investigations, well-designed and controlled clinical research, and analytical epidemiology are invited. Studies related to aetiology, pathogenesis, diagnosis, prevention and treatment are all of interest, but all papers must be based on rigorous hypothesis-driven research. Areas of interest includediseases affectin any structures of the mouth; cancer and pre-cancerous conditions; saliva and salivary glands; bone and hard tissues; relationasip between oral, periodontal, and dental conditions and general health; pain; behaviotal dentistry; chemosensory, developmental, geriatric, and motor disorders.

Randomised trials must adhere to the CONSORT guidelines, and a CONSORT checklist and flowchartmust be submitted with such papers. Please also refer to the notes under section 2.3 above.
Oral Diseases supports the ALLTRIALS initiative and encourages authors submitting manuscripts reporting a clinical trial to register the trials in any of the following free, public clinical trials registries: www.clinicaltrials.gov, http://clinicaltrials.ifpma.org/clinicaltrials/, http://isrctn.org/.
The clinical trial registration number and name of the trial register will then be published with the paper.

Observational studies must adhere to the STROBE guidelines, and a STROBE checklist must be submitted with such papers. Diagnostic accuracy studies must adhere to the STARD guidelines, and a STARD checklist must be submitted with such papers.

Preprint policy: This journal will consider for review articles previously available as preprints on non-commercial servers such as ArXiv, bioRxiv, psyArXiv, SocArXiv, engrXiv, etc. Authors may also post the submitted version of a manuscript to non-commercial servers at any time. Authors are requested to update any pre-publication versions with a link to the final published article.

Review Papers: *Oral Diseases* commissions review papers and also welcomes uninvited reviews. Systematic reviews with or without meta-analyses must adhere to the PRISMA guidelines, and a PRISMA checklist and flowchart must be submitted with such papers. The word limit for Review Papers is 4,000 words, with a maximum of two tables or images and 50 references.

Letters to the Editors: Letters, if of broad interest, are encouraged. They may deal with material in papers published in *Oral Diseases* or they may raise new issues, but should have important implications. Only one letter may be submitted by any single author or group of authors on any one published paper. Letters to the Editors should not include an abstract and are limited to 500 words, with a maximum of 1 figure and 10 references.

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

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

Short Communications: These are brief papers of any topic within the scope of *Oral Diseases*about significant and novel advances that are complete in research endeavor but not suitable for full publications. Short Communications should not include an abstract and are limited to 1000 words, with a maximum of 3 figures and 20 references.

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

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

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

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

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

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Page Charge

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

5.2. Format

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

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

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

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

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

- Title Page
- Structured Abstract
- Main text
- References
- (Figures)
- (Figure Legends)
- (Tables)

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

- A title of no more than 100 characters including spaces
- A running title of no more than 50 characters
- 3-6 keywords
- Complete names and institutions for each author
- Corresponding author's name, address, email address and fax number
- Date of submission (and revision/resubmission)

Abstract: is limited to 200 words in length and should contain no abbreviations. The abstract should be included in the manuscript document uploaded for review as well as separately where specified in the submission process. The abstract should convey the essential purpose and message of the paper in an abbreviated form set out under:

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

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

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

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

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

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

Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

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

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

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

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

5.4. References

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

A sample of the most common entries in reference lists appears below. Please note that a DOI should be provided for all references where available. For more information about APA referencing style, please refer to the APA website. Please note that for journal articles, issue numbers are not included unless each issue in the volume begins with page one.

Journal article

Example of reference with 2 to 7 authors

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

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

Example of reference with more than 7 authors

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

Book edition

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

5.5. Tables, Figures and Figure Legends

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

Detailed information on our digital illustration standards can be found at http://authorservices.wiley.com/bauthor/illustration.asp.

Check your electronic artwork before submitting it: http://authorservices.wiley.com/bauthor/eachecklist.asp.

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

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

Color figures

Color figures may be published online free of charge; however, the journal charges for publishing figures in colour in print. If the author supplies colour figures at Early View publication, they will be invited to complete a colour charge agreement in RightsLink for Author Services. The author will have the option of paying immediately with a credit or debit card, or they can request an invoice. If the author chooses not to purchase color printing, the figures will be converted to black and white for the print issue of the journal.

Guidelines for Cover Submissions

If you would like to send suggestions for artwork related to your manuscript to be considered to appear on the cover of the journal, please follow these general guidelines.

6. AFTER ACCEPTANCE

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

Proof Corrections

The corresponding author will receive an e-mail alert containing a link to a website. A working e-mail address must therefore be provided for the corresponding author. The proof can be downloaded as a PDF (portable document format) file from this site.

Acrobat Reader will be required in order to read this file. This software can be downloaded (free of charge) from the following website: www.adobe.com/products/acrobat/readstep2.html . This will enable the file to be opened, read on screen, and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hard copy proofs will be posted if no e-mail address is available; in your absence, please arrange for a colleague to access your e-mail to retrieve the proofs.

Proofs must be returned to the Production Editor within **three days** of receipt.

As changes to proofs are costly, we ask that you only correct typesetting errors. Excessive changes made by the author in the proofs, excluding typesetting errors, will be charged separately. Other than in exceptional circumstances, all illustrations are retained by the publisher. Please note that the author is responsible for all statements made in their work, including changes made by the copy editor.

Early View (Publication Prior to Print)

Oral Diseases is covered by Wiley-Blackwell's Early View service. Early View articles are complete full-text articles published online in advance of their publication in a printed issue. Early View articles are complete and final. They have been fully reviewed, revised and edited for publication, and the authors' final corrections have been incorporated. Because they are in final form, no changes can be made after online publication. The nature of Early View articles means that they do not yet have volume, issue or page numbers, so Early View articles cannot

be cited in the traditional way. They are therefore given a Digital Object Identifier (DOI), which allows the article to be cited and tracked before it is allocated to an issue. After print publication, the DOI remains valid and can continue to be used to cite and access the article.

Author Services

Online production tracking is available for your article once it is accepted by registering with Wiley-Blackwell's Author Services.