UNIVERSIDADE FEDERAL DE PELOTAS
Faculdade de Odontologia
Programa de Pós-Graduação em Odontologia

Tese

Avaliação quantitativa e qualitativa das alterações salivares na síndrome de ardência bucal (condição sistêmica) e no carcinoma espinocelular oral (progressão e resposta às terapias)

Juan Pablo Aitken Saavedra

Pelotas, 2020

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Tese apresentada, como requisito parcial, para obtenção do grau de Doutor em Odontologia (Clínica Odontológica, ênfase Diagnóstico Bucal), Programa de Pós-Graduação em Odontologia, Faculdade de Odontologia, Universidade Federal de Pelotas.

Orientadora: Profª. Dra. Sandra Beatriz Chaves Tarquinio

Coorientadores: Profª. Drª. Ana Carolina Uchoa Vasconcellos

Prof. Dr. Ricardo Ramires Fernandes

Pelotas, 2020

Universidade Federal de Pelotas / Sistema de Bibliotecas
Catalogaç o na Publicaç o

S111a Saavedra, Juan Pablo Aitken

Avaliaç o quantitativa e qualitativa das alteraç es salivares na síndrome de ard ncia bucal (condiç o sist mica) e no carcinoma espinocelular oral (progress o e resposta s terapias) / Juan Pablo Aitken Saavedra ; Sandra Beatriz Chaves Tarquinio, orientadora ; Ana Carolina Uchoa Vasconcellos, Ricardo Ramires Fernandes, coorientadores. — Pelotas, 2020.

155 f.

Tese (Doutorado) — Programa de Pós-Graduaç o em Clínica Odontológica - nfase em Diagnóstico Bucal, Odontologia, Universidade Federal de Pelotas, 2020.

1. C ncer bucal. 2. Biomarcadores. 3. Sindrome de ard ncia bucal. 4. Saliva. 5. Fat1. I. Tarquinio, Sandra Beatriz Chaves, orient. II. Vasconcellos, Ana Carolina Uchoa, coorient. III. Fernandes, Ricardo Ramires, coorient. IV. Título.

Black : D6

Elaborada por Fabiano Domingues Malheiro CRB: 10/1955

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Data da defesa: 30/04/2020

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Dedicado com todo meu amor e gratidão infinita aos alunos da graduação de Odontologia da UFPel

Agradecimentos

Quero começar meus agradecimentos a Deus. Pela benção de acordar, respirar e poder escrever essas palavras. Gratidão à minha família. Mãe, Pai e Tia Mony Meus irmãos Sergio, Paty, Cone e Catalina e meus sobrinhos Sofia, Evita, Mati, Rafa e Elisa. Vocês são a força, amor, a fé, a certeza. O mais importante. Nunca existiram as distancias, sempre estivemos e estaremos juntos. Obrigado pelo amor infinito e por entender e curtir a minha felicidade. Obrigado Tio Lulo e aos meus avôs, meus anjos do céu.

Sandra, você além de ser a minha orientadora acadêmica, foi a minha guia espiritual neste caminhar. Obrigado por tanto carinho, dedicação, atitude, amizade e em especial, por acreditar em mim e por me ajudar a sentir e acreditar, que as coisas sempre darão certo. Carol, muito obrigado pelo todo teu compromisso, carinho, disciplina e amizade. Aprendi demais contigo. Tua ajuda foi essencial neste processo.

Obrigado a todos os que fizeram parte da tese em cada uma das suas etapas em especial aos pacientes participantes. Aos meus dois maravilhosos grupos de MAP. Ao meu admirado amigo Tom e seus bolsistas, Adriana Fernandes, Diogo e Marcia da química e as minhas orientadas de TCC (Andressa, Bruna, Thais, Caroline, Paula, Ingrid, Bianca, Isadora, Mariana e Janine).

Obrigado à Universidade Federal de Pelotas. Onde quer que eu esteja, vou me sentir parte desta família, com orgulho, amor e gratidão. Obrigado a meu lar, a Faculdade de Odontologia por me receber cada dia com um sorriso. Porque como nunca na minha vida, tinha curtido tanto a rotina de trabalhar em um lugar.

Obrigado ao meu time de patologia. Ivana, Karina, Leandro e Bruno pela ajuda alegre e desinteressada. Obrigado Ana Paula por me ensinar tanto, pela tua coragem e por acreditar em mim e no meu chá de camomila. Adriana, por sempre tentar me integrar no time, por todo o aprendizado, pela tua imensa generosidade e por ter aceito fazer parte da minha banca. Muito obrigado Cassia porque além de ser uma patologista e professora admirada e iluminada, aceitou também fazer parte deste momento tão importante da minha vida.

Aos irmãos que Pelotas me deu para a vida: Carlos, Karine e Andressa. Obrigado Carlos pela amizade que se mantém forte além da distância, pela tua generosidade infinita e desinteressada com todo o mundo e por me ajudar tanto neste caminho. Obrigado Karine por estar sempre comigo, pelo teu sorriso, disposição e carinho infinito. Obrigado Andressa porque você sempre alegrou e melhorou meus dias e foi um suporte essencial todo esse tempo.

Obrigado a todos os professores desta Faculdade que, além de admirar demais e me fazer sentir sempre como um mais do time, viraram grandes amigos: Junior, Torrigol, Josué, Melissa, Piva, Giana, Noeli, Nadia, Foginho, Ezilmara, Thiago, Giovane, Damé, Bia, Lund, Otacílio, Neno, Maisa e Lisa. Obrigado as pessoas do Chile que acreditaram em mim e me deram a oportunidade de estudar um doutorado na princesa do sul.

Obrigado a minha alma mater, a Universidad de Chile, especialmente a Jorge Gamonal e Irene Morales. Obrigado Dr. Lobos, Iris, Ani, Marcela, Andrea, Flaquis, Gonzalo, Xime, Marito, Tino, Gabo e Seba. Obrigado Gonzalo Rojas por ser um referente da vida para mim, por ter aceito fazer parte da minha banca e porque você foi determinante na minha escolha de ser patologista, especialidade que é uns dos maiores presentes da minha vida. Obrigado Ricardo Fernandes por me apresentar e facilitar tanto a chegada a minha Pelotinhas amada com seu mercado e um relógio que nunca funcionou, mas que sempre marcou o horário certo. Pelotas é lugar onde eu já fui mais feliz na minha vida.

Obrigado aos Night Runners, Clube de amigos Ubuntú, meus parceiros musicais, em especial a Fabio Coppola, Clube de Futebol Atlético Portunhol, Hermanos latinos especialmente a Alfonso, Karina e Panamita, aos Parceiros da Caridade e ao Clube de estudos aleatórios.

Aos meus amigos que sempre estiveram e estarão comigo: Catarina, minha família do CDC (Andressa, Ju, Gaby, Cristina e Carla), K-beludas, Karen, Christian, Vitor, Kaio, Rodrigo, Amandinha, Martha, Luane, Fernanda, Glenda, Gabriel, Laylla, Alini, Êmile, Laura, Julia, meu querido time do PET, Carolina, Isabella, Elisa, Laura, Betina, Giulia, Fedalto, Amanda, Marina, Kimberlly, Luís, Douglas, Guilherme, Samille, Geórgia, Ana e Victória e tantos mais que injustamente não conseguirei citar aqui. Obrigado aos mais do que trinta alunos da graduação que tive a benção de orientar em apresentações de trabalhos em congressos de estudantes.

Mesmo com alguns anos de experiência na docência no Chile, cheguei em Pelotinhas para estudar e até pensei que para ser professor de novo (que é o que mais me faz feliz), ia ter que esperar voltar para meu país. Quero agradecer profundamente aos alunos da graduação de odontologia da UFPel. Vocês me fizeram sentir desde o primeiro momento, que além de fazer o meu doutorado aqui, também poderia contribuir na sua formação como cirurgiões dentistas durante esses anos. Ter sentido aquilo, quer dizer que cada passo que eu dei, cada alegria, valeram a pena. Obrigado por me adotar como professor e amigo e por me ajudar a crescer e ser melhor cada dia. Vocês foram sem dúvida o presente mais lindo e mais inesperado que esta aventura me deu e a minha maior motivação para acordar grato e chegar feliz cada dia ao trabalho. Obrigado turmas maravilhosas 2019/1 e turma dos ofícios por ter me homenageado, sem dúvida, o reconhecimento mais importante que já tive na vida. Gratidão infinita a vocês e para todos os estudantes da graduação de Odonto da UFPel que tive a possibilidade e benção de conhecer. Infinita porque não sei se terei tanto tempo nem espaço para agradecer tanto amor envolvido.

“Tirar da vida é fácil, o difícil é tirar da mente e do coração”

Lucas Lemos (Turma dos ofícios, 2019)

Notas Preliminares

O presente trabalho de conclusão de curso foi redigido segundo o Manual de Normas para Dissertações, Teses e Trabalhos Científicos da Universidade Federal de Pelotas de 2019, adotando o Nível de Descrição Tradicional, descrito no referido manual. <https://wp.ufpel.edu.br/sisbi/files/2019/06/manual-2.pdf> Acesso em: 27/09/2019.

O projeto de pesquisa referente a esta Tese foi aprovado dia 28 de novembro de 2016, pela Banca Examinadora composta pelos Professores Doutores Sandra Beatriz Chaves Tarquinio (presidente), Luciana Tovo Rodrigues, Ana Paula Neutzling Gomes e Ana Carlolina Uchoa Vasconcelos (suplente).

Resumo

AITKEN-SAAVEDRA, Juan Pablo. **Avaliação quantitativa e qualitativa das alterações salivares na síndrome de ardência bucal (condição sistêmica) e no carcinoma espinocelular oral (progressão e resposta às terapias).** 2020. <155f>. Tese (Doutorado em Odontologia). Programa de Pós-Graduação em Odontologia, Universidade Federal de Pelotas, Pelotas. 2020.

Introdução: A saliva como meio de diagnóstico de doenças bucais e sistêmicas tem sido objeto de estudo com o intuito de acrescentar sua utilização como exame complementar, considerando as vantagens do seu uso. O carcinoma espinocelular (CEC) é a neoplasia maligna mais comum da cavidade oral e apresenta um comportamento agressivo e invasivo. A detecção precoce de lesões orais potencialmente malignas (LOPM) é essencial para evitar a progressão para o CEC e a detecção de mudanças na composição electrolítica salivar poderia ser de utilidade neste sentido. Além disso, um recente sequenciamento massivo mostrou que dos genes mutados mais associados com o desenvolvimento de CEC, o *FAT1* é o único que codifica uma proteína secretória, potencialmente detectável na saliva e utilizável como biomarcador. A síndrome de ardência bucal (SAB) é uma condição crônica caracterizada por uma sensação de queimação na boca sem achados clínicos ou laboratoriais que justifiquem esse sintoma e pode afetar dramaticamente a qualidade de vida dos afetados. Alterações salivares poderiam determinar a sua etiologia ou refletir o status sistêmico na síndrome. Desta forma, o objetivo deste estudo foi avaliar o potencial uso do FAT1 tecidual e salivar e o perfil bioquímico salivar na progressão de LOPM para o CEC e sua resposta às terapias e traçar o perfil salivar de mulheres com SAB.

Métodos: Este estudo foi dividido em 4 partes 1). Saliva não estimulada (uSRF) foi coletada de 54 pacientes, 18 com CEC (avaliados também 6 meses após terapia), 18 com LOPM e 18 sem lesões bucais. Mediante um espectrômetro de emissão óptica foram determinadas concentrações de 8 eletrólitos. Estes resultados foram complementados com uma revisão sistemática da literatura. 2). A partir desta mesma amostra de pacientes, foram determinados os níveis salivares de FAT1 nativo com a técnica ELISA (Enzyme-Linked Immunosorbent Assay) que se associaram com os níveis teciduais de FAT1 aberrante (das suas respectivas biópsias), submetidos à técnica imunoistoquímica e avaliados por análise semi-quantitativa e determinação computadorizada. Complementando esta análise, níveis teciduais de FAT1 também foram determinados pela técnica imunoistoquímica em um estudo retrospectivo de 72 biopsias (de tecido sadio, displasias de alto e baixo risco e CEC). 3). Foi realizada uma revisão sistemática avaliando e comparando caraterísticas salivares de pacientes com e sem SAB. 4). Foram recrutadas 40 mulheres com SAB e 40 controles. Determinaram-se uSRF, pH, níveis de cortisol, viscosidade e perfil de impacto da SAB na saúde bucal (OHIP-14). Testes de Kruskal-Wallis e Mann-Whitney foram utilizados

para as comparações e o teste de Spearman para as associações entre as variáveis. P <0,05 foi considerado estatisticamente significante.

Resultados: 1) Pacientes com LOPM apresentaram níveis mais altos de Magnésio (Mg) salivar. Informações adicionais sobre o papel dos eletrólitos na progressão do câncer e o seu potencial uso como biomarcadores nesta doença. 2) A análise semi-quantitativa da expressão imunoistoquímica de FAT1 indicou que 2 amostras (11%) no grupo controle e 10 amostras (50%) no grupo CEC, tiveram pontuação 4 na avaliação semiquantitativa (coloração > 50). Resultados semelhantes foram descritos pela determinação computadorizada de FAT1 (os resultados das duas técnicas demonstraram associação forte e positiva). Saliva de pacientes controles apresentaram maiores níveis de FAT1 em comparação com pacientes com LOPM e com CEC antes e após terapia (0,168, 0,162 0,154 e 0,153 nm / mL respectivamente e p <0,05). Foi observada uma correlação negativa e fraca entre os níveis salivares e teciduais de FAT1. 3) A Análise salivar qualitativa, segundo a revisão sistemática realizada, aponta que a SAB poderia estar determinada ou refletir a ação de fatores neuropáticos (também sugeridos por alguns eletrólitos salivares), fatores inflamatórios, emocionais, imunológicos e hormonais. 4) Mulheres com SAB apresentaram em relação ao grupo controle e com diferencias estatisticamente significantes, menor viscosidade (31.1 e 45.01 mPas) menor uSFR (0.35 e 0.61 mL / min) e maiores níveis de cortisol salivar (0.36 e 0.15 µg / dL), os que foram associados positivamente com maiores scores de OHIP-14.

Conclusão: 1). Maiores níveis de Mg salivar estão assiciados às alterações displásicas vistas nas LOPMs. 2) O uso de FAT1 nativo como marcador salivar da progressão ou da resposta à terapia no CEC não é recomendado, de acordo com a metodologia utilizada no presente estudo. A imunomarcação citoplasmática de FAT1, amplamente mais frequente em CEC sugerem seu fenótipo aberrante e poderia estar associado com caraterísticas neoplásicas como agressividade. 3) A análise salivar qualitativa aponta para uma origem multifatorial na SAB. 4) Alterações salivares presentes em mulheres com SAB como menor uSRF e menor viscosidade poderiam estar associadas com a patogênese da síndrome ou ser reflexo do status sistêmico das afetadas. Maiores níveis de cortisol salivar refletem a pior qualidade de vida que mulheres com SAB relatam ter.

Palavras-chave: Câncer bucal; Biomarcadores; Saliva; FAT1; Lesões potencialmente malignas; Sindrome de ardência bucal.

Abstract

AITKEN-SAAVEDRA, Juan Pablo. **Quantitative and qualitative assessment of salivary changes in burning mouth syndrome (systemic condition) and oral squamous cell carcinoma (progression and response to therapies). 2020.**<155p>. Thesis (PhD in Dentistry). Postgraduate Program in Dentistry. Federal University of Pelotas, Pelotas. 2020.

Introduction: Saliva as a means of diagnosing oral and systemic diseases has been the object of study in order to add its use as a complementary exam considering the advantages of its use. Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity and presents an aggressive and invasive behavior. The early detection of oral potentially malignant disorders (OPMD) is essential to prevent progression to OSCC and the detection of changes in the salivary electrolyte composition could be useful in this regard. In addition, a recent massive sequencing showed that of the mutated genes most associated with the development of OSCC, FAT1 is the only one that encodes a secretory protein, potentially detectable in saliva and usable as a biomarker. Burning mouth syndrome (BMS) is a chronic condition characterized by a burning sensation in the mouth without clinical or laboratory findings that justify this symptom and can dramatically affect the quality of life of those affected. Salivary changes could determine its etiology or reflect the systemic status in the syndrome. The objective of this study was to evaluate the potential use of tissue and salivary FAT1 and the salivary biochemical profile in the progression of OPMD to OSCC and its response to therapies and to trace the salivary profile of women with BMS.

Methods: This study was divided into 4 parts 1). Unstimulated saliva (uSRF) was collected from 54 patients, 18 with OSCC (also evaluated 6 months after therapy), 18 with OPMDand 18 without oral lesions. Concentrations of 8 electrolytes were determined using an optical emission spectrometer. These results were complemented with a systematic review of the literature. 2). From this same sample of patients, salivary levels of native FAT1 were determined using the ELISA technique (Enzyme-Linked Immunosorbent Assay) that were associated with tissue levels of aberrant FAT1 (from their respective biopsies), submitted to immunohistochemical technique and evaluated by semi-quantitative analysis and computerized determination. Complementing this analysis, tissue levels of FAT1 were also determined by the immunohistochemical technique in a retrospective study of 72 biopsies (from healthy tissue, high and low risk dysplasias and OSCC). 3). A systematic review was carried out evaluating comparing salivary characteristics of patients with and without BMS. 4). 40 women with BMS and 40 controls were recruited. uSRF, pH, cortisol levels, viscosity and impact profile of SAB on oral health (OHIP-14) were determined. Kruskal-Wallis and Mann-Whitney tests were used for comparisons and the Spearman test for associations between variables. $P < 0.05$ was considered statistically significant.

Results: Patients with OPMD had higher levels of salivary Magnesium (Mg). The semi-quantitative analysis of the immunohistochemical expression of FAT1 indicated that 2 samples (11%) in the control group and 10 samples (50%) and in the OSCC group, had a score of 4 in the semiquantitative evaluation (staining> 50). Similar results were described by the computerized determination of FAT1 (the results of the two techniques demonstrated a strong and positive association). Saliva from control patients showed higher levels of FAT1 compared to patients with OPMD and OSCC before and after therapy (0.168, 0.162 0.154 and 0.153 nm / mL respectively and $p < 0.05$). A weak negative correlation was observed between the salivary and tissue levels of FAT1. Women with BMS showed, in relation to the control group and with statistically significant differences, lower viscosity (31.1 and 45.01 mPas) lower uSFR (0.35 and 0.61 mL / min) and higher levels of salivary cortisol (0.36 and 0.15 µg / dL), that were positively associated with higher OHIP-14 scores.

Conclusion: 1) Higher levels of salivary Mg are associated with the dysplastic changes seen in OPMDs. 2) The use of native FAT1 as a salivary marker of progression or response to therapy in OSCC is not recommended, according to the methodology used in the present study. The cytoplasmic immunostaining of FAT1, widely more frequent in OSCC, suggests its aberrant phenotype and could be associated with neoplastic characteristics such as aggressiveness. 3) The qualitative salivary analysis points to a multidisciplinary origin in BMS. 4) Salivary changes present in women with BMS such as lower uSRF and lower viscosity could be associated with the pathogenesis of the syndrome and be a reflection of the systemic status of those affected. Higher levels of salivary cortisol reflect the worse quality of life that women with BMS report having.

Keywords: Oral cancer; Biomarkers; Saliva; FAT1; Oral potentially malignant disorders, Burning mouth syndrome.

Sumário

1 Introdução

A saliva constitui um dos fluidos corporais mais versáteis e complexos do corpo. É secretada principalmente pelas três glândulas salivares maiores e está composta por uma variedade de eletrólitos, pequenos compostos orgânicos, proteínas, peptídeos, entre outros elementos (XIAO H, 2011). A saliva possui propriedades físicas, químicas, bioquímicas e biológicas fundamentais para manter a saúde bucal (AGUIRRE et al., 1993). A quantidade de saliva produzida por um indivíduo, bem como a sua composição bioquímica e orgânica podem experimentar mudanças em presença de lesões orais e doenças sistêmicas (AGUIRRE et al., 1993) (MAIER; BIHL,1987).

Até a presente data, os métodos convencionais para o diagnóstico de doenças sistêmicas e bucais têm sido úteis e efetivos. No entanto, a ciência médica tem focado em encontrar métodos alternativos para complementar o diagnóstico de doenças de uma forma menos invasiva e menos dolorosa. Neste sentido, a saliva representa uma possibilidade como meio auxiliar de diagnóstico, dada a facilidade de avaliação, e especialmente, devido à baixa complexidade de obtenção de biomarcadores, cuja presença e/ou níveis no conteúdo salivar, podem indicar a existência de um processo patológico, risco do seu desenvolvimento ou até a resposta a um tratamento em particular (BRINKMANN et al, 2011). Atualmente, a biotecnologia emergente ampliou o conhecimento dos biomarcadores salivares, uma vez que as maiorias dos compostos encontrados no sangue estão também presentes na saliva (NRIAGU et al, 2006). Portanto, a saliva em termos de constituição, é funcionalmente equivalente ao sangue e pode refletir o estado fisiológico do corpo. Nos últimos anos, a procura de biomarcadores salivares surgiu como uma potencial abordagem não invasiva em medicina personalizada e de precisão e representa uma possibilidade promissora para detecção precoce, de monitoramento e da avaliação das respostas aos tratamentos de doenças e síndromes sistêmicas e bucais (BRINKMANN et al, 2011).

O carcinoma espinocelular (CEC) é a neoplasia maligna mais comum da cavidade oral. Mesmo com os avanços sobre essa doença, sua sobrevida em cinco anos ainda é de cerca de 50% devido ao seu comportamento agressivo e invasivo

(FERLAY, 2015). Lesões orais potencialmente malignas (LOPM) ou lesões precursoras do CEC, são alterações teciduais que podem assumir o caráter de um tumor maligno a qualquer momento. A detecção e o controle precoce das LOPM são essencial para evitar a progressão para o CEC (VAN DER WAAL, 2009). Neste sentido, as possíveis alterações bioquímicas orais que pacientes com LOPM ou com CEC apresentariam e seriam detectados na saliva, poderiam ser de utilidade neste sentido (SHETTY, S. R, 2015).

Vários estudos foram realizados com o objetivo de investigar as primeiras alterações envolvidas no início e progressão do CEC (LAWRENCE, M, 2015). Nesse contexto, o sequenciamento do genoma do CEC proporcionou uma maior compreensão da complexidade genética e da biologia evolutiva destas células tumorais. Foi estabelecido que os genes mutados mais comumente associados com o CEC são *CASP8, FAT1, NOTCH1, TP53, MLL4 e USP9X* (MARTIN, 2014). A identificação dessas mutações e sua associação com a sobrevivência e hábitos em pacientes com CEC abre a possibilidade de redirecionar terapias antitumorais (PILERI, 2016). Dentre as proteínas codificadas por esses genes, a identificação de FAT1 merece atenção especial, porque devido a sua natureza secretora, pode ser analisada em diferentes fluidos corporais como biomarcador de diversas patologias (LI, 2016). *FAT1* é um gene supressor que codifica uma proteína transmembrana caderina de grande tamanho que tem papel central na morfogênese e migração celular (NISHIKAWA Y; 2011; MORRIS, 2013, URAGUCHI M, 2004). Proteínas secretórias como FAT1, geram um ambiente favorável no espaço extracelular e é essencial para o desenvolvimento de muitos tipos de câncer, podendo atuar também como biomarcadores séricos ou salivares (XUE H, 2008). No câncer hepatocelular, por exemplo, foi demonstrado que a proteína FAT1 é um biomarcador sorológico útil para o diagnóstico e prognóstico nessa neoplasia (LI, 2016). A expressão proteica de FAT1 também pode ser investigada como marcador em outros cânceres com mutações no gene *FAT1*, como o CEC. Considerando que o FAT1 é secretado pelas células e que a maioria das proteínas presentes no sangue também são expressas na saliva (PINEDA-MARTINEZ, 2016), seria muito valioso determinar a expressão salivar do FAT1 nos estágios pré-malignos e no CEC, antes e depois da terapia, com o objetivo de avaliar seu uso como um potencial biomarcador nessa neoplasia. Apesar da sua natureza secretora, a proteína FAT1 não tem sido avaliado na saliva como um biomarcador para CEC.

A síndrome de ardência bucal (SAB) é uma condição oral crônica que pode prejudicar drasticamente a qualidade de vida dos indivíduos afetados (principalmente mulheres adultas) e é caracterizada por a sensação de queimação e sensações bucais desconfortáveis sem alterações clínicas ou achados laboratoriais que justifiquem estes sintomas (CERCHIARI ET AL., 2006; COCULESCU, RADU, & COCULESCU, 2014; FELLER ET AL., 2017). A SAB pode ser um processo primário ou ser atribuído a alguns fatores locais ou comorbidades sistêmicas. Embora a principal causa da SAB não tenha sido totalmente identificada, fatores locais, sistêmicos e psicológicos têm sido associados a sua patogênese (KIM, KIM & KHO, 2018). A avaliação das características salivares das pessoas com SAB pode ajudar na compreensão da patogênese dessa síndrome ou para estimar o estado sistêmico e até determinar a resposta às terapias das pessoas afetadas. (IMURA, SHIMADA, YAMAZAKI E SUGIMOTO, 2016). Algumas alterações salivares podem produzir distúrbios neurológicos da transdução e produzir alterações na percepção sensorial de pacientes com SAB (KÜSTNER & MARQUES, 2002; MOURA ET AL., 2007). Estes pacientes geralmente exibem sintomas de depressão, ansiedade e níveis de estresse psicossocial (Amenábar et al., 2008; Moura et al., 2007), características associadas a alterações nos níveis sistêmicos de cortisol, hormônio que também pode ser avaliado na saliva como um indicador de ansiedade, estresse e qualidade de vida (AMENÁBAR ET AL., 2008). Embora algumas alterações salivares qualitativas e quantitativas tenham sido avaliadas em pessoas com SAB (AMENÁBAR ET AL., 2008; KÜSTNER & MARQUES, 2002; MOURA ET AL., 2007), os resultados ainda são contraditórios. Conhecer o perfil salivar em pacientes com a síndrome, poderia ser de utilidade tanto para entender a etiopatogenia da síndrome, quanto para avaliar o estado sistêmico e até emocional dos afetados. O objetivo deste trabalho foi avaliar quantitativa e qualitativa as alterações salivares na síndrome de ardência bucal (condição sistêmica) e no carcinoma espinocelular oral (progressão e resposta às terapias).

2 Projeto de pesquisa

2.1 Antecedentes e justificativa

2.1.1 Câncer Bucal

O carcinoma espinocelular (CEC) é a neoplasia maligna mais comum da cavidade oral. Caracteriza-se pela taxa de sobrevivência em 5 anos em torno de 50%, o que está diretamente relacionado com seu comportamento agressivo e invasivo. O CEC é um grande desafio para a saúde pública global (MAROCCHIO et al., 2010), porque é o sexto tipo de câncer mais frequente no mundo (FERLAY et al., 2015).

O CEC ocorre principalmente em fumantes e etilistas adultos homens, depois dos 50 anos (THOMPSON, 2006). Clinicamente, o tumor aparece como úlceras endurecidas que não cicatrizam lesões exofíticas, leucoplasia ou eritroplasia, geralmente assintomáticas, mas em fases posteriores pode causar invasão profunda causando dor ou disfagia (NEVILLE; DAY, 2009).

O CEC é um dos tumores de pior prognóstico principalmente pela fase tardia em que é diagnosticado, com comprometimento e metástases linfáticas locais e à distância. O diagnóstico feito em estágios avançados tem consequências para os pacientes e para a sociedade, podendo levar a uma deterioração significativa da qualidade de vida dos afetados em várias esferas, tanto funcionais quanto psicossociais (VAN DER WAAL, 2015).

O sítio mais comumente acometido pelo CEC é a língua, especialmente as superfícies lateral posterior e ventral. O soalho de boca é acometido mais frequentemente nos homens. Outros sítios de envolvimento (em ordem decrescente de frequência) são o palato mole, gengiva, mucosa jugal, mucosa labial e palato duro (NEVILLE; DAY, 2009). Dois terços dos CEC da língua surgem como aumento de volume ou úlceras endurecidas, indolores, da margem lateral posterior; 20% ocorrem nas superfícies anterior lateral ou ventral e somente 4% ocorrem no dorso. Nas investigações epidemiológicas, o carcinoma de soalho de boca representa 35% de todos os cânceres intraorais e parece que sua frequência está aumentando entre as mulheres. Ocorre uma década mais cedo em mulheres do que em homens, porém

ainda é geralmente uma doença de adultos idosos. Dentre todos os carcinomas intraorais, as lesões de assoalho de boca são as mais propensas a originarem-se de uma leucoplasia ou eritroplasia preexistente (VAN DER WAAL, 2015). É também o sítio de câncer oral mais frequentemente associado ao desenvolvimento de uma segunda malignidade primária em outra localização do trato aerodigestivo superior ou em um órgão distante. A área mais comum de envolvimento é a linha média, próximo ao freio lingual. Os carcinomas da gengiva e do rebordo alveolar geralmente são indolores e surgem mais frequentemente na mucosa ceratinizada de um sítio na região posterior da mandíbula (NEVILLE; DAY, 2009). Os tumores da crista alveolar maxilar podem se estender em direção ao palato duro. Se o tumor estiver localizado nas adjacências de um dente, então ele pode simular uma doença periodontal ou um granuloma piogênico (WARNAKULASURIYA, [s.d.]).

2.1.2 Lesões orais potencialmente malignas

As lesões pré-malignas ou lesões precursoras do CEC são alterações teciduais que podem assumir o caráter de tumor maligno a qualquer momento, mas, podem permanecer estáveis por um considerável período de tempo. Em 2005, a Organização Mundial da Saúde (OMS) modificou a terminologia das lesões e condições orais pré-malignas e as denominou lesões com potencial de malignização (VAN DER WAAL, 2005), citando-se nessa classificação, como lesões potencialmente malignas, leucoplasia, leucoplasia verrucosa proliferativa, eritroplasia, queilite actínica, fibrose submucosa, líquen plano e atrofia por deficiência de ferro (SILVEIRA et al., 2009). Histologicamente, tais lesões podem apresentar-se como hiperplasias e displasias com graus variáveis de diferenciação celular (bem, moderadamente ou pouco diferenciadas) (VAN DER WAAL, 2005).

Leucoplasia é um termo clínico utilizado para denominar uma placa branca da mucosa oral, não removível à raspagem, que não pode ser classificada clinicamente como qualquer outra entidade. É a lesão precursora de câncer oral mais frequente da boca. Sua superfície pode ser lisa, rugosa ou verrucosa (VAN DER WAAL, 2005). Acomete principalmente a mucosa jugal e as comissuras labiais, seguidas por mucosa alveolar, língua, lábio, palato duro, palato mole, assoalho de boca e gengiva (NEVILLE; DAY, 2009). A taxa de transformação maligna de leucoplasia varia de 0% a 20% (SCHEPMAN et al., 1998).

A eritroplasia é definida como uma placa ou mancha vermelha que não pode ser classificada clinicamente como qualquer outra entidade. Pode estar associada com uma leucoplasia adjacente, sendo denominada, nesse caso, eritroleucoplasia (NEVILLE; DAY, 2009). Embora seja menos comum do que a leucoplasia, a eritroplasia apresenta maior potencial de malignização (14% a 50%). (MARKOPOULOS; ALBANIDOU-FARMAKI; KAYAVIS, 2004).

As lesões potencialmente malignas podem ter alterações epiteliais denominadas displasias, as quais apresentam potencial de malignização. A displasia é observada em quase todos os casos de eritroplasia e até 30% dos casos de leucoplasia oral (SILVEIRA et al., 2009). No entanto, há um componente subjetivo no diagnóstico histológico (NEVILLE; DAY, 2009). Uma possibilidade para otimizar o diagnóstico das diferentes fases de lesões orais poderia ser identificar biomarcadores que caracterizam cada estágio de progressão da neoplasia para em caso de detecção de displasia epitelial, prevenir a progressão para CEC.

Segundo a OMS, as mudanças do epitélio podem ser divididas em celulares e arquiteturais. As mudanças arquiteturais são: estratificação irregular, perda da polaridade das células basais, epitélio em forma de gota, aumento do número de mitoses, ceratinização prematura de células individuais, presença de mitoses em camadas superficiais, pérolas de ceratina nos ninhos epiteliais. As mudanças celulares são: variações em tamanho e forma celular e nuclear, aumento da relação núcleo-citoplasma, presença de figuras mitóticas atípicas, aumento do número e tamanho dos nucléolos e hipercromatismo nuclear.

Foi proposta uma classificação binária, dividindo as lesões em “alto risco” e “baixo risco”, dependendo do número e tipo de alterações presentes em cada uma delas. Segundo isto, foram classificadas como displasias de “alto risco” aquelas com pelo menos 4 alterações arquiteturais e 5 alterações celulares e de “baixo risco” aquelas com menos de 4 alterações arquiteturais e 5 alterações celulares (KUJAM et. al. ,2006).

É essencial a detecção e controle precoce de lesões potencialmente malignas para assim, evitar a progressão para o CEC. Até agora, a biópsia das lesões é o único mecanismo para avaliar as alterações celulares associadas com displasia e com sua eventual progressão para o CEC. O desenvolvimento de métodos modernos para

monitorar a progressão e controle de lesões tais como biomarcadores na saliva poderiam nos permitir ter uma avaliação eficaz, não invasiva, de baixo custo e fácil de coletar, alternativa que poderia significar uma maior adesão ao tratamento e controle das lesões potencialmente malignas e facilitar o desenvolvimento de políticas públicas para a prevenção do câncer oral. No entanto, para serem usados corretamente, os biomarcadores salivares devem ser confiáveis, biologicamente relevantes e possuir concentrações mensuráveis para implementá-las como método diagnóstico de uso permanente. Até a presente data não existe marcador de prognóstico clínico de CEC em saliva na prática odontológica clínica com todas estas características.

2.1.3 Genética do Câncer

O desenvolvimento do CEC é um processo sequencial, com acúmulo de alterações genéticas, epigenéticas, metabólicas e hormonais, devido a exposição consecutiva a agentes cancerígenos (HADDAD; SHIN, 2008). O sequenciamento do genoma do câncer proporcionou uma maior compreensão da complexidade genética e da biologia evolutiva das células tumorais (ABBEY et al., 1995). Na maioria dos casos a transformação e metástase são clonais, provavelmente, derivados a partir de células individuais. No ano 2000, Perou et al. demonstraram por exemplo, que o câncer da mama correlaciona-se com diferenças nos padrões de expressão gênica global que, por sua vez, refletem em aspectos do comportamento biológico dos tumores (PEROU et al., 2000). Este e outros estudos subsequentes (SØRLIE et al., 2001) (VAN'T VEER et al., 2002) (BERTUCCI et al., 2006), demonstram correlações detalhadas entre características histopatológicas, moleculares e clínicas.

O estudo mais recente, utilizando sequenciamento massivo em tumores de 50 pacientes diferentes com CEC, mostrou três subtipos moleculares com base nas mutações encontradas nos tumores da cavidade oral. O primeiro grupo tem mutações predominantemente no gene *CASP8*, *FAT1* e/ou *NOTCH1*. Um segundo grupo possui mutações em *TP53* e, um terceiro grupo, majoritariamente heterogêneo, com mutações em *MLL4*, *USP9X*, entre outros genes (INDIA PROJECT TEAM OF THE INTERNATIONAL CANCER GENOME CONSORTIUM, 2013). Cada um desses três subtipos moleculares foi associado com a sobrevivência e hábitos em pacientes afetados por CEC, o que abre a possibilidade de redirecionar as terapias antitumorais, realizando diagnóstico em estágios iniciais o qual, traz benefícios clínicos aos

pacientes (INDIA PROJECT TEAM OF THE INTERNATIONAL CANCER GENOME CONSORTIUM, 2013). Dentre as proteínas codificadas por estes genes, merece especial atenção a identificação de FAT1, já que devido a sua natureza secretória (LI et al., 2016), poderia ser analisada em diferentes fluidos corporais como biomarcador de diferentes patologias. No caso de câncer hepatocelular, por exemplo, FAT 1 demostrou ser um biomarcador sanguíneo útil do seu diagnóstico e prognóstico (LI et al., 2016) podendo também ser investigado como marcador em outros tipos tumorais.

2.1.4 FAT1

FAT1 é parte de uma superfamília de caderinas, composta por quatro proteínas gigantes de 500 a 600 kd de peso e tem similaridades estruturais em invertebrados e mamíferos. FAT1 é uma proteína transmembrana de tipo I e está composta por 34 repetições de caderinas, cinco repetições do tipo EGF, um domínio A-G de laminina na região extracelular e uma cauda citoplasmática que é muito diferente nas caderinas clássicas (GRIFANTINI et al., 2011)(DUNNE et al., 1995). FAT1 é capaz de suprimir de forma potente o crescimento de células tumorais *in vitro* e *in vivo.*

2.1.5 Mutações em FAT1 e CEC.

FAT1 funciona como um gene supressor tumoral e suas mutações, especialmente deleções, desempenham um papel fundamental no desenvolvimento e progressão do CEC (NAKAYA et al., 2007). Um estudo recente indicou que *FAT1*, transfectado em células de CEC, reduziu significativamente a proliferação celular e aumentou a taxa de apoptose. Efeitos antitumorais semelhantes também foram observados *in vivo* (FAN et al., 2015). Além disso, mutações somáticas não sinônimas são frequentes em vários tipos de tumores humanos como glioblastoma multiforme, câncer de cólon e CEC. Quase todas as mutações têm sido localizadas em domínios funcionais já conhecidos. A frequência e a localização das alterações em *FAT1* sugerem que estas mutações podem ter consequências funcionais (MORRIS et al., 2013). Quando o gene *FAT1* é inativado por uma mutação, poderia levar a uma ativação da via Wint β-catenina e permitir o desenvolvimento tumoral. FAT1 pode interagir com β-catenina e sequestrá-la na periferia da célula regulando, assim, a sua atividade de transcrição. Se não é sequestrada na membrana celular, β-catenina se liga ao fator de células T (TCF), proteínas que translocam-se para o núcleo e ativam

genes Wnt, promovendo a proliferação celular (MORRIS et al., 2013). Relata-se uma forte intensidade de marcação da membrana para β-catenina em displasias leves, moderadas e severas (LO MUZIO et al., 2009). A função da β-catenina na adesão celular e sua translocação para o núcleo estariam relacionadas com proliferação e invasão do CEC (REYES et al., 2015). *FAT1* tipo selvagem pode antagonizar potentemente a transcrição mediada por β-catenina. Também se demonstrou que a expressão aumentada de β-catenina acelera o crescimento celular promovendo a progressão do ciclo celular, efeitos que são reprimidos quando é feita uma co-transfecção de *FAT1*. Portanto, as mutações em *FAT1* são uma das principais causas de ativação da via Wnt em vários tumores humanos, incluindo o CEC (URAGUCHI et al., 2004).

2.1.6 FAT1 e biomarcador na saúde humana.

O entendimento da composição do espaço extracelular também ajuda a compreender a complexa dinâmica de invasão tumoral e biologia do câncer (LIOTTA et al., 1980). Proteínas secretoras são essenciais para gerar um ambiente na matriz extracelular favorável para o desenvolvimento de muitas doenças (PALTRIDGE; BELLE; KHEW-GOODALL, 2013) (XUE; LU; LAI, 2008). Além disso, estas proteínas podem distribuir-se para o sangue periférico e, então tornarem-se uma fonte de estudo para fins de diagnóstico e prognóstico das doenças (QI et al., 2009). Nos últimos anos, avanços na análise do proteoma do plasma e saliva humanos forneceram uma oportunidade considerável para a descoberta de biomarcadores clínicos (ANDERSON et al., 2004) (JEONG et al., 2009)(NANJAPPA et al., 2014). Realizou-se uma análise das proteínas segregadas a partir de populações de células homogéneas, e foi identificada, no caso de câncer hepatocelular, por exemplo, que FAT1 e DKK3, seriam biomarcadores do soro, úteis tanto para o diagnóstico e prognóstico desta doença (LI et al., 2016).

2.1.7 A saliva e suas características

A saliva é composta por uma variedade de eletrólitos, pequenos compostos orgânicos, proteínas, peptídeos e polinucleotídeos. Possui propriedades físicas, químicas, bioquímicas, biológicas, atividade antibacteriana e antiviral, lubricidade e

reparação de mucosa (CASTAGNOLA et al., 2011; HUMPHREY; WILLIAMSON, 2001). As proteínas salivares estão associadas com muitas dessas funções e a sua concentração é bastante estável (entre 0,5 e 3 mg/ml), No entanto, esta concentração total, pode ser maior na presença de infecções orais, doenças sistêmicas, e depois da exposição à radioterapia (AGUIRRE et al., 1993) (MAIER; BIHL,1987). A composição bioquímica salivar, bem como os seus níveis de pH, também podem sofrer variações dependendo da saúde geral e bucal das pessoas.

A saliva tem uma faixa de pH de 6,3 a 7,3 em pessoas saudáveis. Um pH salivar inferior de 7,0 indica geralmente acidemia. Tem sido relatado que pessoas com CEC, possuem pH mais ácido e que pessoas em tratamento, apresentam uma saliva mais alcalina do que aquelas sem tratamento (RAMYA et al., 2015). Uma das possíveis explicações deste fenômeno poderia ser pelo metabolismo anaeróbico de glicose em condições de hipóxia criadas pelo CEC, onde é possível encontrar maior acidificação salivar, o que facilitaria o crescimento e a sobrevivência das células neoplásicas. Devido à elevada absorção de glicose pelas células tumorais e a subsequente glicólise anaeróbia associada, aumentaria a produção de ácido láctico, o que também poderia explicar a acidificação salivar (RAMYA et al., 2015). Até o presente momento, tem sido relatadas mudanças nos níveis de pH na progressão da doença periodontal (NATER et al., 2007), mas, evidências a respeito de seus níveis no CEC, são ainda escassas. Tem-se comparado o pH salivar de pessoas sem e com o CEC, mas até agora não há registros de estudos prospectivos que caracterizem o pH ao momento do diagnóstico de CEC e depois de um tempo, o que poderia ser de utilidade para o avaliar o prognóstico do CEC. Assim, as mudanças salivares sobre os oligoelementos na saliva poderiam também ser de utilidade com este mesmo propósito.

Oligoelementos como Cobre (Cu) e Zinco (Zn) poderiam ter um papel importante na formação e desenvolvimento de neoplasias malignas. O Cu participa no metabolismo celular sendo parte de várias enzimas como a tirosinase e citocromo-oxidase, envolvidas principalmente nas reações de oxidação. O nível sérico médio de Cu tem sido relatado significativamente maior nos soros de pacientes com lesões pré-malignas e malignas bucais (TADAKAMADLA; KUMAR; GP, 2011). A razão para o aumento dos níveis de Cu no soro poderia ser devido à sua capacidade de induzir uma enzima a base de proteína intrínseca no tecido conjuntivo que degrada colágeno e elastina, fator importante na etiopatogenia do CEC (TADAKAMADLA; KUMAR; GP,

2011). Mudanças nos níveis de Cu, tem sido relatados também na saliva de pacientes com CEC em comparação com aqueles sem lesões (SHETTY et al., 2015).

Alterações nos níveis de Ferro (Fe) também têm sido relatadas na saliva de pacientes com CEC, o que poderia relacionar-se com a sua utilização na síntese de colágeno (ANURADHA; DEVI, 1993). Mudanças salivares nos níveis de Fe poderiam conduzir às alterações na vascularização epitelial, processo determinante na patogenia do câncer. O Zinco atua como um cofator para a enzima superóxido dismutase, que é uma parte do sistema antioxidante primário de todos vertebrados. Alguns estudos têm revelado níveis mais baixos de Zn em soro de pacientes portadores de doenças potencialmente malignas. Os níveis salivares de Zn, também podem sofrer variações nos casos de pacientes com lesões orais potencialmente malignas.

Em doenças como a Fibrose Cística, a análise do oligoelementos avaliados por espectroscopia na saliva de pacientes acometidos, mostram uma diferença significativa no nível de Sódio (Na), Potássio (K), Vanádio (V), Cromo (Cr), Selênio (Se), e Arsênio (As) quando são comparados com indivíduos saudáveis (VIEIRA et al., 2011). Os estudos recomendam que nessa doença deve ser considerada a composição bioquímica salivar como método complementar de avaliação de seu diagnóstico e prognóstico. No caso do CEC, é necessária a realização de estudos adicionais.

2.1.8 Biomarcadores em saliva

Até a presente data, os métodos convencionais para o diagnóstico do câncer têm sido úteis e efetivos. No entanto, a ciência médica tem focado em encontrar métodos alternativos para diagnosticar esta doença de uma forma menos invasiva e menos dolorosa. Neste sentido, a coleta de várias amostras de saliva durante o dia, tal como demonstrado por estudos recentes, pode ser muito útil. Este fluido, além de proporcionar proteção aos tecidos da cavidade oral e participar em processos fisiológicos orais, representa uma possibilidade de agir como elemento cuja coleta funcione como meio auxiliar de diagnóstico, dado o seu baixo custo econômico, facilidade de avaliação e baixa complexidade de obtenção de biomarcadores, cuja presença e/ou níveis no sangue indiquem a existência de um processo patológico, o

risco de desenvolver ou resposta a um tratamento em particular. Atualmente, foram identificados biomarcadores na saliva cuja concentração está relacionada com a progressão de estados de algumas doenças crônicas como diabetes (AITKEN et al., 2015), síndrome de Sjögren, câncer do pâncreas, da mama, do pulmão e Alzheimer. Neste último caso, tem sido determinado que os níveis de detecção da α/β amilóide 1-42 na saliva são indicativos da progressão da doença e a sua utilização para a intervenção terapêutica em estágios iniciais (BERMEJO-PAREJA et al., 2010). Em outro estudo, diferenças estatisticamente significativa foram estabelecidas entre pacientes com CEC e indivíduos saudáveis tratados com as concentrações mais elevadas das interleucinas IL-8, IL8-B, detectadas na saliva (BRINKMANN et al., 2011). A saliva tem sido utilizada há décadas como um meio de monitoramento do risco cariogênico (BAUGHAN et al., 2000) e progressão da doença periodontal (KORNMAN et al., 1997) (SOCRANSKY et al., 2000). Atualmente, a biotecnologia emergente ampliou o conhecimento dos biomarcadores salivares, uma vez que as maiorias dos compostos encontrados no sangue estão também presentes neste fluido (NRIAGU et al, 2006). A saliva poderia, então, ser uma fonte permanente de monitoramento e detecção precoce de certas doenças ou na auxiliar na determinação de suas progressões.

Até o momento atual não existe marcador de prognóstico clínico para o CEC na prática odontológica clínica. Considerando que das mutações mencionadas no mais recente sequenciamento massivo do CEC, *FAT1* é o único gene que codifica uma proteína de secreção, potencialmente identificável na saliva (LI et al., 2016b), torna-se relevante sua avaliação como proteína e biomarcador no CEC. FAT1 já foi encontrada no soro como biomarcador de câncer (LI et al., 2016a), mas ainda não em saliva. Desta forma, o presente trabalho busca identificar, caracterizar e determinar os níveis de FAT1 na saliva de pacientes com CEC e com displasias de alto e baixo risco, bem como correlacionar tais achados com a expressão de FAT1 em tecidos provenientes de biópsias dos mesmos pacientes. Assim, em caso de uma associação positiva, os níveis de FAT1 poderiam ser potencialmente utilizados como biomarcadores salivares de progressão do CEC, podendo auxiliar na definição de um tratamento personalizado e mais efetivo e contribuir sensivelmente com a prevenção do desenvolvimento de CEC, seu tratamento e também com a melhora da qualidade de vida dos pacientes.

2.2 Objetivos

2.2.1 Objetivo Geral

Identificar FAT1 e determinar seus níveis teciduais e salivares em indivíduos com displasias de baixo e alto risco e carcinoma espinocelular de boca, ao momento de diagnóstico e após 6 meses de tratamento, correlacionando–os entre si.

2.2.2 Objetivo específico estudo retrospectivo

Avaliar e comparar a expressão tecidual de FAT1 em espécimes de displasias de alto e baixo risco, carcinoma espinocelular e de proliferações epiteliais benignas (papilomas) e relacionar esses valores com as características clínicas e hábitos de fumo e álcool dos pacientes acometidos.

2.2.3 Objetivos específicos estudo prospectivo

- Traçar o perfil bioquímico da saliva em pacientes com displasias de alto e baixo risco, carcinomas espinocelulares e proliferações epiteliais benignas (papilomas) ao momento da biopsia e após 6 meses do tratamento.
- Avaliar expressão tecidual de FAT1 em espécimes de displasias de alto e baixo risco, carcinoma espinocelular intrabucal e proliferações epiteliais benignas (papilomas) dos pacientes recrutados.
- Avaliar expressão de FAT1 salivar de pacientes com displasias de alto e baixo risco, carcinomas espinocelulares e proliferações epiteliais benignas (papilomas) ao momento da biopsia e após 6 meses do tratamento.
- Correlacionar expressão de FAT1 tecidual com a expressão de FAT1 salivar de pacientes com displasias de alto e baixo risco, carcinomas espinocelulares intrabucal e proliferações epiteliais benignas (papilomas)
- Comparar a expressão salivar de FAT1 ao momento da biopsia e após 6 meses de tratamento.

2.3 Metodologias

2.3.1 Desenho do estudo.

O estudo será dividido em duas fases. Primeiro, será avaliada a expressão tecidual de FAT1 em 80 amostras (biópsias) pertencentes ao serviço de Anatomia Patológica do Centro de Diagnóstico das Doenças da Boca (CDDB) da Faculdade de Odontologia da Universidade Federal de Pelotas (Ufpel), das quais, 20 serão de displasias de alto risco, 20 de baixo risco, 20 de CEC intrabucal e 20 relativas a proliferações epiteliais benignas (papilomas), de acordo com o protocolo da OMS (WARNAKULASURIYA, 2009). A expressão tecidual de FAT1 de displasias de alto e baixo risco serão comparadas entre elas e com a sua expressão nos espécimenes de CEC. Nesses últimos casos (CEC), a expressão tecidual de FAT1 será também associada com os hábitos de álcool e tabaco e estadiamento do câncer (TNM).

A segunda parte do estudo será prospectiva. Será feita uma amostra por conveniência, constituída por 80 pacientes com indicação de biópsia devido à presença de lesões intraorais com suspeita clínica de displasias de alto e baixo risco, CEC e proliferações epiteliais benignas (papilomas), seguindo o protocolo da OMS (THOMPSON, 2006). Cirurgiães dentistas capacitados realizarão as biópsias e a coleta de saliva não estimulada nesses mesmos pacientes. As coletas salvares serão feitas no mesmo dia, mas antes de fazer a biópsia. Nos casos que sejam confirmados os diagnósticos histológicos de displasias de alto e baixo risco, CEC e proliferações epiteliais benignas (papilomas), se determinará a expressão tecidual e salivar de FAT1, valores que serão correlacionados ao momento do exame inicial. A expressão salivar de FAT1 também será avaliada 6 meses após tratamento, níveis que serão comparados. Será realizada também, uma caracterização bioquímica salivar ao momento do exame inicial e após 6 meses do tratamento. Amostras salivares e teciduais de papilomas serão utilizadas como controle por representarem proliferações epiteliais, mas de natureza benigna (VAN DER WAAL, 2009).

2.3.2 Primeira parte: Estudo Retrospectivo

2.3.2.1 Amostras

Serão selecionados dos arquivos do CDDB da Faculdade de Odontologia da Universidade Federal de Pelotas (UFPel), 80 blocos de parafina, dentro dos quais, 20 serão de displasias de alto risco, 20 de baixo risco, 20 de CEC intrabucal e 20 lesões hiperplásicas epiteliais não neoplásicas (Papilomas). Os diagnósticos desses laudos correspondentes aos blocos selecionados, serão conferidos pela equipe de patologia oral da UFPel para sua correta classificação. Uma vez conferidos os diagnósticos, será feita a avaliação de FAT1 tecidual. Nos casos de CEC, os resultados serão associados com os hábitos de álcool e tabaco e estadiamento do câncer (TNM), segundo o protocolo de Greene (GREENE et. al., 2002), de acordo com a informação de cada ficha desses pacientes. (Anexo 1)

2.3.2.2 Determinação de FAT1

Para determinar a presença tecidual de FAT1, os blocos, serão recolhidos em lâminas carregadas positivamente, em cortes com espessura de 3 mm. Mais tarde, serão desparafinados em xilol e reidratados, com álcoois descendentes até água destilada. Será realizada reação imunoistoquímica utilizando o complexo de estreptavidina-avidina-biotina (ABC) para permitir sua visualização. Será identificada FAT1 de acordo com as instruções do fabricante (FAT1, SIGMA). Os blocos serão incubados em média com 100 ul do anticorpo anti-FAT1. As lâminas serão bloqueadas com BSA a 1% em PBST. As secções serão pré-incubadas com soro de cavalo durante 20 minutos à temperatura ambiente e incubadas durante 30 minutos. Em seguida serão agregados os anticorpos primários com 1:200 de diluição, pronto para usar (UTR), respectivamente, numa câmara úmida a 37 ° C. As secções serão posteriormente lavadas com PBS durante 5 min e incubadas com anticorpo secundário biotinilado durante 30 min a 37 °C. A reação será finalmente visualizada com diaminobenzidina (DAB) e contra-coradas com hematoxilina. Segundo a porcentagem de positividade, as amostras serão classificadas em 4 grupos. O primeiro grupo (escore 0) será aquele sem marcação, o segundo (escore 1), com positividade entre 1 e 25%, o terceiro (escore 2), com positividade entre 25 e 50% e o quarto grupo (escore 3), com positividade celular maior do que 50%. A quantificação de FAT1 será avaliada por exame microscópico de luz com ampliação de 400x segundo protocolo estabelecido por Caldeira (CALDEIRA et al, 2015).

2.3.3 Segunda parte: Estudo Prospectivo

2.3.3.1 Recrutamento de pacientes.

Serão recrutados 80 pacientes de ambos os sexos maiores de 30 anos que consultam de maneira espontânea ou são encaminhados até a clínica do CDDB da Faculdade de Odontologia da UFPel, que apresentem lesões clinicamente compatíveis lesões potencialmente malignas (displasias de alto e baixo risco), papilomas e CECs. Os indivíduos que concordarem em participar vão assinar um consentimento informado o qual cumpre com as recomendações da Declaração de Helsinki (DOMJÁN; KAKUK; SÁNDOR, 2014).

2.3.3.2 Aspectos éticos

O projeto de pesquisa, feito em conformidade com as recomendações da Declaração de Helsinki (DOMJÁN; KAKUK; SÁNDOR, 2014), sendo apresentado ao Comitê de Ética em Pesquisa da Faculdade de Odontologia da UFPel. Cada participante no projeto deverá assinar um termo de consentimento informado por escrito, em que manifestam que a sua participação é livre, voluntária e informada. Os dados obtidos de cada indivíduo serão tratados em sigilo absoluto e serão utilizadas apenas para fazer a pesquisa, sem fins lucrativos. O nome e informações pessoais nunca serão identificados publicamente. Além disso, os participantes terão o direito de recusar ou de renunciar a participação no estudo, a qualquer momento do mesmo. Embora não seja considerada uma recompensa financeira para os voluntários, eles terão o direito de ser informados sobre os resultados da pesquisa.

2.3.3.3 Instrumentos e procedimentos clínicos

Cirurgiões dentistas previamente treinados vão coletar saliva não estimulada dos pacientes em um tubo de centrífuga de 50 ml Falcon ®, pré-pesado e rotulado de acordo com o protocolo descrito por Navazesh (NAVAZESH et al., 1992). Os voluntários não deverão fumar, escovar os dentes ou consumir alimentos uma hora antes da coleta. Após 5 minutos de um estado anterior de relaxamento, os pacientes serão convidados a assumir na “posição de cocheiro” descrita por Schultz e utilizada por Navazesh e depositarão saliva por 5 minutos no tubo Falcon ®. Durante a coleta, os indivíduos deverão permanecer sentados, quietos e não deverão falar. A amostra de saliva será mantida em um recipiente a 5 ° C para o transporte para o laboratório do Centro de Diagnóstico de Doenças da boca (CDDB) da Faculdade de Odontologia

da Universidade Federal de Pelotas, destino final das amostras salivares onde serão armazenadas segundo a resolução CNS número 441 de 2011. Imediatamente será medido e registrado o pH salivar. O restante da saliva será congelada a -80 C, em tubos eppendorf ® de 2 ml, para ser submetida à análise posterior (determinação dos níveis salivares de FAT 1 e análise bioquímica salivar).

A saliva deve ser mantida a -80 ° C antes da análise devido ao fato de que as propriedades das proteínas e eletrólitos salivares são mantidas a essa temperatura (RAO et al., 2009). Posterior esse analise, a saliva não utilizada será descartada.

A saliva será coletada dos pacientes que assinem o termo de consentimento livre e esclarecido antes de ser feita a biópsia, cuja análise laboratorial só será feita após conferido o diagnóstico histológico de displasia de baixo ou alto risco, CEC e papiloma.

2.3.3.4 Biópsias

As biópsias indicadas em pacientes com diagnóstico clínico compatível com lesões potencialmente malignas, CEC e papiloma de acordo com o protocolo da OMS (THOMPSON, 2006), serão executadas por cirurgião dentistas. As amostras de tecido serão encaminhadas ao CDDB da Faculdade de Odontologia da UFPel em tubos plásticos de 50 ml com formol em seu interior. Após a macroscopia, os cortes histológicos serão corados com hematoxilina e eosina e posteriormente, patologistas bucais da Faculdade de Odontologia da UFPel farão o diagnóstico histopatológico da lesão.

As displasias serão classificadas como displasias de “alto risco” quando apresentarem pelo menos 4 alterações arquiteturais ou 5 alterações celulares, e de “baixo risco” aquelas com menos de 4 alterações arquiteturais e menos de 5 alterações celulares. As mudanças arquiteturais são: estratificação irregular, perda da polaridade das células basais, epitélio em forma de gota, aumento do número de mitoses, ceratinização prematura de células individuais, presença de mitoses em camadas superficiais, pérolas de ceratina nos ninhos epiteliais. As mudanças celulares são: variações em tamanho e forma celular, variações em forma e tamanho nuclear, aumento da relação núcleo citoplasma, presença de figuras mitóticas atípicas, aumento do número e tamanho dos nucléolos e hipercromatismo nuclear (KUJAM et. al. 2006).

2.3.3.5 Análise do pH salivar

O pH será avaliado com o medidor de pH (ELICO- medidor de pH, Hydeabad, Índia), previamente padronizado e calibrado utilizando água destilada. O pH será medido diretamente sobre as amostras e os resultados serão tabulados.

2.3.3.6 Análise bioquímica salivar

Para traçar o perfil bioquímico, a saliva será avaliada por espectrometria de emissão óptica, com plasma de argônio acoplado indutivamente (ICP-OES), a partir das amostras que ficaram congeladas a -80 °C depois da medição de pH salivar. Para a remoção da porção orgânica, a cada alíquota de 1 mL de saliva, será adicionando 5 mL de HNO_3 e 5 mL de água deionizada e aquecida em forno de microondas analítico fechado. Soluções padrões de Zinco, Cobre e Ferro serão preparadas com reagentes de grau analítico e os volumes finais ajustados com água destilada e deionizada. A quantificação dos elementos será realizada pela análise direta da amostra de saliva sem conteúdo orgânico, utilizando um espectrofotômetro de emissão óptica com plasma de argônio acoplado indutivamente. O análise bioquímica será feita na Faculdade de Química Forense, da UFPel, Campus Capão do Leão.

2.3.3.7 Determinação dos níveis salivares de FAT 1

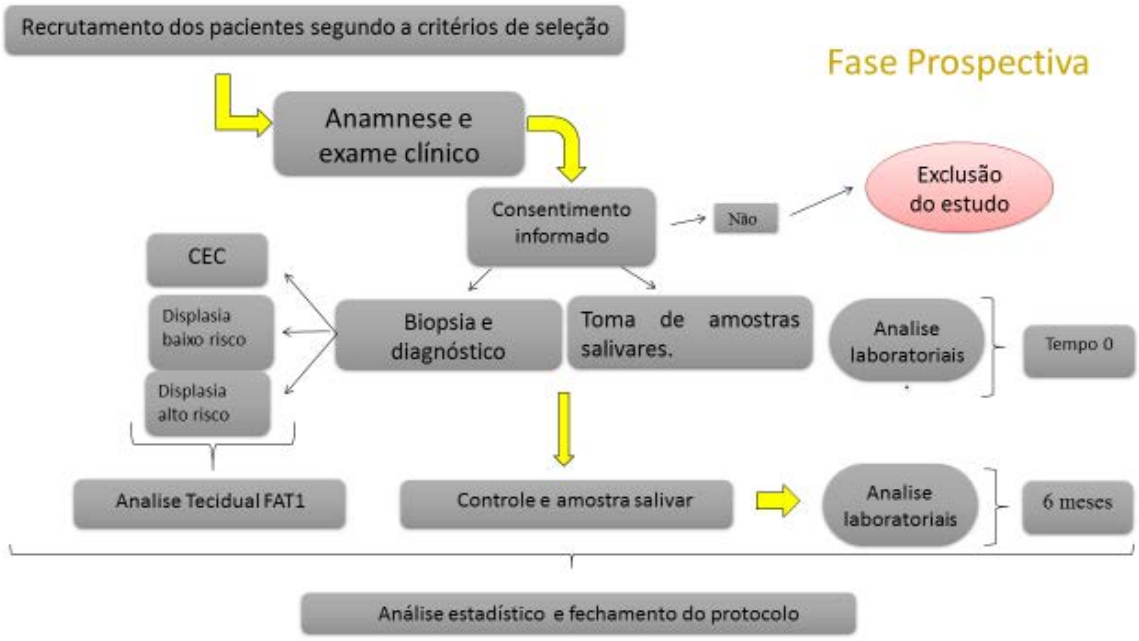
Das amostras salivares coletadas, serão extraídos 100 mL para determinação de FAT1. Estas frações das amostras de saliva serão analisadas em um teste de análise imunoenzimática quantitativa (ELISA) para FAT1 (GenWayBiotech, Inc.CA, EUA). Placas de ELISA (Falcon Becton Dickinson, Hershey, EUA) serão sensibilizadas com IgY de galinha contra FAT1 humana (dilução 1: 450), num volume de 100 ul/poço, em tampão de bicarbonato 0,05 M (pH 9,6). Após a incubação, a placa será lavada e depois bloqueada com 200 ul/poço de albumina de soro tamponado 2 horas, a 37°C. Em seguida, 100 ul de FAT1 recombinante em diluições seriadas e amostras diluídas de saliva (10/01) serão adicionadas e incubadas durante 1 h a 37 °C. Após a incubação, a placa será lavada e, em seguida, serão adicionados IgY de galinha contra FAT1 conjugado num volume de 100 ul / em solução com tampão de bicarbonato 0,05 M (pH 9,6), o que será incubando durante 1 hora a 37°C. Subsequentemente, a mistura será revelada com e as placas serão lidas a 450 nm utilizando um leitor de placas de ELISA. Esta análise salivar será feita no laboratório

do Centro de Diagnósticos de Doenças da Boca (CDDB), na Faculdade de Odontologia da Universidade Federal de Pelotas, sob a supervisão do responsável pelo projeto Profa. Dra. Sandra Beatriz Chaves Tarquinio.

2.3.4 Análise estatística

Para a realização da análise estatística, o método estatístico será escolhido com base na aderência no modelo de distribuição normal e igualdade de variâncias. Para todos os testes será considerado o valor $p<0,05$ como estatisticamente significativo. Dependendo da distribuição normal ou não das amostras serão feitas as comparações entre a expressão tecidual de FAT1 nos diferentes espécimes, sendo utilizados o teste T ou Man-Whitney, respectivamente. Os dados referentes aos valores dos níveis da FAT1 tecidual e salivar serão correlacionados usando teste de Pearson ou Spearman, respectivamente se as amostras têm distribuição tiverem distribuição normal ou não, para associar níveis de FAT1 em saliva e tecidos das displasias e CEC. Para a análise dos dados cuja resposta ou desfecho é apresentado em categorias com ordenação (estadiamento clinico, porcentagem de positividade de FAT1 tecidual e hábitos), será utilizado um modelo de regressão logística ordinal, para estabelecer probabilidade de ocorrência de um evento, neste caso, CEC. Será utilizado o software Stata 11.0.

2.3.5 Organograma fase prospectiva



2.3.6 Orçamento

FONDECYT 11140281: “GENETIC ALTERATIONS OF ORAL EPITHELIAL MUCOSA DURING MALIGNANT TRANFORMATION”. Prof. Ricardo Fernández-Ramires. ramiresfernandez@gmail.com Phone: +56229781718 (Anexo)

O projeto foi aprovado pelo Comitê de Ética da Faculdade de Odontologia da Universidade de Chie (Anexo 6.4)

Item	Quantidade	Valor
Medidor de pH (ELICO-Hydeabad, Índia)	1	6000
Imunoenzimo (ELISA) quantitativa para FAT1 (GenWayBiotech, Inc.CA, EUA).	1	4000
Tubo de centrífuga de 50 ml Falcon	100	1000
FAT1 Imunoistoquimica, SIGMA	1	3000
Material de consumo (Luvas, sacos de lixo e outros)		2000
	Total	16.000

2.3.7 Cronograma

1. Apresentação do projeto
2. Compra materiais
3. Sel. espécimenes
4. Expressão FAT1 em espécimenes
5. Comparação da expressão FAT1
6. Primeiro artigo
7. Recrutamento de pacientes
8. Caraterização BQ da saliva tempo 0
9. Determinação FAT1 salivar tp 0
10. Determinação FAT1 teciduais
11. Caraterização BQ saliva 6 meses
12. Det.FAT1 salivar 6 meses
13. Analise dos resultados
14. Fechamento do protocolo
15. Escrever artigos

Atividade	Ano: 2016		Ano: 2017		Ano: 2018		Ano: 2019	
	Semestre		Semestre		Semestre		Semestre	
	1	2	1	2	1	2	1	2
1		X	X					
2			X					
3			X					
4			X					
5			X					
6			X					
7			X	X	X			
8			X	X	X			
9					X			
10					X			
11				X	X	X		
12					X	X		
13						X	X	
14							X	X
15							X	X

2.3.8 Referências

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3 Relatório do trabalho de campo

Tendo em vista as metodologias propostas no projeto de pesquisa, algumas modificações foram necessárias, em função das sugestões da banca e das limitações experimentais que surgiram no decorrer do trabalho. Após realizar as mudanças sugeridas pela banca avaliadora da qualificação, o projeto de pesquisa foi submetido ao comitê de ética em pesquisa (CEP) da Faculdade de Odontologia da Universidade Federal de Pelotas (UFPel). Em decorrência do patrocínio por uma fundação chilena (Fondecyt 11140281), com financiamento do projeto intitulado “genetic alterations of oral epithelial mucosa during malignant tranformation”, concedido ao professor Ricardo Fernández-Ramires), o mesmo teve que ser avaliado pelo Comitê Nacional de Pesquisa em Brasilia. Durante o processo, que durou aproximadamente um ano até o projeto ser aceito, foram desenvolvidas várias outras pesquisas no centro de diagnóstico de doenças da boca (CDDB), as quais já haviam sido previamente aprovadas pelo CEP local e relacionavam-se à utilização da saliva como fonte de biomarcadores como reflexo da saúde sistêmica e bucal, tendo gerado artigos publicados, trabalhos de conclusão de curso, desdobramentos em projetos aceitos e cadastrados que começaram a se desenvolver em 2020, tendo, inclusive, sido premiado um trabalho com o primeiro lugar na 56 semana academica odontologica da UFPel (Caracterização sistêmica e uso de farmácos em mulheres pelotenses com Síndrome da Ardência Bucal), sob orientação do doutorando. Especialmente relevante foi o projeto realizado em mulheres com síndrome de ardência bucal (SAB), devidamente cadastrado no Registro Brasileiro de Ensaios com o número (RBR-774xbd) e cuja publicação “Efeito de um substituto salivar caseiro à base de Camomila (Matricaria chamomilla) e Linhaça (Linum usitatissimum) no alívio dos sintomas da Síndrome da Ardência Bucal” encontra-se atualmente em vias de aceitação (The Journal of Alternative and Complementary Medicine). Este trabalho teve importante repercussão na Faculdade de Odontologia da UFPel (FO/UFPel). Atualmente, o uso da mistura de camomila e linhaça além de fazer parte do protocolo de tratamento no CDDB em pessoas com SAB com excelentes resultados, fez que o nosso interesse em trabalhar com a saliva como biomarcador no câncer bucal, tivesse

um objetivo mais abrangente, incorporando esta síndrome como parte essencial da nossa pesquisa de doutorado. A revisão bibliográfica (cadastrada devidamente no PROSPERO) e o estudo de casos e controles apresentados nesta tese como capítulos 6 e 7 e que fazem parte do projeto guarda-chuva da SAB acima indicado, compõem as sessões dedicadas a esta temática no presente trabalho.

Uma vez que foi aceito nosso trabalho: “Detecção de FAT1 tecidual e salivar e sua avaliação como um preditor da progressão das displasias de baixo e alto risco para o carcinoma espinocelular oral (CEC) ”, com o número de parecer 2.476.399 pelo comitê de ética nacional, começamos o seu desenvolvimento no dia 30 de janeiro de 2018. Foi tarefa difícil, numa primeira etapa, atingir o nosso objetivo de avaliar o número de pacientes com CEC que a nossa pesquisa precisava e acompanhá-los durante 6 meses até obter uma segunda amostra salivar. Inclusive, dois dos pacientes avaliados na fase inicial, faleceram antes da segunda coleta salivar. Mesmo assim, após um ano de acompanhamento, foi possível atingir o número de pacientes que a pesquisa precisava, cada um com duas amostras salivares que ficaram congeladas até a análise laboratorial, a qual precisou ser realizada em duas etapas, pois o primeiro Kit de ELISA que recebemos, trouxe um reagente estragado e tivemos que esperar por mais 3 meses até que o fornecedor mandasse um novo produto (Mybiosource® Catalog Numer MBS905038).

Durante o tempo que os pacientes foram acompanhados, foi desenvolvido o estudo imunoistoquimico. Após várias provas laboratoriais para estabelecer a concentração e protocolo ideais, obtivemos os primeiros e promissores resultados. Nesta fase do projeto, foi decidido utilizar amostras de tecido sadio ao invés de papilomas (como foi proposto no projeto inicial), pois refletimos sobre o fato de que o grau de alteração epitelial que apresentam essas lesões, mesmo benignas, poderia alterar significativamente os nossos resultados e objetivos buscados.

Nosso projeto inicial também tinha como objetivo, traçar o perfil bioquímico dos pacientes com displasias de alto e baixo grau e com CEC para avaliar o potencial uso dos eletrólitos salivares como biomarcadores da progressão e resposta às terapias nesta doença. Embota tivesse sido proposto inicialmente realizar estas análises na Faculdade de Química Forense, da UFPel, Campus Capão do Leão, os testes foram realizados finalmente na Universidade Federal de Santa Maria – UFSM, pois esta contava com a tecnologia para realizar em forma simultânea as análises de vários

eletrólitos mediante a espectrometria de emissão óptica, o que avaliamos ser benéfico para o estudo num menor prazo de tempo. Este objetivo além de ser atingido, foi acrescido de uma revisão sistemática.

Mesmo sendo esta parte do nosso trabalho bem inovadora, pelo fato de ser o primeiro estudo a avaliar os níveis salivares de FAT1 como possível biomarcador de CEC e também como um reflexo de sua resposta às terapias associadas, houve limitações no desenvolvimento da pesquisa, como a impossibilidade de associar diretamente a expressão imunoistoquímica do FAT1 e os níveis salivares dessa proteína, uma vez que os anticorpos utilizados nas duas fases foram direcionados a diferentes tipos de FAT1, sendo um mutado e outro nativo não mutado, respectivamente. Esta limitação ocorreu porque o kit de ELISA disponível no mercado para avaliar a presença da proteína em fluidos corporais FAT1 era o de sua forma nativa e o anticorpo utilizado no estudo imunoistoquímico para detectar a mesma proteína era o de sua forma aberrante. Mesmo assim, os resultados deste trabalho, além de ser inovadores, foram correspondentes ao tipo de proteína que cada kit avaliava.

Em termos gerais, mesmo mudando algumas linhas de nossa proposta inicial, de projeto, acreditamos que ter incorporado uma síndrome de difícil manejo, deu robustez ao nosso trabalho. Os quatro artigos que fazem parte desta tese e que esperamos sejam publicados uma vez corregidos e avaliados pela banca avaliadora, somam-se a mais doze realizados e publicados durante o processo de doutoramento, vários dos quais apontam a utilização da saliva como reflexo ou como parte fundamental da etiologia tanto de doenças sistêmicas quanto locais. Estes resultados abrem também a possibilidade de continuar a linha de pesquisa focada nas caraterísticas salivares e a sua utilização na área de diagnóstico bucal na UFPel, como proposta de atuação conjunta com a Universidade do Chile.

4 Artigo 1

Use of salivary biochemical profile to assess the progression of potentially malignant disorders to oral squamous cell carcinoma and its response to therapies: A case control study and a systematic literature review[1]

Juan Aitken-Saavedra[a,b]
Ana Carolina Uchoa Vasconcellos[c]
Diogo La Rosa Novo[d]
Marcia Foster Mesko[d]
Karine-Duarte da Silva [b]
Sandra Beatriz Chaves Tarquinio[c*]

[a] Department of Oral Pathology and Medicine, School of Dentistry, University of Chile. Santiago, Chile

[b] Graduate Program in Dentistry, Federal University of Pelotas, Pelotas, RS, Brazil.

[c] Center of Diagnosis of Diseases of the Mouth, Federal University of Pelotas. Pelotas, Rio Grande do Sul, Brazil

[d] Center of Chemical, Pharmaceutical and Food Sciences, Federal University of Pelotas. Capão do Leão, Rio Grande do Sul, Brazil

Corresponding author.
Sandra Beatriz Chaves Tarquinio, PhD
Email: sbtarquinio@gmail.com
Address: R. Gonçalves Chaves, 457 - Centro, Pelotas - RS,
Postal address: 96015-560 | Phone: +55-53-981112894 | Fax 56-2-9781748

[1] Artigo formatado segundo as normas do periódico Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology.

Abstract

Background: Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity. Early detection and control of oral potentially malignant disorders (OPMD) is essential to prevent progression to OSCC. The study aims to determine electrolyte concentration in the saliva of patients without oral lesions, with OPMD, and with OSCC at the moment of the diagnosis and after therapy. In addition, the data in this research are compared to those obtained from a systematic review.

Methods: Unstimulated saliva flow rate from 18 patients with OSCC (who were also evaluated six months after therapy), 18 with OPMD, and 18 without oral lesions was collected. A biochemical analysis was performed to evaluate the salivary concentrations of potassium (K), phosphorus (P), sodium (Na), calcium (Ca), magnesium (Mg), zinc (Zn), cooper (Cu), and iron (Fe). Kruskall Wallis test was performed, and $P < 0.05$ was interpreted as statistically significant. The systematic review retrieved 10 studies that associate salivary electrolyte levels with progression, presence, and response to therapies in OSCC.

Results: High Mg levels were found in the saliva of patients with OPMD ($p<0.05$). No differences were observed in the other elements evaluated among groups. The systematic review revealed that one article indicated a decrease and three papers reported an increase in salivary Na levels in OPMD and OSCC. Two articles indicated a decrease in salivary K levels in OSCC, and two other papers reported high Mg levels in OPMD and OSCC.

Conclusion: According to our results, high salivary Mg levels can be useful as biomarkers and can indicate the presence of OPMD. Further studies on the role of salivary electrolytes in the progression of OSSC are needed.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity. Even with the significant advances and studies concerning this disease, its five-year survival remains approximately 50% due to its aggressive and invasive behavior.[1] Oral potentially malignant disorders (OPMD) are tissue changes that may precede OSCC,[2] and their early detection is essential to prevent progression to OSCC.[2,3]

Medical science has focused on finding alternative methods for diagnosing OSCC at early stages. In this sense, the use of saliva as a source of biomarkers can be useful because of its advantages, such as facility of evaluation, low complexity of obtaining, and its simple storage.[4] Salivary biochemical compositions may vary according to the general and oral health of individuals, and its assessment may be useful in determining the local and systemic status of patients[4]. Although the study on salivary biomarkers represents an alternative and complementary tool to the solid biopsy for diagnosis and prognosis in various types of cancers, the impact of saliva as a font of biomarkers remains limited, requiring further research to discover the best scenario for clinical uses.[5] Some electrolytes, which can be directly or indirectly evaluated in saliva, play an important role in various physiological metabolic processes in living tissues.[6] More than 25% of enzymes in the human body must be activated by metal ions to perform their metabolic functions.[7] Research on salivary electrolytes and their relationship with OSCC and OPMD is still scarce and contradictory.

Considering the possible oral biochemical alterations that patients with premalignant and malignant lesions can present and be detected in saliva,[8] the study aims to evaluate the salivary concentrations of potassium (K), phosphorus (P), sodium (Na), calcium (Ca), magnesium (Mg), zinc (Zn), cooper (Cu), and iron (Fe) simultaneously in patients without lesions, with OPMD, and with OSCC (pre and post therapy) to evaluate their salivary concentration levels as possible oral cancer predictors of progression and/or therapy responses associated with OSCC. For the first time a salivary electrolyte analysis in relation to OSCC, patients were evaluated after therapy. In addition, we compared our data with those obtained from a systematic literature review.

Materials and Methods

This study comprised two phases. Phase 1 involved a systematic review whose main outcome is the use of salivary electrolytes as biomarkers and progression predictors related to OPMD and OSCC and/or therapeutic responses associated with OSCC. Phase 2 involved a case control study whose aim is to biochemically characterize the saliva of patients without oral lesions, with OPMD, and with OSCC (at the moment of the diagnosis and after therapy) for evaluating salivary electrolyte levels.

Phase 1: Systematic Review

Protocols and sources of information

This systematic review was conducted according to the guidelines of the Cochrane Handbook for Systematic Reviews of Interventions,[9] following the four-phase flow diagram of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement.[10] The literature search was carried out by two independent reviewers (J.P.A.S and A.C.U.V) in January 2020. The following databases were screened: PubMed (National Library of Medicine), Scopus (Elsevier), and Web of Science (Thomson Reuters). In addition, the reference lists of the selected articles were searched manually.

Electronic searches and search strategy

The search strategy is described in **Supplementary Appendix 1**. The main outcome was case-control studies that consider the use of salivary electrolytes as predictive biomarkers or progression indicators and/or as responses to therapy in patients with OPMD or OSCC. No restrictions regarding language use and publication date were observed. Letters to the editor and editorials commenting about other published articles were excluded.

Study selection

The study characteristics were independently extracted by two reviewers (J.P.A.S and A.C.U.V). Any disagreement on the eligibility of the included studies was resolved through discussion and consensus. In case of disagreement, a third reviewer (S.B.C.T) decided whether the article/s should be included. All article titles and abstracts found were analyzed and selected in accordance to the eligibility

criteria. If titles/abstracts were unavailable or did not provide sufficient information to decide whether to include or exclude the articles, full text versions were retrieved. Full-text articles were then obtained, and the same eligibility criteria as described above were applied. Duplicated articles were discarded.

Data extraction

The following information was extracted from each included article: author/s and publication year, the country where the study was undertaken, gender and average age of participants, habits (tobacco and alcohol), site of lesion, and main finding of salivary electrolyte levels. Due to the high degree of heterogeneity in terms of different studies and methodologies, conducting a meta-analysis was considered inappropriate.

Study types

Studies were excluded for the following reasons: A) studies that used other biological media, such as blood or other body fluids instead of saliva as electrolyte source in association with OPMD and/or OSCC; B) reviews, personal opinions, book chapters, and conference summaries; C) associations between salivary electrolytes and OSCC or OPMD in vitro studies or in vivo animal studies; D) studies with insufficient information on the criteria for the diagnosis of OSCC; E) Studies that do not consider the histological diagnosis of epithelial dysplasia for OPMD.

Risk of bias assessment

The first two authors systematically assessed the quality of individual studies by using JBI Meta-Analysis of Statistics Assessment and Review Instrument (JBI-MAStARI).[11] The questionnaire consists of 10 questions that were answered with yes, no, unclear, or not applicable. The studies were classified as follows: high methodological quality (> 5 “yes” responses), moderate methodological quality (3–4 “yes” responses), or low methodological quality (0–2 “yes” responses).

Phase 2: Case Control study

Study design, sampling and setting

The present study was approved by the Research Ethics Committee of the National Research Ethics Commission (approval code: No. 2.262.681) and was conducted in accordance with the guidelines of the Declaration of Helsinki. A total of 54 adults, of both sexes, referred to the Diagnostic Center for Oral Diseases (DCOD) of School of Dentistry - Federal University of Pelotas, from 2016 to 2018, were selected. The sample was divided into four groups: 1 and 2) 18 patients who had the histological diagnosis of OSCC (which were evaluated before and after therapy), 3) 18 patients with lesions with the histopathological diagnosis of dysplasia (and clinical diagnostic of OPMD), and 4) 18 patients without oral lesions. All volunteers were evaluated by a dentist specializing in oral pathology. To be included in the experimental group, the patients must have indication for biopsy for appearing lesions compatible with the histological diagnosis of epithelial dysplasias or OSCC. To be part of the control group, patients should not have any evidence of oral lesions. The exclusion criteria for all the groups are as follows: a history of radiation therapy in the head and neck areas; chronic thyroid disease; known Sjogren's disease; those who had prior surgery of the salivary glands; contact allergies; pregnant women; and individuals who currently use antibiotics, corticosteroids, or antifungals. The diagnoses corresponding to the selected blocks of participants post biopsy were confirmed by a professional with experience in oral pathology (S.B.C.T). OPMD was histologically classified according to the binary classification system of oral epithelial dysplasia.[3] The OSCC staging was performed considering the following three criteria: T (size of the primary tumor), N (spread of the disease to regional lymph nodes), and M (presence of metastasis).[13] Data related to disease and systemic disorders and drug use were obtained from questionnaires. Smoking and alcohol habits were recorded. Individuals were classified as smokers if they reported to have smoked more than 100 cigarettes in their lifetime and who currently smoke every day.[12] The Eleventh Revision of the International Classification of Diseases (ICD-11) was used as the criteria for defining alcohol habits.[11]

Salivary collection

Saliva was collected from patients who gave written informed consent prior to the biopsy, and salivary electrolyte evaluation was performed only after confirming the histological diagnoses of OSCC, OPMD, and normal tissues. Previously trained dentists collected unstimulated saliva from patients in a 50 mL pre-weighed Falcon® centrifuge tube. Unstimulated saliva flow rate (uSRF) was collected between 9 am and 11 am from patients who have not eaten, smoked, or exercised any oral hygiene 90 min before the procedure.[13] Saliva collection was performed between 9 am and 11 am for five minutes. Subsequently, the tubes with saliva were kept in a container at 5 °C for transport to the laboratory of DCOD/UFPel. Each tube was later weighed through gravimetry. A specific weight of 1.005 g/mL was assigned to the fluid, and the calculated total volume was expressed in milliliter per minute to determine uSFR. Saliva was centrifuged at 2500 rpm for 10 min at −5 °C, and supernatants were stored frozen at −80 °C until the biochemical analysis. The same salivary collection procedure was repeated six months after the initial diagnosis in patients with OSCC. All saliva samples were only used for the purposes of this investigation. After laboratory analysis, the samples were discarded according to resolution CNS 441 of 2011.[14]

Salivary biochemical analytical examination

Salivary electrolyte concentration was determined using an inductively coupled plasma optical emission spectrometer (Spectro CIROS CCD, Spectro Analytical Instruments, Kleve, Germany), equipped with a cross flow nebulizer coupled to a double pass scott type spray chamber. Instrumental performance was optimized following the instructions of the manufacturer and previous work published in the literature.[15] The equipment was externally calibrated using K, P, Na, Ca, Mg, Zn, Cu, and Fe (1.0–10000 μg L^{-1}) reference solutions, which were prepared by diluting a stock solution (1000 mg L^{-1}, Merck) in 5% HNO3. The same stock solution was used in the recovery tests for evaluating the accuracy of the determination step. Argon 99.996% (White Martins, São Paulo, Brazil) was used for plasma generation, nebulization, and as auxiliary gas. For the elemental determination, 100 μL of saliva supernatant was diluted 80 times prior to the sample introduction into the equipment. This dilution factor was previously optimized to minimize interferences during the determination step. Elemental concentration in saliva was determined by inductively

coupled plasma optical emission spectrometry. The wavelengths selected in the determination step were 589.592 nm for Ca, 766.490 nm for K, 317.933 nm for Ca, 324.752 nm for Cu, 238.204 nm for Fe, 285.213 nm for Mg, 213.857 nm for Zn, and 214.914 nm for P. The results were relatively expressed as µg of the elemental per mL of saliva. Limit of quantification was calculated from the mean of the curve blank values plus 10 times the standard deviation obtained for 10 replicates of the curve blank.

Data analysis

Descriptive and quantitative data analyses were performed using the Statistical Package for the Social Sciences for Windows 22.0 (SPSS, Inc., Chicago, IL, USA). To determine whether the variables have a normal distribution, the Shapiro Wilk test was applied. Kruskall Wallis test was conducted for the comparison of salivary electrolyte concentrations and uSFR among groups and between sex habits and type of therapy received. A $p < 0.05$ was interpreted as significant, and the level in confidence intervals was 95%.

Results

Phase 1

A total of 881 references were identified in the three electronic databases. Two references were identified through other sources. After the removal of 51 duplicates, 832 titles/abstracts were read. From those primarily elected, 26 articles met the eligibility criteria and were included. After a thorough reading of these articles and the exclusion of those that contained insufficient information, 10 full texts were finally retrieved for analysis. The flow chart of the study is presented in **Figure 1**.

All articles were published in English, between 2002 and 2018. Considering the 10 elected articles, 364 OSCC, 305 OPMD, and 260 control cases were evaluated. All the articles evaluated salivary electrolytes in patients with OSCC compared with a control group. Specifically, one article evaluated salivary electrolytes in patients with OSCC and those who received different types of therapies after diagnosis. Six articles evaluated patients with OPMD. According to the articles that specified the sex of evaluated patients, from the 197 OSCC cases, 130 were men (66%), and 67 were women (34%); from the 30 OPMD cases, 19 were

men (63.3%), and 11 were women (36.7%); from the 100 control cases, 59 were men (59%), and 41 were women (41%). The average age established according to the articles that indicated this parameter was 60.3 (35–72) for OSCC, 50.2 (23–64) for OPMD, and 48.5 (37–70) for control patients. Four articles specified the site of the lesions. The sites mostly affected by OSCC and OPMD were tongue with 55% and 25% cases, respectively. Five articles considered the habits of tobacco and/or alcohol. The percentages of patients with these habits varied between 12.5% and 76.5% for tobacco and between 0% and 13.5% for alcohol in OSCC patients. The only article that made reference to habits in patients with OPMD indicated that 100% smoked and nobody (0%) drank.

Considering salivary electrolyte levels, one article indicated a decrease and three an increase in the Na index in patients with OSCC and OPMD (when compared with their control group). Another article revealed low salivary of this electrolyte levels after six weeks of radiotherapy in patients with OSCC. Two articles indicated a decrease in salivary K levels in OSCC patients, and another reported an increase in this electrolyte after six weeks of radiation. Two articles presented high salivary Mg levels in patients with OSCC, and one study indicated low Mg levels in patients with OSCC and OPMD. One article reported that Zn salivary levels are low in patients with OPMD and OSCC. Another study revealed that such levels are higher in patients with OPMD and OSCC than the control group. All information about the characteristics of the included studies is presented in **Table 1**.

The risk of bias analysis revealed that the main problem of the studied articles is the identification and handling of the confounding factors among cases and the control to interpret the results. Eight studies were classified as high methodological quality, and two as moderate methodological quality. Exposure measures and their form of assessment had little risk of bias. **Supplementary Appendix A2** presents the risk of bias of the selected studies by JBI-MAStARI.

Phase 2

Sociodemographic data

Fifty-four patients participated in this study 27 men (50%) and 27 women (50%). Participants' age varied between 31 and 89 years. The mean age was 56.3 years (±14.6); 42.2 (±16.6) in the control group, 58.2 (±13.9) in the OPMD group, and

60.1 (±12.6) in the OSCC group. Twenty-four (44.4%) were smokers: four (22.2%) from the control group, six (33%) from the OPMD group, and 14 (77%) from the OSCC group. Twenty-two (40.7%) were social alcoholics: eight (39%) from the control group, seven (39%) from the OPMD group, and seven (39%) from the OSCC group. The most frequent site of lesions in patients with OSCC was the lateral border of the tongue with nine (50%) cases, followed by soft palate with four (22.2%) cases. The most frequent site of lesions in patients with OPMD was the buccal mucosa with nine (50%) cases, followed by the lateral border of the tongue with five (27.8%) cases. Regarding the OPMD cases, 16 (88.9%) were clinically leukoplakias, and two (11.1%) were erythroleukoplakias. Ten (55.6%) were classified as low risk dysplasia and eight (44.4%) as high-risk dysplasia. Considering the OSCC cases, according to the TNM classification of OSCC, five (27.7%) patients were classified as T1N0, eight (44.4%) as T2N0, one (5.6%) as T1N1, one (5.6%) as T2N1, one (5.6%) as T2N2, and two (11.2%) as T4N2. Regarding the type of treatment performed, 12 patients (66.7%) were treated with surgery alone, and six (33.3%) with surgery in addition to chemotherapy and/or radiotherapy. The baseline characterization of the study population is summarized in **Table 2.** The uSFR averages were 0.645 mL/min for the control group, 0.514 mL/min for OPMD, 0.541 mL/min for OSCC, and 0.454 mL/min for OSCC after therapy, without significant statistical differences ($p > 0.05$).

Salivary electrolytes

Seventy-two salivary samples were analyzed, one from each control and OPMD patients and two from patients with OSCC (before and after therapy). A highly significant increase ($p = 0.041$) was found in the salivary Mg levels in the OPMD group. Details of the mean, SD, and median of all evaluated salivary electrolyte levels in evaluated groups are shown in **Table 3 and Figure 2**. No differences were observed for the levels of any electrolyte when comparing sex, habits and type of therapy received ($p > 0.05$).

Discussion

Many metabolic disorders, oral precancerous conditions, and oral cancers are accompanied by alterations in the concentration of one or more trace elements, being their identification helpful not only in the establishment of its early diagnosis and treatment but also in the prognosis and in the understanding of these diseases' progress.[16]

K, Na, Ca, Cl, and P are the most concentrated biochemical elements in saliva due to their importance in the electrolytic balance of this fluid. In our study, and agreeing with the referred data,[17] K, Na, Ca, and P were the most concentrated electrolytes in all the evaluated groups. In sociodemographic and habits terms, the present study agrees with evidence that indicates that there would be no differences regarding electrolytes salivary levels according sex[18] and the smoking and alcohol habits.[19] In relation to the latter, a decrease in Na levels associated with acute[20] and non-social consumption (reported by the participants in this study) of alcohol was described. Differences in electrolytes salivary levels could be determined by another factors such as oral health. Mg was the only electrolyte that showed different salivary levels among the studied groups. Its salivary concentration in the OPMD group was higher than in the other groups. Mg is a mineral required for a wide variety of physiological functions and biological activities, such as activation or inhibition of enzymes and regulation of cellular proliferation, progression, and differentiation.[21] Currently, much attention is paid to the role of Mg in neoplastic biology because of its involvement in processes such as high proliferation rate, low cell death rates, de-differentiation, and invasion. This scenario becomes complex if Mg is considered to affect the various stages of tumorigenesis and to be related to certain tissue changes, such as neoangiogenesis. Moreover, the availability of Mg may change in response to treatment modalities such as chemotherapy.[7] In the evaluation of Mg concentration and its relationship with different kinds of cancer, factors such as the presence of this electrolyte in foods in the daily diet should be considered. In this regard, studies have generally demonstrated the beneficial effects of Mg intake in relation to its effects and cancer, such as an inverse relationship between high Mg levels in drinking water and less chance of dying because of breast cancer[22] or ovarian cancer.[23] However, another evidence indicates that no association exists between the risk developing bladder cancer[24] or gastric cancer[25] and the total dietary caloric intake of Mg.

The evidence of salivary and serum Mg levels and their relationship with OSCC development remains scarce. Sample reports are that reduced Mg levels in serum may be related to greater progression of head and neck cancers.[26] If the findings of the present study on salivary Mg levels are compared with the systematic review, the evidence is contradictory: Shpitzer et al. (2007)[27] and Carausu et al.

(2016)[28] indicated that salivary Mg levels are higher in patients with OSCC or OPMD than those in the control group. By contrast, Aziz et al. (2018)[29] showed that Mg levels are low in the plasma and saliva of OSCC patients. Shpitzer et al.[27] and Aziz et al.[29] established that salivary and serum Mg levels are equivalents and can serve as biomarkers of the carcinogenesis process. Therefore, the real role of Mg in OSCC tumor cells comes in discussion. A 2018 finding provided interesting explanations about the increasement observed in the salivary Mg levels in patients with OPMD. This study showed that salivary Mg levels in individuals with nasopharyngeal carcinoma were significantly higher than in healthy individuals. This finding is correlated with the fact that the transient potential of the melastatin receptor, which is a member of subfamily 7 (TRPM7), an important Mg transporter, is overexpressed in tumor tissues. The authors concluded that the salivary Mg level within a certain range can act as a risk factor for the progression of tumorigenic activities in head and neck carcinoma.[30]

The OSCC may influence the availability of plasma Mg, which has a modulating effect on angiogenesis, facilitating and enhancing the process.[21] By contrast, OSCC can also inhibit endothelial cell migration and proliferation, which is sensitive to extracellular Mg concentrations.[31] Previous studies revealed that Mg enhances angiogenesis (consequently collaborating with tumor growth and metastasization) by increasing nitric oxide and vascular endothelial growth factor production.[32] In animals submitted to a low Mg diet, a return to normal diet induces an almost explosive tumoral growth that can be explained, at least in part, by a potentiation of angiogenesis.[33] Regarding the treatment of OSCC, chemotherapy may induce Mg deprivation, reducing the portion of growing tumor cells.[21] However, such an evaluation was impossible to conduct in the present study because the percentage of patients with OSCC who received chemotherapy was very low.

The salivary levels of other trace elements evaluated in this study showed no significant differences among the evaluated groups. Another research reported the same result, indicating that Na, K, Ca, Fe, P salivary levels and even Mg levels were not useful as biomarkers in the diagnosis of OSCC8. Nevertheless, an evidence claimed high Fe serum levels[34] or high Cu salivary levels in patients with OSCC.[35] According to the present systematic review, Zn salivary levels in patients with OPMD and OSCC can decrease[21] or contrastly be higher in patients with OSCC and OPMD

than in patients in the control group.[36] Zn, due to its function in extracellular matrix metalloproteinases, can be associated with tumorigenesis and show different salivary or serum levels in OSCC patients.[37] Finally, although the present study could not find any differences, in another study suggested that serum Zn levels can be used as biomarkers in OSCC.[38] indicating that the present study must be improved. According to the present systematic review, high concentrations of Na salivary levels in patients with OSCC are reported in certain articles,[27,39] that could reflect dehydration dehydration due to smoking and alcohol consumption. Another article indicated low salivary Na levels after six weeks of radiotherapy in patients with OSCC; this phenomenon may be a consequence of the demineralization related to this kind of therapy.[40] Another evidence revealed that after radiotherapy, OSCC patients had low uSRF.[41] However, the present study did not find any differences among different groups regarding uSRF nor regarding the levels of any electrolyte. This result can be explained by the low percentage of people who received radiotherapy (which causes a decrease in salivary flow) and the different times of exposure to that therapy among different volunteers. The decrease in salivary flow is reported in patients receiving radiotherapy, but no differences in this aspect were found between patients with and without OSCC,[42] thus agreeing with the results of the present study.

According to our results, Mg levels were higher in the saliva of people with OPMD. The reason for this observation needs further explanation, but one hypothesis can be raised, that is, the increased angiogenesis in OPDM can be related to the high Mg levels, which did not continue to increase in OSCC because this electrolyte can be an initial stimulus for the endothelial proliferation in cancer progression. Factors other than Mg levels can also be involved in the maintenance of the neovascularization observed in OSCC. Further studies are necessary to confirm this theory. An analysis of the association between the Mg levels in different body fluids with the microvessel density in OPDM, OSCC, and control groups is also suggested.

Certain topics have been pointed as limitations of the present study. For example, the electrolyte levels analyzed in the blood and saliva can be simultaneously investigated to establish an association between them and suggest the use of saliva as a source of biomarkers; these possibilities were indicated in our

systematic review,[28] but impossible to perform in the case control study. Further research complemented with in vivo studies must be conducted to assess the local neoplastic behavior of OSCC, according to different concentrations of salivary electrolytes. Despite these limitations, the present study is the first attempt to evaluate the salivary electrolyte concentrations in OSCC before and after treatment. This research also suggests that Mg can be an electrolyte that is important in OPMD and probably in its progress to cancer. Moreover, the lack of solid data in the literature reinforces the importance of deepening the investigations about this subject. Further studies can provide additional information about the role of Mg or other electrolytes in OSCC or cancer progression. The use of salivary electrolytes as biomarkers of the OSCC could be useful in the early detection of the disease or as a tool for evaluating the effect of current therapies in oral cancer.

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Figure 1.

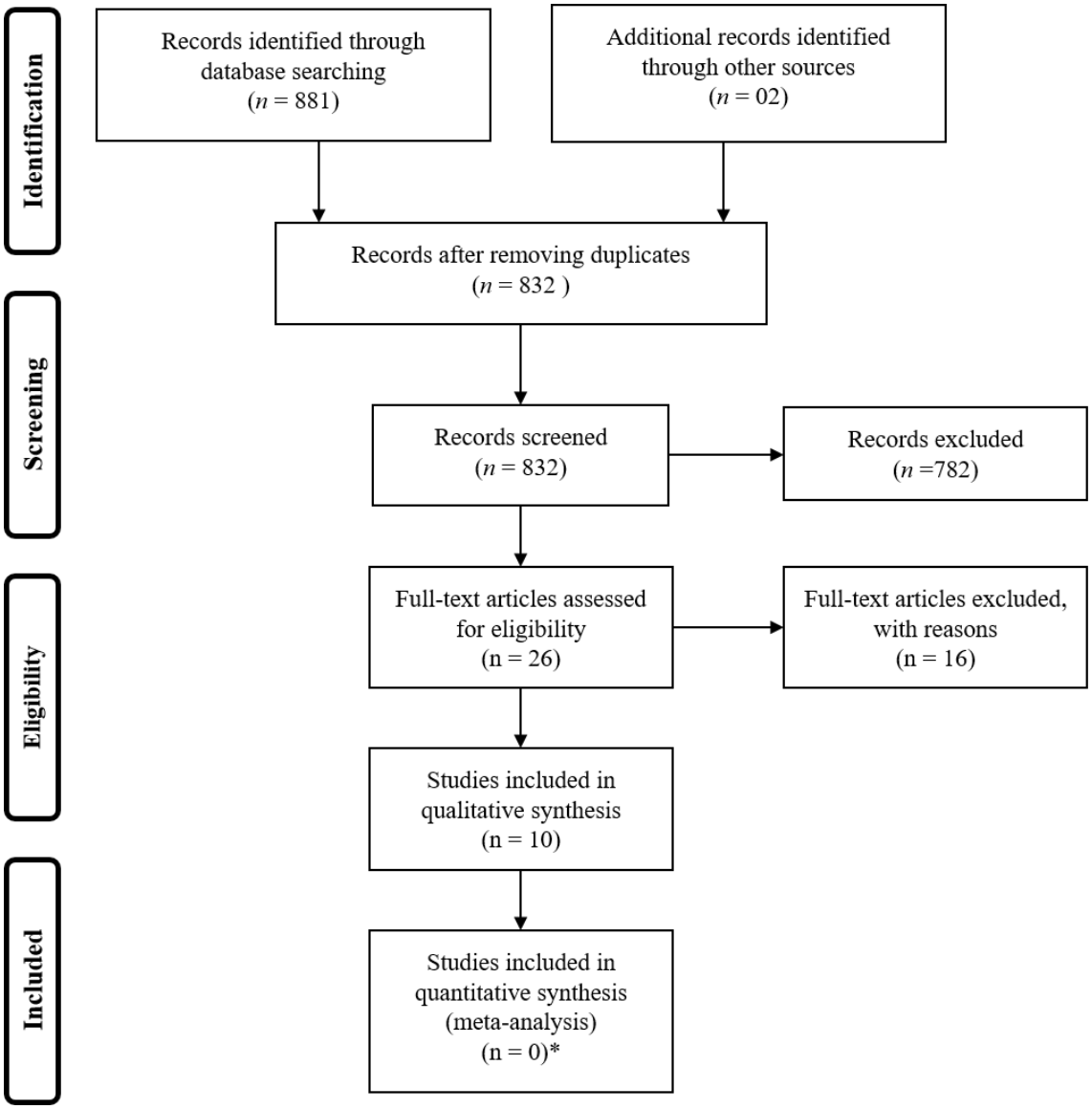


Figure 1. Search flowchart according to the PRISMA Statement

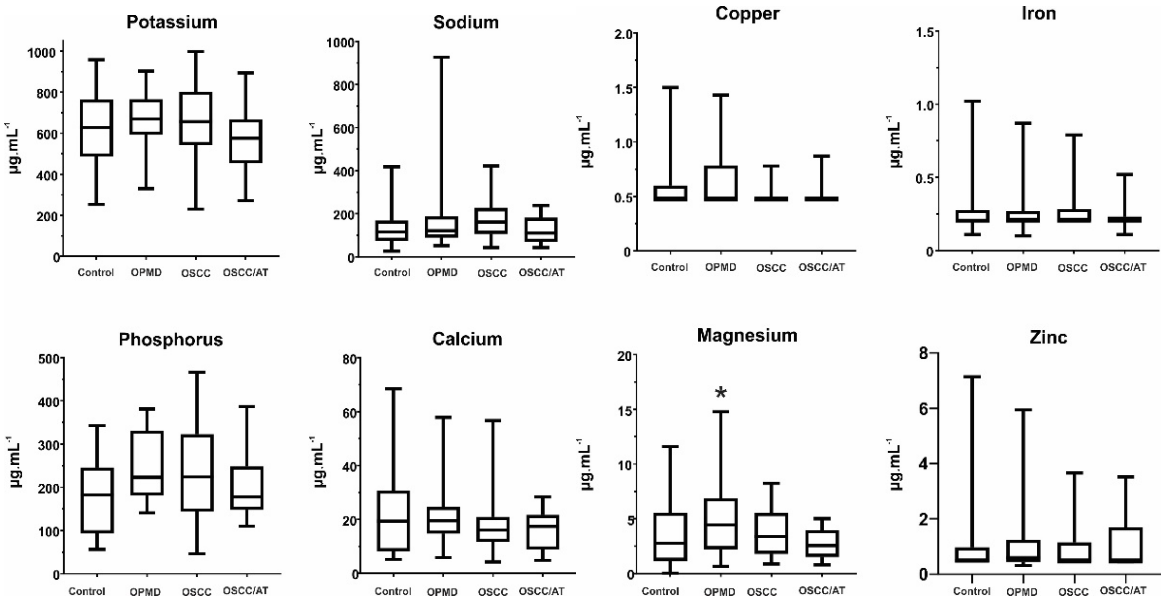


Figure 2. OPMD: Oral potentially malignant disorders; OSCC: Oral squamous cell carcinoma; OSCC/AT: After therapy; *: Statistically significant

Table 1. Review of previous studies associating the use of salivary electrolytes as biomarkers or progression predictors and/or responses associated with OSCC

Study	Method and Patient	Sex | Age		Site	Habit		>	<
		Male: Female	Range		Smoke	Alcohol		
Girja KP et al. (2002) [19] - India	1) 15 Control	10:5	(40−65)	N/S	0 (0%)	0 (0%)	N/S	Na and K in OSCC and OPMD
	2) 15 OPMD	9:6	(40−64)	N/S	15 (100%)	0 (0%)		
	3) 15 OSCC	12:3	(45−65)	Bucal Mucosa: 13 86.7%) Tongue: 2 (13.3%)	15 (100%)	0 (0%)		
Shpitzer T et al. (2007) [20] - Israel	1) 25 Control	N/S	Median 68 ± 17	N/S	2 (8%)	0 (0%)	Na, Ca, P, and Mg in OSCC	K in OSCC
	2) 25 OSCC	12:13	(30−86) Median (50 ± 15)	T: 25 (100%)	N/S	N/S		
Chitra S et al. (2008) [21] - India	89 OSCC divided into three groups treated with:						K in six weeks of radiation1),	Na in six weeks of radiation
	1) three weeks of radiation	54:35	Median (50 ± 15)	Bucal Mucosa: 28 (31.5%) Alveolus: 19 (17.1%) Tongue: 13 (14.6%) Floor of the mouth: 11 (12.4%) Retromolar trigone: 8 (9%), 5 (5.6%)	Chewing tobacco: 48 (53.9%), Smoke: 29 (32.6%)	N/S		
	2) six weeks of radiation	N/S	N/S	N/S	N/S	N/S		
	3) three weeks of radiation + tocopherol	N/S	N/S	N/S	N/S	N/S		
Fuchs PN et al. (2011) [22] - Croatia	1) 24 OSCC	20:4	(60 ± 2.5)	Tongue: 4 (16.7%) Sublingual area: 61 (66.6%) Soft palate: 4 (16.7%)	21 (12.5%)	N/S	Na and Cl in OSCC	N/S
	2) 24 Control	9:15	(24 ± 3.7)	N/S	9 (37.5%)	N/S		
Ayinampudi BK et al. (2012) [23] - India	1) Six Control	3:3	(38−52) Median (45 ± 5.1)	N/S	0 (0%)	0 (0%)	Cu and Zn in OPMD and OSCC	N/S
	2) 20 OMPD	15:5	(23−62) Median (45 ± 12.1)	N/S	N/S	N/S		
	3) 10 OSCC	5:5	(35−65) Median (52 ± 11.5)	N/S	N/S	N/S		

Table 1 (Cont). Review of previous studies associating the use of salivary electrolytes as biomarkers or progression predictors and/or responses associated with OSCC

Study	Method and Patient	Sex | Age		Site	Habit		>	<
		Male: Female	Range		Smoke	Alcohol		
Dziewulska A et al. (2013) [9] - Polonia	1) 30 Control	25:5	(37−70)	N/S	Smoke or Ex smoke: 17 (56.7%)	N/S	Na in OSCC	N/S
	2) 34 OSCC	27:7	(35−72)	T: 11 (32.4%) Floor of the mouth: 8 (23.5%) Tonsil: 6 (17.6%) Others: 9 (26.5%)	Smoke or Ex smoke: 26 (76.5%)	N/S		
Shetty SR et al. (2014) [24] - India	1) 65 Control	Unspecified sex distribution (20−60)		N/S	N/S	N/S	Zn OSCC and OPMD	
	2) 115 OMPD	N/S		N/S	N/S	N/S		
	3) 50 OSCC	N/S		N/S	N/S	N/S		
Shetty SR et al. (2015) [8] - India	1) 50 Control	Unspecified sex and age distribution. Matched in age and gender.		N/S	N/S	N/S	Cu in OPMD and OSCC	Zn in OPMD and OSCC
	2) 100 OMPD	N/S		N/S	N/S	N/S		
	3) 50 OSCC	N/S		N/S	N/S	N/S		
		Unspecified sex distribution:						
Carausu E et al. (2016) [25] - Romania	1) 28 Control	N/S	51.9 (± 17.1)	N/S	N/S	N/S		
	2) 43 OPMD	N/S	52.1 (± 15.5)	N/S	N/S	N/S	Mg in OSCC and OPMD	-
	3) 35 OSCC	N/S	55 (± 16.3)	N/S	N/S	N/S		
Aziz NZ et al. (2018) [26] - India	1) 17 Control	Unspecified sex and age distribution		N/S	N/S	N/S		Mg in OSCC -
	2) 17 OPMD	N/S	N/S	N/S	N/S	N/S		
	3) 17 OSCC	N/S	N/S	N/S	N/S	N/S		

Table 1. OSCC: oral squamous cell carcinoma; OPMD: oral potentially malignant disorders; oral submucous fibrosis; OLP: oral lichen planus; OL: oral leukoplakia; K: potassium; P: phosphorus, Na: sodium, Ca: calcium, Mg: magnesium, Zn: zinc, Cu: copper, Fe: iron. Cl: Chloride; > Higher salivary levels of this element with statistically significant differences than the control group or than the group indicated; <: lower salivary levels of this element with statistically significant differences than the control group or than the group indicated.
N/S: Not specified

Table 2. Baseline characterization of the study population

Group	Gender male: female	Age (average, standard deviation [SD])	Site	Dysplasia classification	Habit		TNM**	Type of therapy	uSRF* (average, SD) (mL/min)
					Smoke	Alcohol			
Control (*n* = 18)	6:12	49.2 (± 16.6)	N/A	N/A	4	8	N/A	N/A	0.645 (± 0.31)
OPMD (*n* = 18)	7:11	58.2 (± 13.9)	Buccal mucosa: 9 Tongue: 5 Alveolar ridge Mucosa: 2 Palate: 2	Low risk: 10 High risk: 8	6	7	N/A	N/A	0.514 (± 0.12)
OSCC (*n* = 18)	14:4	60.1 (± 12.6)	Tongue: 9 Soft palate: 4 Retromolar trigone: 3 Floor of the mouth: 2	N/A	14	7	T1N0: 5 T2N0: 8 T1N1: 1 T2N1: 1 T2N2: 1 T4 N2: 2	N/A	0.541 (± 0.29)
OSCC/AT (*n* = 18)	S/I	S/I	S/I	N/A	S/I	S/I		Surgery: 12 Surgery + Chemotherapy /Radiotherapy: 6	0.454 (± 0.31)

Table 2: OSCC: oral squamous cell carcinoma; OSCC/AT: oral squamous cell carcinoma after therapy; OPMD: oral potentially malignant disorders; uSFR*: unstimulated salivary flow rate; ** TNM: T (size of the primary tumor), N (spread of the disease to regional lymph nodes), and M (presence of metastasis); N/A: Not applicable; S/I: Same information with the OSCC group.

Table 3. Mean and SD of salivary electrolyte levels in patients without oral lesions, with OPMD, and with OSCC before and after therapy

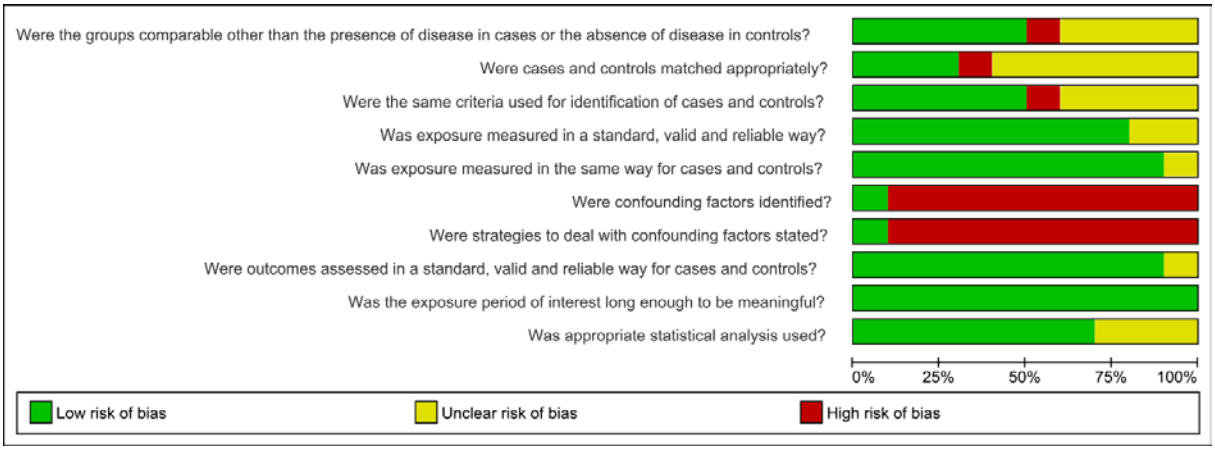
Group	K $\mu g\ L^{-1}$	P $\mu g\ L^{-1}$	Na $\mu g\ L^{-1}$	Ca $\mu g\ L^{-1}$	Mg $\mu g\ L^{-1}$	Zn $\mu g\ L^{-1}$	Cu $\mu g\ L^{-1}$	Fe $\mu g\ L^{-1}$
Control (*n* =18)	612.6 (± 192.2)	186.7 (± 91.1)	135.4 (± 95.6)	21.8 (± 16.8)	3.51 (± 3.0)	1.15 (± 1.63)	0.61 (± 0.29)	0.27 (± 0.21)
OPMD (*n* =18)	661.8 (± 158.9)	248.3 (± 82.2)	183.8 (± 202.8)	22.9 (± 14.2)	5.41 (± 4.1)	1.11 (± 1.34)	0.64 (± 0.27)	0.26 (± 0.17)
OSCC (*n* =18)	651.3 (± 213.4)	237.2 (±109.6)	171.9 (± 92.6)	17.5 (± 11.5)	3.71 (±2.3)	0.87 (± 0.82)	0.51 (± 0.82)	0.26 (± 0.15)
OSCC/AT (*n* =18)	573.3 (± 161.7)	199.3 (± 76.7)	127.8 (± 63.1)	16.5 (± 7.2)	2.71 (± 1.3)	1.04 (± 0.97)	0.52 (± 0.11)	0.23 (± 0.95)
P-value	0.459	0.136	0.469	0.359	**0.041***	0.917	0.132	0.908

Table 3: K: Potassium; P: Phosphorus; Na: Sodium; Ca: Calcium; Mg: Magnesium; ZN: Zinc; Cu: cooper; Mg, Fe: Iron; OPMD: Oral potentially malignant disorders; OSCC: Oral squamous cell carcinoma; AT: After therapy; *: Statistically significant

Supplementary Appendix 1: Search strategy

Database	Keywords used	Number of articles found
PubMed	(((“Cancer of Mouth” OR “Mouth Cancers” OR “Oral Cancer” OR “Cancer, Oral” OR “Cancers, Oral” OR “Oral Cancers” OR “Cancer of the Mouth” OR “Mouth Cancer” OR “Cancer, Mouth” OR “Cancers, Mouth” OR “Squamous Cell Carcinoma of the Head and Neck” OR “Squamous Cell Carcinoma, Head and Neck” OR “Carcinoma, Squamous Cell of Head and Neck” OR “Head and Neck Squamous Cell Carcinoma” OR “Condition, Precancerous” OR “Conditions, Precancerous” OR “Precancerous Condition” OR “Condition, Preneoplastic” OR “Preneoplastic Condition” OR “Preneoplastic Conditions” OR “Conditions, Preneoplastic” OR “Leukoplakias, Oral” OR “Oral Leukoplakia” OR “Oral Leukoplakias”)) AND (“Trace Element” OR “Element, Trace” OR “Elements, Trace” OR “Biometals” OR “Biometal” OR “Trace Minerals” OR “Mineral, Trace” OR “Minerals, Trace” OR “Trace Mineral” OR “Electrolytes” OR “Ions” OR “Anions +” OR “Cations +” OR “Polyelectrolites” OR “Phosphorus” OR “Sodium” OR “Calcium” OR “Magnesium” OR “Zinc” OR “Cooper” OR ”Iron”)) AND (“Saliva” OR “Salivas”)	95
Scopus	(“Cancer of Mouth” OR “Mouth Cancers” OR “Oral Cancer” OR “Cancer, Oral” OR “Cancers, Oral” OR “Oral Cancers” OR “Cancer of the Mouth” OR “Mouth Cancer” OR “Cancer, Mouth” OR “Cancers, Mouth” OR “Squamous Cell Carcinoma of the Head and Neck” OR “Squamous Cell Carcinoma, Head and Neck” OR “Carcinoma, Squamous Cell of Head and Neck” OR “Head and Neck Squamous Cell Carcinoma” OR “Condition, Precancerous” OR “Conditions, Precancerous” OR “Precancerous Condition” OR “Condition, Preneoplastic” OR “Preneoplastic Condition” OR “Preneoplastic Conditions” OR “Conditions, Preneoplastic” OR “Leukoplakias, Oral” OR “Oral Leukoplakia” OR “Oral Leukoplakias”) AND (“Trace Element” OR “Element, Trace” OR “Elements, Trace” OR “Biometals” OR “Biometal” OR “Trace Minerals” OR “Mineral, Trace” OR “Minerals, Trace” OR “Trace Mineral” OR “Electrolytes” OR “Ions” OR “Anions +” OR “Cations +” OR “Polyelectrolites” OR “Phosphorus” OR “Sodium” OR “Calcium” OR “Magnesium” OR “Zinc” OR “Cooper” OR ”Iron”) AND (“Saliva” OR “Salivas”)	741
Web of Science	TS=(“Cancer of Mouth” OR “Mouth Cancers” OR “Oral Cancer” OR “Cancer, Oral” OR “Cancers, Oral” OR “Oral Cancers” OR “Cancer of the Mouth” OR “Mouth Cancer” OR “Cancer, Mouth” OR “Cancers, Mouth” OR “Squamous Cell Carcinoma of the Head and Neck” OR “Squamous Cell Carcinoma, Head and Neck” OR “Carcinoma, Squamous Cell of Head and Neck” OR “Head and Neck Squamous Cell Carcinoma” OR “Condition, Precancerous” OR “Conditions, Precancerous” OR “Precancerous Condition” OR “Condition, Preneoplastic” OR “Preneoplastic Condition” OR “Preneoplastic Conditions” OR “Conditions, Preneoplastic” OR “Leukoplakias, Oral” OR “Oral Leukoplakia” OR “Oral Leukoplakias” AND TS=(“Trace Element” OR “Element, Trace” OR “Elements, Trace” OR “Biometals” OR “Biometal” OR “Trace Minerals” OR “Mineral, Trace” OR “Minerals, Trace” OR “Trace Mineral” OR “Electrolytes” OR “Ions” OR “Anions +” OR “Cations +” OR “Polyelectrolites” OR “Phosphorus” OR “Sodium” OR “Calcium” OR “Magnesium” OR “Zinc” OR “Cooper” OR ”Iron” AND TS= ("Saliva" OR “Salivas”)	45

Supplementary Appendix 2: Assessment of risk of bias



5 Artigo 2

Can the immunohistochemical and salivary expressions of FAT1 be used as a biomarker of oral squamous cell carcinoma?[2]

Juan Aitken-Saavedra[1,2], Ana Carolina Uchoa Vasconcellos[3], Wellington Luiz De Oliveira da Rosa[4], Carlos Enrique Cuevas-Suarez[5], Laylla Galdino dos Santos[6], Ricardo Ramires-Fernandez[7], Sandra Beatriz Chaves Tarquinio[8]

1 Graduate program in dentistry. Federal University of Pelotas. Pelotas, Brazil

2 Department of Oral Pathology and Medicine, School of Dentistry, University of Chile, Santiago, Chile

3 Center of Diagnosis of Diseases of the Mouth, Federal University of Pelotas. Pelotas, Brazil

4 Department of Restorative Dentistry, School of Dentistry, Federal University of Pelotas, Pelotas, RS, Brazil

5 Dental Materials Laboratory, Autonomous University of Hidalgo State, Pachuca, México

6 Graduation student in Dentistry. Federal University of Pelotas. Pelotas, Brazil

7 School of Dentistry, Universidad Mayor, Santiago, Chile

8 Center of Diagnosis of Diseases of the Mouth, Federal University of Pelotas. Pelotas, Brazil

Corresponding author.

Sandra Beatriz Chaves Tarquinio

Gonçalves Chaves Street, 457 / Rm 607

Centro District – Zipcode: 96015-560

Pelotas, RS, Brazil

Telephone number: +55 53981112894

Email: sbtarquinio@gmail.com

[2] Artigo formatado segundo as normas do periódico Oral Oncology.

Abstract

Objectives: To evaluate use of Cadherin-Related Tumor Suppressor Homolog (FAT1) levels in tissues and saliva as biomarkers of progression and response to therapies in oral squamous cell carcinoma (OSCC). **Materials and Methods:** Unstimulated salivary flow rate (uSRF) was collected from 54 patients: 18 with OSCC (also evaluated 6 months after therapy), 18 with OPMD and 18 without oral lesions.). Native FAT1 salivary levels were determined by enzyme-linked immunosorbent assay (ELISA) and associated with the FAT1 staining (determined by immunohistochemistry from their respective biopsies and analyzed by semi-quantitative and computerized analysis). FAT1 staining were also determined in another 72 samples (of healthy tissue, high and low risk dysplasias and OSCC). Kruskal-Wallis and Mann-Whitney tests were used for comparisons and the Spearman test for associations between variables. $P < 0.05$ was statistically significant. **Results:** Semi-quantitative analysis of FAT1 stained indicated that, 2 samples in the control group (11.1%), 3 in the low-risk dysplasia group (16.7%), 5 in the high-risk dysplasia group (27.8%), and 10 in the OSCC group (55.6%) had a score of 4 (stain> 50). A strong correlation was observed between computerized and semi-quantitative evaluation of FAT1 stained ($Rho2=0,73$; $p < 0.001$). Control patients' saliva showed higher levels of FAT1 compared to patients with OPMD and OSCC before and after therapy (0.168, 0.162 0.154 and 0.153 nm / mL respectively; $p < 0.05$). A weak negative correlation was observed between the salivary and tissue levels of FAT1. **Conclusion:** Use of native FAT1 as a salivary marker of progression or response to therapy in OSCC is not recommended. The cytoplasmic immunostaining of FAT1 showed in tumor cells in present study suggests its aberrant phenotype and could be associated with OSCC characteristics such as aggressiveness.

Key words: Oral cancer; Biomarkers; Saliva; FAT1; Potentially malignant lesions;

Introduction

Oral Squamous Cell Carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity. Despite several studies on OSCC, the 5 years-survival rate of this neoplasm are still around 50%, because their aggressive and invasive behavior [1,2]. Oral potentially malignant disorders (OPMD) lesions or precursor lesions of OSCC, are tissue alterations that may assume the character of a malignant tumor at any time. Early detection and control of potentially malignant lesions is essential to avoid progression to OSCC [3,4].

Several studies have been performed with the objective of investigating the first changes involved in cancer initiation and progress [3,4,5]. In this context, the sequencing of the OSCC genome provided a greater understanding of the genetic complexity and evolutionary biology of tumor cells and their development. It has been established that the mutated genes most commonly associated with OSCC are *CASP8, FAT1, NOTCH1, TP53, MLL4 and USP9X* [5]. The identification of these mutations and their association with survival and habits in patients with OSCC opens the possibility of redirecting antitumor therapies [5]. Among the proteins encoded by these genes, the identification of FAT1 deserves particular attention, due to its secretory nature, which can be analyzed in different body fluids as biomarker of several pathologies [6]. Cadherin-Related Tumor Suppressor Homolog (*FAT1*) gene is a tumor suppressor in humans, encode a very large transmembrane cadherin protein [7] and have the pivotal role in cell morphogenesis and migration. When mutated, FAT1 is unable to inhibit WNT / β-catenin signaling, causing an increasement in proliferation, accelerating cell migration and invasion, decrease in cell adhesive strength in OSCC and finally, lost their function of tumor progression suppression [8-11].

Secretory proteins such as FAT1 generate a favorable environment in the extracellular matrix, essential for the development of many types of cancer and they can act as serum or salivary biomarkers, being useful, as a complementary method for diagnosis and prognosis of some neoplasms [12,13,14]. In hepatocellular cancer for example, it has been shown that protein FAT1 is a helpful serological biomarker for diagnosis and prognosis in this neoplasm [6]. The protein expression of FAT1 could also be investigated as a marker in other cancers with mutations in FAT1 gene, as OSCC. Considering the fact that FAT1 is secreted by cells and that most of the proteins present in the blood are also expressed in the saliva [15], it would be very valuable to

determine salivary expression of FAT1 in both pre-malignant stages and in OSCC, before and after therapy, aiming to assess its use as a potential salivary biomarker in this cancer. Saliva collection is a low-invasive, less expensive method, with the possibility of evaluating large volumes of fluid and several times a day, as well as it can be a potential source of biomarkers [16]. Despite their secretory protein nature, FAT1, has not been evaluated in saliva as a biomarker for OSCC until nowadays, and its tissues expression must be evaluated. The aim of our study was to evaluate the potential use of FAT1 as a biomarker of OSCC progression by associating its levels in saliva with tissue expression in the same patients.

Materials and Methods

The present study was approved by the Research Ethics Committee of the National Commission (approval code: No. 2.262.681) and was conducted in accordance with the guidelines of the Declaration of Helsinki. The sample size calculation was performed based on articles that indicate the percentage of *FAT1* mutations performed by sequencing in patients with OSCC [17,18]. For the level of confidence of 95% and power of 80%, being necessary 18 individuals in each group. This study comprised two phases, whose study design, sampling, and setting are described below:

Phase 1

It was performed an evaluation and comparison of the immunohistochemical expression of FAT1 among 18 samples of healthy tissues (obtained from patients with indication of removing impacted third molars and without clinical or histological evidence of inflammation), 18 of low-risk dysplasias, 18 high-risk dysplasias and 18 of OSCC. The diagnoses of the selected cases were confirmed by a professional with experience in oral pathology (S.B.C.T). OPMD were histologically classified according to the binary classification system of oral epithelial dysplasia [4]. The OSCC staging was performed according to the TNM classification, which uses three criteria: T (size of the primary tumor), N (spread of the disease to regional lymph nodes) and M (presence of metastasis) [19]. Data about medical history and drug use were obtained from questionnaires. Smoking habits were also recorded. The individuals were

classified as smokers when they reported to smoke more than 100 cigarettes in their lifetime and who currently smoke every day [20].

Phase 2

Fifty-four patients participated in this case-control study that aimed to associate salivary and tissue levels of FAT1. This phase study samples were divided in 4 groups. Control and OSCC group patients were the same as in the phase 1 study. Salivary samples were obtained from these two groups and from a new group of OPMD patients with 10 (55.6%) classified as low-risk dysplasia and 8 (44.4%) as high risk-dysplasia. For OSCC patients two salivary samples were obtained, one at the first clinical evaluation and other, 6 months after the therapy. All volunteers that were submitted to salivary collection, were evaluated by a dentist specializing in oral pathology (J.P.A.S). Exclusion criteria for all the groups included: a history of radiation therapy in the head and neck area, chronic thyroid disease, known Sjogren's disease, those who had prior surgery of the salivary glands, contact allergies, pregnant women and individuals in current use of antibiotics, corticosteroids or antifungals. Data about medical history and drug use and smoke habit were also recorded.

Phase 1: Immunohistochemical study

Samples were selected from the files of Diagnostic Center for Oral Diseases (DCOD) - School of Dentistry, Federal University of Pelotas, in order to evaluate the immunohistochemical stain of FAT1 in the 72 samples of phase 1, and in the 18 OPDM new cases of phase 2, as described above. Tissues were submitted to immunohistochemical technique using the antibodies for FAT1 (Anti-Cadherin-related tumor suppressor homolog precursor) according to the protocol established by the manufacturer (HPA023882; Sigma-Aldrich, St. Louis, MO, USA). Sections, mounted on silanized sheets were deparaffinized in xylol and hydrated in a decreasing ethanol solution. Antigen retrieval was performed with a TRIS-EDTA solution (pH 9.0) in a 96 °C water bath for 30 min. The hydrogen peroxide blocking, protein blocking, and detection steps were performed with ready-to-use solutions provided in the kit (Spring BioScience, Pleasanton, CA, USA). The reaction was revealed with 3,3′ diaminobenzidine (Spring Bioscience Corporation, CA, USA) and was counterstained with Harris' hematoxylin.

Quantification of immunoreactivity of FAT1

Semi-quantitative analysis of FAT1

Five high-power fields (400X magnification) were analyzed separately by two pathologists (S.B.C.T) and (J.P.A.S) using an Olympus Microscope (Model CX21, Nanjing, Jiangsu, China) for each silanized slide, analyzing the intratumoral regions for OSCC and epithelium for the other groups. Positivity was graded as 1 (no staining observed), 2 (1%–25% with positive stain cells), 3 (26%–50% with positive stain cells), 4 (>50% with positive stain cells), according to the studies performed by Shinriki et al [21] and Caldeira et al [22]. The discrepancies between the evaluators were discussed together, until getting a consensus.

Computerized determination of FAT1 expression

A total of 360 images (four of each sample with 200x magnification) were scanned and stored in uncompressed tiff format (marked image file format) with RGB class of 24 bits and resolution of 640 x 480 pixels using a LEICA microscope (Model DFC7000T, Wetzlar Germany). For the quantification of cells positively labeled for FAT1 was used Image-Pro Plus 4.5 program (Media Cybernetics, Silver Spring, EUA). The image segmentation of the stained cell was based on RGB parameters of 8 bits per channel: red (100-210), green (85-190) and blue (80-180). The statistical analysis was made according to a mean of the results obtained from the 4 photographs obtained of each sample [23].

Phase 2 Enzyme-linked immunosorbent assay (ELISA)

A total of 54 adults were selected from DCOD of School of Dentistry - Federal University of Pelotas, during the period of 2016 to 2018. The sample was divided into four groups, as described above.

Salivary collection procedure

Saliva was collected from patients who gave written informed consent prior to the biopsy, and salivary evaluation was performed only after confirmation of histological diagnosis of OSCC or OPMD or healthy tissue. Previously trained dentists collected unstimulated saliva from patients in a 50 mL pre-weighed Falcon ® centrifuge tube. For the evaluation of unstimulated salivary rate flow (uSRF), saliva was collected between 9 am and 11 a.m. The patients may not have eaten, smoked or performed

any oral hygiene 90 min before the procedure [24]. After 5 min, the tubes with saliva were kept in a container at 5 °C for transport to the laboratory of DCOD/Federal University of Pelotas. Salivary samples were kept at -80°C. The same salivary collection procedure was repeated 6 months after the initial diagnosis in patients with OSCC. After laboratory analysis, the samples were discarded according to resolution CNS 441 of 2011 [25].

Enzyme-linked immunosorbent assay (ELISA) technique

On the day of the test, the saliva samples were thawed completely, vortexed, and centrifuged at 1500 rpm for 15 min at 4 °C. The supernatants were kept at room temperature and analyzed in duplicate using Human FAT tumor suppressor homolog 1 (Drosophila) (FAT1) ELISA Kit (Mybiosource® Catalog number MBS905038, San Diego, CA, USA). A spectrophotometers (MFA), whose measuring capacity was 450 nm, were used to determine absorbance. The test protocol was carried out in accordance with the manufacturer's specifications

Statistical analysis

Descriptive and quantitative data analysis was performed using the Statistical Package for the Social Sciences for Windows 22.0 (SPSS, Inc., Chicago, IL, USA). To determine whether the variables had a normal distribution, the Shapiro Wilk test was used. Kruskall Wallis test was used to compare the computerized determination of FAT1 expression among the four groups. The comparison among frequency of semi-quantitative positivity of FAT1 among the different groups were analyzed using Chi-Square test and Tukey's test. Mann Whitney test was used to compare FAT1 staining according to sex, location of lesions and habits in OPMD group. The same variables plus the type of therapy received were compared to FAT1 staining in the OSCC group. The spearman test was used for the association between the salivary and tissue levels of FAT1 and also to associate values of the semi-quantitative and computerized determination of FAT1 in tissue samples. Values of $p < 0.05$ were considered statistically significant.

Results

Phase 1

Sociodemographic data

Samples of seventy-two patients were included in this Immunohistochemical study. There were 42 men (58.3%) and 30 women (42.7%). Participants' age varied between 19 and 87 years. The mean age was 56.60 years (± 18.29). Twenty-three (32%) were smokers, 27 (37.5%) no smokers and 22 (30.6%) without information about this habit. For the low-risk dysplasia group, the three common sites were buccal mucosa (6/33.3%), followed by tongue (5/27.8%) and retromolar trigone (3/16.7%). For high-risk dysplasia cases, tongue was the more frequent site (9/50%), followed by soft palate (4/22.2%) and retromolar trigone (3-16.7%) The more frequent sites of OSCC lesions were tongue (9/50%), floor of the mouth (3/16.7%) and soft palate (3/16.7%). Regarding OSCC cases, according to TNM classification, 5 (27.7%) patients were classified as T1N0, 8 (44.4%) as T2N0, 1 (5.6%) as T1N1, 1 (5.6%) as T2N1, 1 (5.6%) as T2N2 and 2 (11.2%) as T4N2. Regarding the type of treatment performed, twelve patients (66.7%) were treated with surgery alone, and 6 (33.3%) with surgery in addition to chemotherapy and/or radiotherapy. The details of sociodemographic data of Immunohistochemical study are summarized in **Table 1**.

Table 1. The baseline characterization of the phase 1 study

Group	Gender Men: Women	Age (Average SD)	Site	Smoke Y=Yes; N=No WI:Without Information	TNM**	Type of therapy
Control (n=18)	10:8	32.5 (± 15.3)	Retromolar Trigone: 18	Y: 2; N: 16; WI: 0	N/A	N/A
Low-risk dysplasia (n=18)	8:10	54.3 (± 13.4)	Oral Mucosa: 6 Tongue: 5 Retromolar Trigone: 3 Soft Palate: 2 Gum: 2	Y: 5; N: 8; WI: 5	N/A	N/A
High-risk dysplasia (n=18)	8:10	60.3 (± 15.5)	Tongue: 9 Soft Palate: 4 Retromolar Trigone: 3 Floor of the Mouth: 2	Y: 6; WI: 12	N/A	N/A
OSCC (n=18)	16:2	65.1 (± 11.9)	Tongue: 9 Floor of the Mouth: 3 Soft Palate: 3 Oral Mucosa: 2 Retromolar Trigone: 1	Y: 10; N: 3; WI: 5	T1N0: 5 T2N0: 8 T1N1: 1 T2N1: 1 T2N2: 1 T4 N2: 2	Surgery: 12 Surgery + Chemotherapy /Radiotherapy: 6

Semi-quantitative analysis of FAT1 staining

In the control group, 8 samples (44%) were classified with score 1, (38.9%) with score 2, 1 (5.6%) with score 3 and 2 (11.1%) with score 4. In the low-risk dysplasia group, 7 samples (38.9%) were classified with score 1, 4 (22.2%) with score 2, 4 (22.2%) with score 3 and 3 (16.7%) with score 4. In the high-risk dysplasia group, 4 samples (22.2%) were classified with score 1, 4 (22.2%) with score 2, 5 (27.8%) with score 3 and 5 (27.8%) with score 1. In the OSCC group, 2 samples (11.1%) were classified with score 1, 3 (16.7%) with score 2, 3 (16.7%) with score 3 and 10 (55.6%) with score 4. In the OPMD group that was part of the phase 2 study, 6 samples (33.3%) were classified with score 1, 5 (27.8%) with score 2, 4 (22.2%) with score 3 and 3 (16.7%) with score 4. Of 10 low-risk dysplasia from OPMD cases, 4 (40%) were classified with score 1, 2 (30%) with score 2, 3 (30%) with score 3 and 1 (10%) with score 4. Of 8 high-risk dysplasia from OPMD cases, 2 (25%) were classified with score 1, 3 (37.5%) with score 2, 1 (12.5%) with score 3, and 2 (25%) with score 4. The variability of the frequency between the groups was not statistically significant (Chi 2 test; $p = 0.076$) however, A Tukey's test showed differences between the medians of the control group with the OSCC group ($p = 0.02$). The details of semi-quantitative analysis of phase 1 FAT1 stain are shown in **Figure 1A**.

Computerized determination of FAT1 staining

The average values of computerized determination of FAT1 for control group was 894.1 (±1588), for low-risk dysplasia was 1958.5 (±2934), for high risk dysplasia was 3445.8 (±4349) and for OSCC was 5989.1(±4956). For OPMD group that was part of phase 2 study, the value was 2619 (±3352.6): 2323 (±1805) for low-risk dysplasia cases and 2989 (±2143) for high-risk dysplasia cases. The details of Computerized determination of FAT1 staining are summarized in **Figure 1B**. A strong correlation was observed between computerized determination and semi-quantitative analyses of FAT1 staining ($Rho2=0,73$; $p <0.001$). **(Figure 2)**. There were no differences in FAT1 staining determined by semi-quantitative and computerized analysis when they were compared with the variables related to habits and with location of lesions inside OPMD and OSCC group ($p>0.05$).

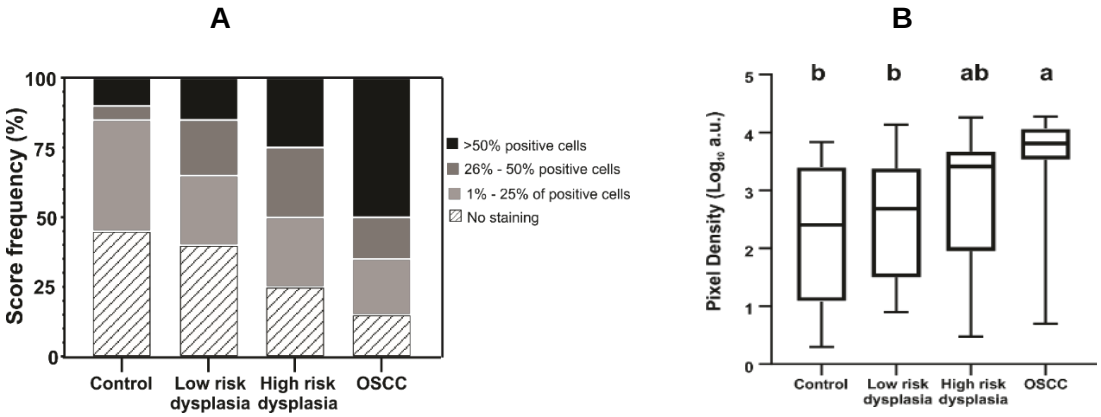


Figure 1. Semi-quantitative analysis and computerized determination of FAT1 stain (Phase 1). (A): The variability of the frequency between the groups was not statistically significant (Chi 2 test; p = 0.076). The medians of the scores of the different groups were analyzed with the Kruskal-Wallis test (p = 0.010). A *Tukey's test* showed differences between the medians of the control group with the OSCC group (p = 0.02). (B) Same lower-case letters indicate that there are no statistically significant differences (p>0.05).

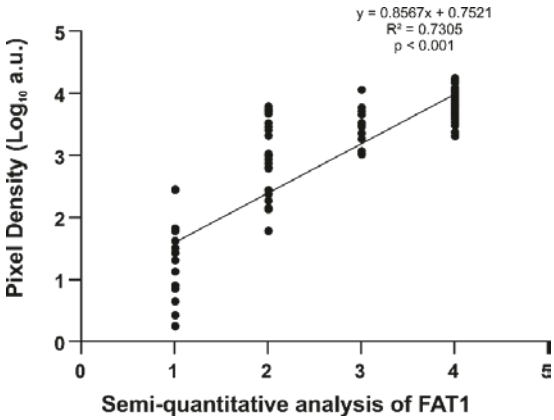


Figure 2. Association between semi-quantitative and computerized determination of FAT1 staining. (Phase 1). According spearman test, a strong correlation was observed between values of the computerized determination and semi-quantitative FAT1 stain (p <0.001).

Computerized determination of FAT1 staining

The immunohistochemical FAT1 staining in the different studied groups are illustrated and described in **Figure 4**, that summarized the main findings.

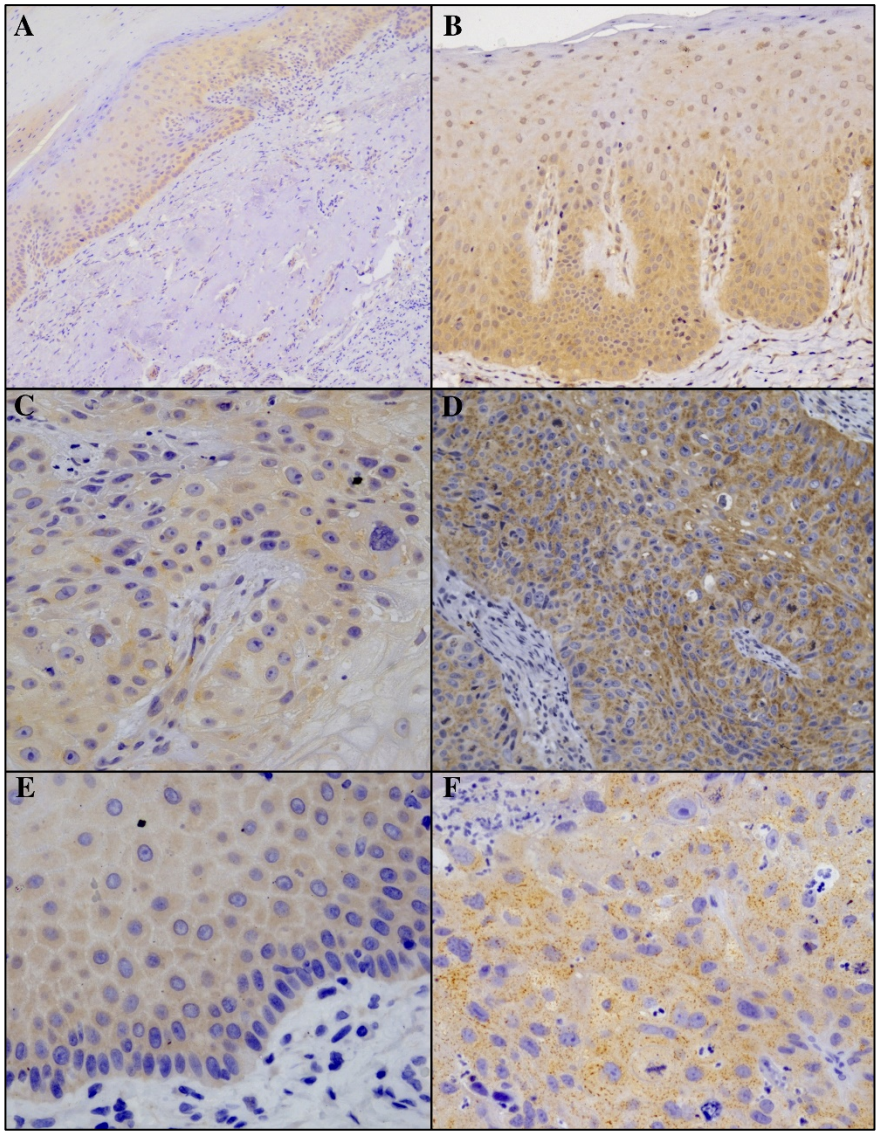


Figure 4. Immunohistochemical staining of FAT1 protein in samples of healthy non-tumor tissue, OPDM (low and high risk) and in OSCC. FAT1 immunohistochemical staining in the studied groups (1A-1F). (A) low-risk dysplasia (100X); (B) high-risk dysplasia showing staining in the epithelium and being more pronounced specially in the its basal third (400x); The majority of epithelial neoplastic cells reveal immunoreactivity for OSCC, but with different intensity, (C) showing less pronounced immunohistochemical staining (200X) and (D), higher immunohistochemical expression, which represent most of the OSCC cases (200X). The expression of FAT1 is demonstrated with cytoplasmatic staining in healthy and tumoral tissues. (E) the non-tumoral epithelial cells showed diffuse and sparse staining (200X), and (F) tumor OSCC cells presented a distinct granular pattern with dotted appearance (200X).

Phase 2

Sociodemographic data

As the control group and OSCC have already been characterized. The OPMD group is detailed as follow: Seven were men and 11 women. The mean age was 58.2

years (± 13.9). Sixteen cases (88.9%) were clinically leukoplakias and 2 (11.1%) erythroleukoplakias. The most frequent site was the buccal mucosa with 9 cases (50%), followed by the lateral border of the tongue with 5 cases (27.8%). Six (33%) were smokers.

Salivary FAT1 levels (ELISA)

The average of salivary FAT1 levels were 0.168 (±0.076) nm mL / for the control group, 0.162 nm/ mL (±0.057) for OPMD, 0.154 (±0.059) mL / min for OSCC and 0.153 (±0.076) mL /min for OSCC after therapy, with significant statistical differences ($p<0.05$). Data are illustrated in **Figure 4A**. There were no differences in salivary FAT1 levels when they were compared to habits, site of lesions and sex inside OPMD neither when comparing salivary FAT1 levels according to the same variables plus the type of therapy received, in the OSCC group ($p>0.05$).

Association between salivary and tissue FAT1 levels

A weak negative correlation was observed between FAT1 salivary levels and values of computerized determination of FAT1 stain in tissue samples ($p <0.001$). Figure 5 B.

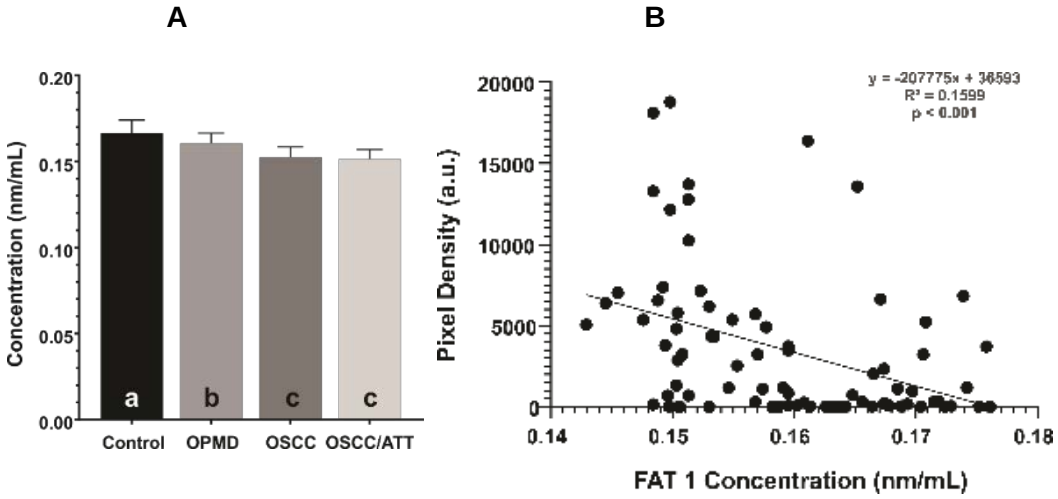


Figure 5: **FAT1 Salivary levels according diagnosis and OSCC after therapy and association between salivary levels and values of computerized determination of FAT1 staining (Phase 2).** In the Figure A, same lowercase letters indicate that there are no statistically significant differences ($p>0.05$). Figure B shows that there is a weak negative relationship between salivary and tissue levels of FAT1 and quantitative analysis of FAT1 stain in respective samples ($p <0.01$).

Discussion

Although several studies have revealed different rates of *FAT1* mutations in OSCC [26] and the secretory nature of this protein [6], it had not yet been evaluated in saliva as a potential biomarker for this tumor.

Before discussing about our main findings, it is important to consider the sociodemographic profile of this study patients, as well that the development of OSCC from OPMD and their association with risky lifestyle habits as smoking [3,4,27]. The knowledge of these factors, together with the identification of possible biomarkers can be important to understand the tumoral biology [16]. In the present retrospective study and also in the clinical phase, patients showed similarities regarding age, more frequent site of lesions and habits, when compared to the reported literature [1,2,3,28]. On the other hand, there were no differences in FAT1 staining evaluated by the two techniques used in this study, when the OPMD group was compared inside itself. Thus, tissues with characteristics associated with a higher risk factors (being female, location on the tongue and smoking habit) did not present more frequent FAT1 staining that the tissues associated with less risk features for the development of OSCC [4]. Also, no differences in FAT1 staining were observed when comparing sociodemographic variables, smoke habit and associated therapy within the OSCC group. These results are consistent with studies which indicated that the rate of mutations for FAT1 is not significantly different between smokers and nonsmokers with OSCC [29]. They are also compatible with studies that indicated that other mutated genes (not *FAT1*) such as Cyclin 1 (CCND1) and Mitogen-Activated Protein Kinase 2 (MAP4K2), could be altered, according to the site of the neoplasm, associating higher mutation rates in tongue OSCC, when compared to other sites [30].

Even though FAT1 can be considered a potential biomarker of OSCC progression from OPMD, the majority of the studies that evaluated mutations in this gene do not include these lesions in their analyses. However, there is a recent immunochemical evidence indicating increased staining of FAT1 protein in OPMD compared to normal tissues [31]. In the present study, there was observed a strong and increasing tendency of FAT1 expression demonstrated by the rising values of the quantitative and semi-quantitative analyses, when compared healthy tissues with dysplasias and OSCC, using the immunohistochemical method. Healthy tissues

showed lower immunohistochemical staining of FAT1 when compared to high-risk dysplasias and OSCC, with statistically significant differences in the computerized determination, and according to the semi-quantitative analysis. Moreover, half of the OSCC samples were positive for FAT1 (compared to 10% in healthy tissues). These results reinforce the evidence that indicates that the percentage of *FAT1* mutation in OSCC (even with frequencies ranging from 7% to 80%), is estimated close to 50% [32].

To better understand our immunohistochemical findings it is important to have in mind the mechanism of action of the FAT1 protein in the development of OSCC. In healthy cells, the interaction of wild-type FAT1 with β-catenin prevents the nuclear location of β-catenin and decreases its transcriptional activity [33]. This phenomenon may suggest that the higher percentages of positive immunostaining in OSSC neoplastic cells for cytoplasmatic FAT1 in our study corresponded to the mutated protein, which is unable to inhibit WNT / β-catenin signaling, causing an increasement in proliferation. In fact, the antibody used in the phase 1 study for FAT1 in the immunohistochemistry technique detected an atypical protein, what justify the described findings. It has been described that the main intracytoplasmic domain of FAT1 in OSCC differing of a benign tissue specimen [7]. FAT1 would be located at the boundaries of cells in normal and well-differentiated cells, but would show a diffuse cytoplasmic and nuclear distribution in poorly differentiated OSCCs [7]. In cells with normal tumor suppressing function of FAT1, the encoded proteins do not accumulate in the cytoplasm frequently, which could explain the lower immunostaining of the FAT1 protein observed in our healthy tissue samples. In contrast, in colorectal cancer, 95.7% of neoplastic samples showed cytoplasmic positivity for FAT1, only 4.3 % showed plasma membrane positivity and less than 5% of normal colon samples showed moderate or strong cytoplasmic positivity for FAT1, while most of them were negative or weak [34]. Considering breast cancer, an cytoplasmatic intense expression of FAT1 has also been associated with increased cell migration and invasion [8]. A similar phenomenon could have happened in our OSCC samples, which showed a frequent granular cytoplasmic immunostaining pattern for FAT1 and less common, more diffuse and sparce cytoplasmatic expression in normal tissues.

Although the present study indicated a rising immunochemical expression of FAT1 when comparing normal, dysplastic and tumor tissues, there was not any association between those findings and the salivary levels of FAT1, when they were evaluated in the same patients. According to our results, we expected that just like in tissue samples, the OSCC patients' saliva would have shown higher levels of FAT1. It was curious that patients without OSCC or any evidence of oral lesions had revealed a higher concentration of salivary FAT1, when compared to patients with OSCC. As previously reported, the cytoplasmic accumulation of FAT1 and the frequent positive immunostaining for this protein shown in neoplastic tissues may be a reflection of its aberrant expression of various types of cancers, including OSCC [36]. This phenomenon may suggest that, in our study, the protein identified in saliva is a native (wild-type) FAT1 protein. In fact, the Elisa kit available in the market and used in the present study detected a native and non-mutated encoded protein. Therefore, we could justify and understand the weak negative relationship between the salivary concentrations of FAT1 and its tissue expression in quantitative analyses. The hypothesis firstly suggested was that the FAT1 accumulated in the cytoplasm of normal cells, could be secreted in the extracellular environment and, in theory, could be identified in saliva. However, more studies are needed to confirm this suggestion, searching levels of secreted mutated FAT1 (salivary or serum) and associating them with the constitutive protein.

In addition, salivary levels of FAT1 did not show significant changes after therapy in OSCC patients, as well as, in the comparison of them with the immunohistochemical analysis between different groups, according to gender and habits, as commented before. These results reinforce the fact that the OSCC is a neoplasm of multifactorial etiology, determined not only by the punctual mutations of FAT1. Mutations in this gene, apparently would not depend on the common risk factors associated with the development of the OSCC or also on the site of lesion, but being related to other factors as genetic mutations, for example [27].

Another interesting finding of the present study was the association between the values of the semi-quantitative evaluation and the ones delivered by the computer program. Even the correlation was not perfect as had been demonstrated in another study [23], we could consider that both types of analyzes are complementary. The computerized evaluation provides values according to the intensity of pixels observed,

what can be especially interesting in OSCC cases, where the staining pattern was more pronounced than in the presented healthy tissues. This evaluation can complement the semi-quantitative analysis, and it could also indicate different biological behavior. However, both quantitative methods did not suppress the qualitative evaluation in immunohistochemistry and the morphologic observation of OSCC specimens made by exploited pathologists.

Finally, to the best of our knowledge, the present work is the first in assessing salivary FAT1 levels as a possible OSCC biomarker and also as a reflex of its response to associated therapies. There were limitations in the development of this research, such as the impossibility of directly associate the FAT1 immunohistochemical expression and salivary levels of this protein, since the antibodies used in both phases were directed to different types of FAT1, being one mutated and other native, non-mutated, respectively. Even though the referred association could not be established, the frequent and higher cytoplasmic concentration of the FAT1 showed in tumor cells in our study, could also indicate aggressiveness of the OSCC, just as it had been pointed [35;36], suggesting its aberrant phenotype. Moreover, FAT1 may be also involved in the mechanisms of migration and invasion of OSCC and, therefore, may be an important target for the development of new therapeutic strategies [37]. Our findings suggest that the use of native FAT1 as a salivary marker of progression or as a response to OSCC-related therapy is not recommended, because even it was observed differences in their levels between patients with and without OSCC, there was no association of these levels with clinical-pathological characteristics nor with tissue immunostaining of this protein. Further studies are needed to look for biomarkers that will help us with the early diagnosis of this complex and aggressive disease.

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6 Artigo 3

Salivary characteristics in burning mouth syndrome: A systematic review[3]

Juan Aitken-Saavedra[a,b]
Sandra Beatriz Chaves Tarquinio[c]
Matheus Kinalski[b]
Daniela Haubman Pereira[d]
Marcella Wohlfahrt Martins[d]
Ana Carolina Uchoa Vasconcelos[c]

[a] Department of Oral Pathology and Medicine, School of Dentistry, University of Chile. Santiago, Chile

[b] Graduate Program in Dentistry, Federal University of Pelotas. Pelotas, Rio Grande do Sul, Brazil

[c] Center of Diagnosis of Diseases of the Mouth, Federal University of Pelotas. Pelotas, Rio Grande do Sul, Brazil

[d] Graduation student in dentistry. Federal University of Pelotas. Pelotas, Brazil

*** Corresponding author.**

Ana Carolina Uchoa Vasconcelos[c], PhD

E-mail: carolinauv@gmail.com

Address: R. Gonçalves Chaves, 457 - Centro, Pelotas - RS,

Postal address: 96015-560 | Phone: +55-53-981112894 | Fax 56-2-9781748

[3] Artigo formatado segundo as normas do periódico Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology.

Abstract

Background: Burning Mouth Syndrome (BMS) is a chronic oral condition characterized by a burning sensation, without clinical changes or laboratory findings that justify this symptom. The objective of this systematic review was to evaluate salivary characteristics on BMS.

Methods: Two reviewers conducted a bibliographic search on four databases (PubMed, Web of Science, Scopus and Cochrane). Case-control studies were included.

Results: Twenty-eight studies were included. Twenty-four articles (85.7%) evaluated organic salivary biomarkers, 15 (53.6%) salivary flow rate (SRF) and 6 (21.4%) salivary electrolytes. Eighteen organic biomarkers showed higher levels in BMS patients and within these, the two most indicated were α-amylase and cortisol. Eight organic biomarkers, including Tumor Necrosis Factor-alpha (TNF-α) and IL-6, showed lower levels in BMS patients. According SRF, four articles indicated that BMS patients have less unstimulated (uSRF) and six did not establish differences between cases and controls. Two articles indicate that stimulated (sSRF) is lower in BMS patients nevertheless, seven didn't show differences. Sialochemical analysis revealed that sodium (Na), chlorine (Cl), potassium (K), sodium (Na), calcium (Ca), showed higher levels in patients with BMS according to three studies, but two do not establish differences.

Conclusion: The qualitative salivary characteristics in BMS suggest that the syndrome has neuropathic inflammatory, emotional, immune and hormonal involvement. SRF would not be associated with BMS in terms of pathogenesis or as a reflection of the systemic state.

Keywords: Burning mouth syndrome, Biomarkers, Saliva, Hyposalivation.

Introduction

Burning Mouth Syndrome (BMS) is a chronic oral condition that dramatically impacts the quality of life of those affected (mainly adult women) and is characterized by burning and uncomfortable sensations in the mouth, without clinical alterations or laboratory findings that justify these symptoms.[1-4] BMS may be a primary process or arising from some pathological conditions. Although the etiology of primary BMS has not been fully identified, local, systemic, and psychological factors have been associated.[5,6]

Saliva has essential properties to maintain healthy and balanced conditions within the oral cavity.[9] It has been described in patients with BMS some salivary changes in pH, salivary flow or even in the concentrations of some ions such as magnesium (Mg), sodium (Na), potassium (K) or chlorine (Cl) and organic biomarkers.[10,11,12]. These salivary alterations can reflect the systemic state of BMS patients and could determine its pathogenesis. In addition, saliva has some advantages as a diagnostic fluid such as the easiness of obtaining sufficient volume to perform a variety of analyzes repeatedly, being a non-invasive method well accepted by patients.[8]

The evidence of qualitative and quantitative salivary characteristics in patients with BMS are still contradictory.[9-12] The evaluation of these characteristics and their association with the BMS could help to better understand the etiology and even assess the systemic status of patients affected by the syndrome. For this reason, and given that to our knowledge, there is no survey of information on this aspect, the objective of this systematic review was to compare through case-control studies, salivary characteristics between patients with and without BMS.

Materials and Methods

Protocols and sources of information

This systematic review was conducted according to the guidelines of the Cochrane Handbook for Systematic Reviews of Interventions, following the four-phase flow diagram of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement[14] and is registered in PROSPERO under the number CRD42019140389. The literature search was carried out by two independent

reviewers (J.P.A.S and A.C.U.V) in December 2019. The following databases were screened: PubMed (National Library of Medicine), Scopus (Elsevier), Web of Science (Thomson Reuters) and Cochrane. In addition, the reference lists of the selected articles were searched manually.

Electronic Searches and search strategy

The search strategy is described in **Supplementary Appendix 1**. The main outcome was: Salivary changes related to BMS. The authors used the Review Manager, Version 5.3. Copenhagen: (The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). The results were synthesized following the Cochrane Collaboration statistical guidelines.

Study selection

Study characteristics were extracted independently by two reviewers (J.P.A.S and A.C.U.V). Any disagreement on the eligibility of studies included was resolved through discussion and consensus, and in case of disagreement, a third reviewer (S.B.C.T) decided whether the article should be included. There were no restrictions regarding articles' language or date of publication. Letters to the editor and editorials commenting about other published articles were excluded. When the title/abstract was not available or did not provide sufficient information for a decision on inclusion or exclusion, the full text was retrieved. Duplicated articles were discarded.

Data extraction

The following information was extracted from each included article: authors and year of publication, the country where the study was undertaken, gender and average of age of participants and main finding of salivary changes in relation to BMS. Due to the high degree of heterogeneity in terms of different studies and methodologies, it was considered inappropriate to conduct a meta-analysis.

Types of study to include

Case-control studies were included in the review. Studies were excluded for the following reasons: Phase 1: Title and Summary: Studies that used other biological media, such as blood or other body fluids instead of saliva regarding with BMS; Reviews, personal opinions, letters to the editor, book chapters, and conference summaries; Associations between saliva and burning mouth syndrome in experimental

studies (in vitro or in vivo animal studies) Phase 2: Full article: Studies with insufficient information about the definition of the groups to be studied or that do not establish criteria for diagnosing BMS.

Assessment of risk of bias

The first two authors were systematically assessing the quality of individual studies using JBI-MAStARI (JBI Meta-Analysis of Statistics Assessment and Review Instrument). The questionnaire consists of ten questions that were answered with yes, no, unclear, or not applicable. The studies were classified as follows: high methodological quality (>5 ‘‘yes’’ responses), moderate methodological quality (3–4 ‘‘yes’’ responses), or low methodological quality (0–2 ‘‘yes’’ responses)[15].

Results

The search flow according to the Prisma statement is summarized in **Figure 1**. A total of 1009 references were identified in the four electronic databases. Three references were identified through other sources. After the removal of 204 duplicates, 808 titles/abstracts were read in stage 1. Of these, 754 articles didn’t meet the eligibility criteria and were excluded. Fifty-four full-text references were assessed and 26 were excluded after applied exclusion criteria, totalizing finally for this systematic review, 28 articles.

The findings regarding author, year of publication, country, number of cases, gender and age average are summarized in **Table 1**. The studies were published between 1993 and 2019. The countries with the largest number of articles in descending order, were Brazil with seven (one in partnership with Spain), Croatia, Korea, Israel with three each and Italy with two. Finland, Spain, Serbia, Turkey, the United Kingdom, the United States, Japan, France, Iran, and Sweden had one each. Even though all articles indicated the number of patients evaluated, 4 did not specify the age range or the gender of the patients. A total of 849 patients with BMS were evaluated. Of this, 669 patients specified gender: 569 were women and 100 were men. A total of 756 cases belonged to control group. Of this, 662 cases specified gender being 569 were women and 92 were men. Sample sizes ranged from 9 to 91 in the BMS group and from 9 to 90 in the control group. The average age of the evaluated groups varied from 50 to 69 years in the BMS group and from 39.5 to 68.3 years in the control group.

There is no exclusive criterion for establishing the diagnosis of BMS according to the articles evaluated. Eight articles used the International Classification of Headache Disorders[6] to establish BMS diagnosis.[13,26,28,30,31,36,37,38] The others (20 articles) diagnosed BMS according to the presence of typical symptoms: daily and symmetrical burning pain sensation of the oral mucosa, lasting for at least 3 months, with constant or increasing intensity, that endures more than 2h during the day and improves during eating or drinking, having no interference with sleep, and unaccompanied by any dental, oral, or medical clinical signs. Fourteen articles indicate some blood tests.[11,12,13,23,24,25,26,28,29,30,31,36,37,38]

The main salivary findings of the systematic review are summarized in **Table 2**. Regarding assessed salivary characteristics, twenty-four articles evaluated organic biomarkers, fifteen saliva flow rate (SRF) and five salivary electrolytes. The sum of the total samples that evaluated organic biomarkers salivary was 668 and 576 for the BMS and control group, respectively. Eighteen organic biomarkers showed higher levels or greater salivary activity in BMS patients compared to the control group ($p < 0.05$). Of these biomarkers, the two most indicated were α-amylase and cortisol in three articles each. The other biomarkers were: Nerve growth factor (NGF), Tryptase, Cystatin SN, Immunoglobulin G (IgG), Immunoglobulin M (IgM), Immunoglobulin A (IgA), Kallikrein-13, α-enolase, 17β-estradiol, glandular kallikrein, neurotrophin P75NTR, Thiocyanate, Interleukin 2 (IL-2), Interleukin 18 (IL-18), Interleukin 6 (IL-6) and Opiorphin. Eight organic biomarkers showed lower levels or less salivary activity in BMS patients compared to control group ($p < 0.05$): Morning salivary dehydroepiandrosterone (DHEA), neurotrophic factor (BDNF), Chondroitin sulfate (CS), IgA, Substance P (SP), Cortisol, Tumor Necrosis Factor-alpha (TNF-α) and IL-6. Eleven organic biomarkers didn't show differences in levels or salivary activity in BMS patients compared to control group ($p < 0.05$). The two most indicated were IL-6 in three and TNF-α in two articles. The other biomarkers were: Cathepsin G, Opiorphin, Progesterone, Interleukin1β (IL-1β), Interleukin 8 (IL-8), IL-2, CGRP, Neurokinin A (NKA) and Sialic Acid. In addition, three articles referred biomarkers to in general manner: one article indicates more total proteins, another less total proteins, and another indicates that there are no differences in the role of major parotid glycoproteins.

Regarding SFR, the sum of the total samples that evaluated unstimulated saliva flow rate (uSRF) and/or stimulated saliva flow rate (sSRF) was 439 and 434 patients

in the BMS group and in the control group, respectively. uSRF averages for patients with BMS and control ranged from 0.08 to 0.34 ml/min and 0.46 to 0.83 ml/min, respectively. sSRF in BMS patients with and in the control group ranged from 0.46 to 1.17 ml/min and 0.63 to 1.23 ml/min, respectively. Four articles established that BMS patients had lower uSRF in comparison with the control group ($p<0.05$) however, 6 didn't show significant statistical differences for this parameter. Two articles indicated that sSRF was lower in BMS patients ($p<0.05$), however, 7 didn't show significant statistical differences for this parameter.

The sum of the total sample that evaluated salivary electrolytes levels was 208 and 193 patients for the BMS group and the control group, respectively. Three articles indicate that salivary levels of potassium (K), Chlorine (Cl), phosphorus (P), sodium (Na) and calcium (Ca) are higher in patients with BMS when compared with control group ($p<0.05$) and 2 articles indicate that there are no significant statistic differences regarding salivary electrolytes levels of K, P, Na, Ca, magnesium (Mg), zinc (Zn) cooper (Cu), iron (Fe), Chlorine (Cl), Boron (B), Cadmium (Cd), Aluminum (Al), Manganese (Mn), Selenium (Se), Antimony (Sb), Molybdenum (Mo), Vanadium (V), Strontium (Sr), Chromium (Cr), Titanium (Ti), Lithium (Li), Cobalt (Co), Thallium (Tl): Sulfur (S), Plumbum (Pb), Nickel (Ni), Arsenic (As), Beryllium (Be), Bismuth (Bi) when compare case and control groups. One study established that patients with BMS have lower salivary levels of Mg when compared to control patients ($p<0.05$).

The risk analysis of biases revealed that the main problem of the articles studied is to identify and deal with the confounding factors between cases and control to interpret the results. Sixteen (57.1%) studies were classified as high methodological quality, 9 (32.1%) as moderate methodological quality and 3 (10.7%) as low methodological quality. Exposure measures and their form of assessment had little risk of bias. **Supplementary Appendix A2** contains the risk of bias of the selected studies by JBI-MAStARI.

Discussion

Salivary characteristics found in people with BMS could help to better understand its pathogenesis, estimate the systemic state of affected individuals and even determine the response to therapies. Even Brazil was the country with the most articles published in this systematic review, the vast majority of studies come from

Europe, where the population is older and there are more women in the range most associated with BMS [20]. Regarding the gender and age of patients with SMC, this systematic review shows a clear predilection for women in their 50s and 60s.[34] This agrees with retrospective and cohort studies published elsewhere, in which data showed that women in those same decades of life are the most affected by the syndrome [3,4]. It is important to note that women were also predominant when the control group was analyzed, although a wide variation in age was observed.

The search for organic biomarkers as a complementary method of diagnosis or evaluation of BMS systemic status has been an incentive for science in recent years.[33,38] Even the organic biomarkers evaluated, which present higher or lower levels in saliva are different in nature, several of them point to a similar etiology. Increased salivary levels of neurotrophin P75NTR glandular, kallikrein, α-enolase and NGF and tryptase activity, as well as decreased SP activity in BMS patients could be indicative of the neuropathic etiology of the syndrome.[28] Besides that, the alpha-enolase proteins IL-18 and KLK13, overexpressed in saliva of patients with BMS, are possibly associated with peripheral nerve damage, a consequence of oral mucosa inflammation process that it promotes. IL-18 is a proinflammatory cytokine which acts as an inflammatory mediator in body fluids and induces chronic inflammation in different diseases such as rheumatoid arthritis and KLK13 is involved in inflammatory response by activating kinins, regulating inflammatory process, enhance the vascular permeability.[33]

There is also enough evidence that indicates a possible inflammatory etiology in BMS. The cystatin, for example, may reflect a defensive reaction to continuous inflammation, a phenomenon that may be one of the conditions underlying the development of BMS. Lower CS levels concentration or higher glandular kallikrein activity, important hormones in regulating local blood pressure and inflammatory response in saliva of BMS patients, could also be involved with a similar etiology of the syndrome.[22] TNF-α and IL-6 are potent modulators of corticotropin-releasing hormone, which produce increased hypothalamic-pituitary-adrenal axis activity suggesting a relationship between inflammatory and hormonal factors in BMS etiology. These conclusions are also suggested in a study that showed higher salivary IL-6 levels in BMS patients[21] and others that showed that a decrease in TNF-α and IL-6 are associated with a relief in the burning sensation.[13] Other organic biomarkers could

suggest or be associated with an emotional involvement in the syndrome. Changes in salivary levels of salivary opiorphin (natural painkiller and antidepressant), may reflect emotional imbalances associated with the syndrome.[35] Interleukin-2 (IL-2), can induce depressive symptoms and also suggest an emotional involvement in BMS pathogenesis.[21] On the other hand, higher levels of salivary cortisol,[12,29,34] hormone responsible for regulating physiological stress, metabolic and some immunological functions can indicate anxiety and stress in BMS patients.[12,13] The presence of higher levels of salivary α-amylase (an enzyme necessary to digest nutrients through hydrolysis of starch into glucose and maltose) has also been associated with psychological stress, probably due to its direct action on the sympathetic autonomic nervous system.[18,19,34]

Regarding trace elements, the evidence is contradictory. Electrolytes are required in small concentrations as essential components of biological enzyme systems or structural portions of biologically active constituents. Bioelements, for example Cu and Zn are involved in vital biochemical activities, such as different redox and free radical formation, and the maintenance of proton cell homeostasis.[18,19] The central role of salivary composition in taste perception is well established. The salivary ionic environment is considered critical in taste transduction and may also be important in sensory complaints.[18] In this sense, studies have shown higher salivary levels of some salivary electrolytes in BMS patients (K, P, Cl, Ca, Na), which could reinforce the idea of the relationship between neuropathy pain and saliva characteristics.[11,18] In addition, the role of Mg in BMS may be related to its antidepressant effects and there is sufficient information to associate psychological factors in the etiology of BMS. Although it has been suggested that salivary Mg levels may have an impact on BMS symptoms,[24] evidence is limited. The possible role of altered salivary electrolytes in BMS may gain more credibility through other studies that clearly demonstrate that ionic imbalances, such as hypertonic conditions, increase pain and activate small-diameter sensory neurons.[19]

Regarding the evaluation of SRF the present systematic review shows that the amount of saliva produced, stimulated or not, would not be associated with the syndrome. The decrease in uSRF in BMS patients reported in some studies[7,23,29,36] can promote the lack of chemical and physical protection of the oral mucosa, facilitating the establishment of BMS 10. However, most articles included in this review indicated

that there are no differences for both uSRF[7,23,29,36] and sSRF[11,12,16,20,28,29,31] between people with and without BMS. Regarding SRF, even though it is important to note that the decrease in salivary flow in patients with BMS may be a consequence of the drugs used to treat the systemic diseases associated with the syndrome,[36] articles evaluated generally do not specify or standardize this aspect.

From the information extracted from the evaluated articles, it was possible to make relevant conclusions regarding the association between salivary characteristics and BMS. Quantitative salivary characteristics (SRF evaluation) could indicate that they are not associated with the syndrome in terms of pathogenesis or reflex of the systemic state. On the other hand, qualitative salivary analysis points to a multidisciplinary origin of the syndrome. The organic biomarkers evaluated suggest that BMS can be determined or reflect neuropathic (also suggested by some salivary electrolytes), inflammatory, emotional, immunological and hormonal factors. The lack of standardization, both for establishing the BMS diagnosis and for the use of medications in affected patients (which could have consequences especially in quantitative salivary variables evaluated), should be factors to improve in future researches. In this sense, and although more studies with more standardized variables are needed, qualitative salivary characteristics can be useful in assessing both the etiology and the systemic state, the progression and even the orientation of more effective therapies of this still, enigmatic syndrome.

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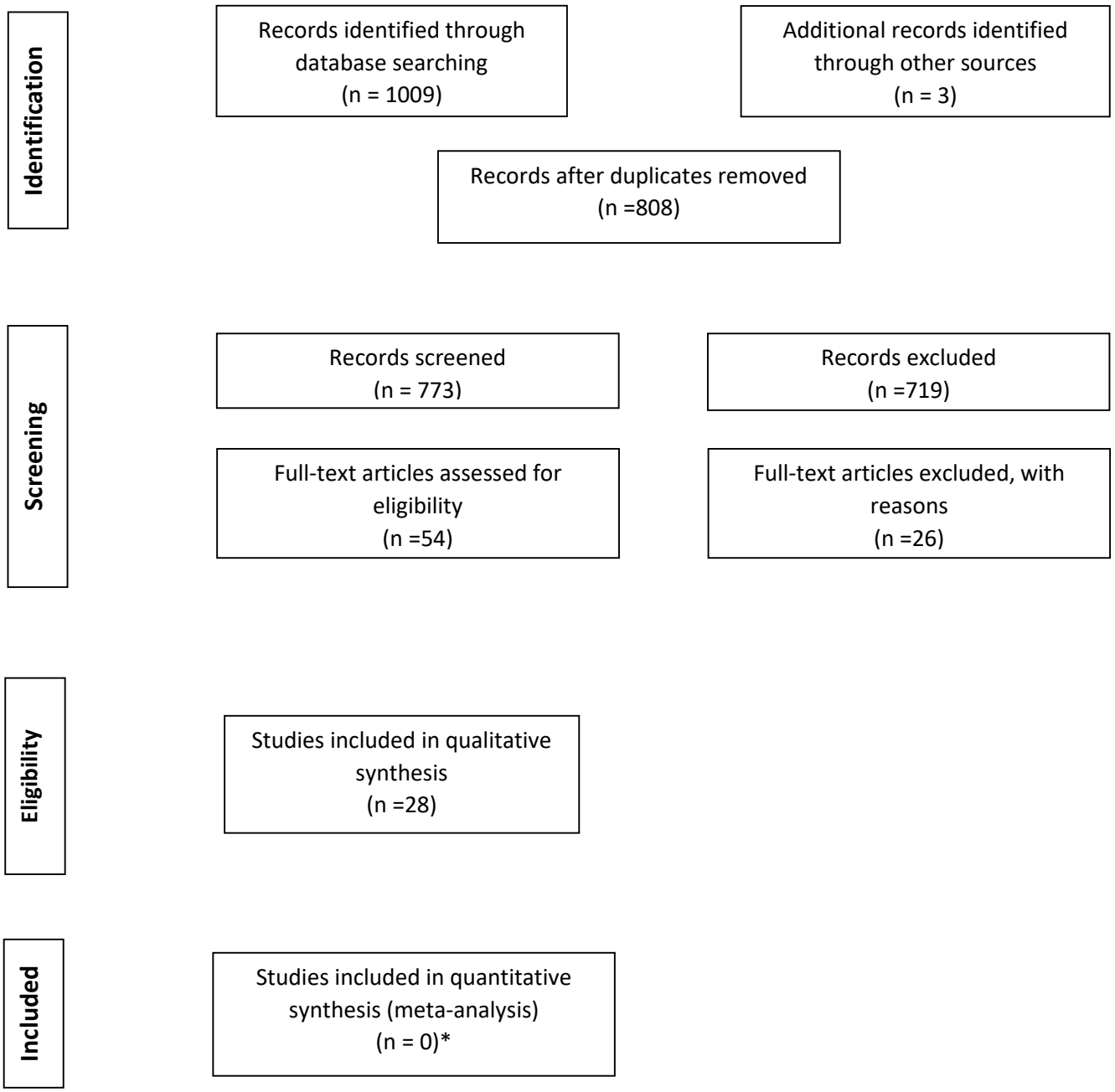


Figure 1. Search flowchart according to the PRISMA Statement

Table 1. The baseline characterization of systematic review

Author	Year	Country	N (BMS)	Women BMS	Men BMS	Average age (SD) BMS	N (Control)	Women Control	Men Control	Average age (SD) Control
Tammiala-Salonen, T.et al[16]	1993	Finland	9	-	-	50	9	-	-	50
Lundy, F. T.et al[17]	1997	UK	10	7	3	61 (±9)	10	7	3	56 (±9)
Hershkovich et al[18]	2004	Israel	91	-	-	58.1 (±11)	90	62	28	57.16 (±16)
Granot, M. et al[19]	2005	Israel	35	24	22	58.7 (±15,4)	19	13	6	48.3 (±9.4)
Marques Soares, M. et al[20]	2005	Brazil, Spain	40	37	3	63.12 (±11,8)	40	37	3	63.12 (±11.8)
Simčić, D. et al.[21]	2006	Croatia	30	-	-	55-65	30	-	-	55-65
de Moura, S. et al[11]	2007	Brazil	24	23	1	62.8	24	23	1	62.8
Loeb L.et al[22]	2007	Brazil	15	-	-	-	15	-	-	-
Amenabar, J. et al[12]	2008	Brazil	30	24	6	61.63 (±10.77)	30	24	6	61.63 (±10.77)
Fernandes, C. et al[23]	2009	Brazil	30	30	0	-	30	30	0	n
Pekiner, F. et al[24]	2009	Turkey	30	19	11	54.23 (±12.78)	30	21	9	50.87 (±6.23)
Suh, K. et al[25]	2009	Korea	40	40	0	61.6 (±10.1)	20	20	0	65.1 (±9)
Zidverc-Trajkovic, J et al[26]	2009	Serbia, Hungary	78	51	27	64.6 (±10.9)	16	-	-	-
Boras, V. et al [27]	2010	Croacia	26	26	0	65.6	28	22	0	50
Borelli, V.et al[28]	2010	Italy	20	17	3	69	20	16	4	68.3
Kim, H. et al[29]	2012	Korea	30	30	0	65.7 (±4.9)	20	20	0	68.1 (±2.3)
De Souza, F. et al[13]	2014	Brazil	30	29	1	62.13 (±12.74)	32	31	1	61.59 (±12.84)
López-Jornet, P[30]	2014	Spain	28	22	6	58.8 (±10.2)	30	17	13	50 (±17.2)
Lee, Y. et al[31]	2015	Korea	33	27	6	65.3 (±11.1)	30	27	3	61.1 (±9)
Imura, H. et al[7]	2016	Tokyo, Japan	15	15	0	55.2 (±6.05)	30	30	0	52.3 (±3.49)
Boucher, Y. et al[32]	2017	France	21	19	2	58.5 (±11.7)	21	19	2	58.9 (±11.5)
Ji, E. et al[33]	2017	USA	19	-	-	-	19	-	-	-
Nosratzehi, T. et al[34]	2017	Iran	30	26	4	63.8 (±1.5)	30	26	4	39.5 (±13.3)
Salarić, I.et al[35]	2017	Croatia, UK	29	24	5	67.5	29	20	9	67.3
Acharya, S et al[36]	2018	Sweden	56	56	0	67.8	56	56	0	67.9 (±3)
Cabras, T et al[37]	2019	Italy	16	16	0	66 (±10)	14	14	0	66 (±10)
Krief, G.et al[38]	2019	Israel	20	20	0	66.6	20	20	0	67.4
Morandini L. et al [40]	2019	Brazil	14	14	0	60.71 (±10.25)	14	14	0	62.92 (±10.15)

SD: Standard Desviation
N: Number

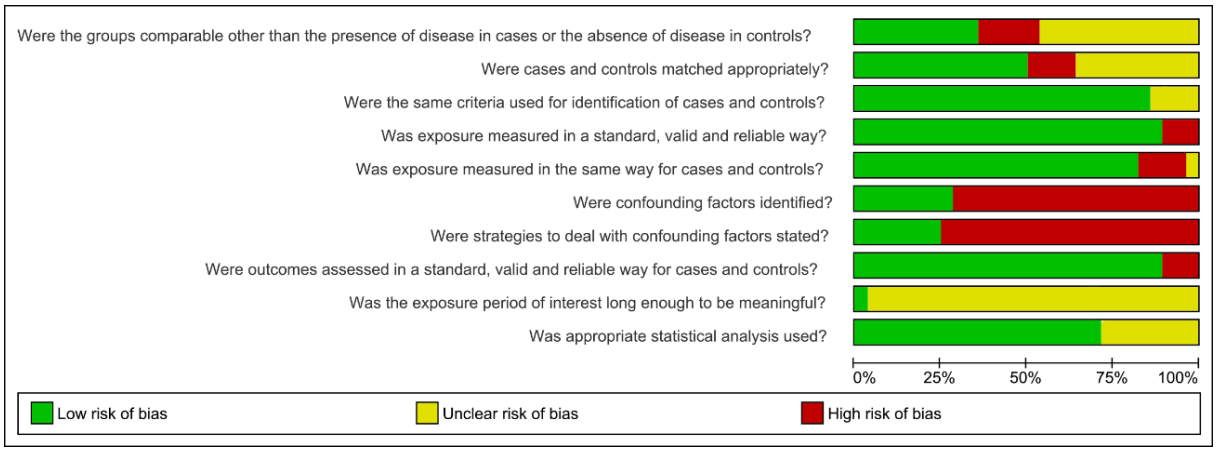
Table 2. Assessed salivary characteristic according country and main finding

Assessed salivary characteristic	Country	Main finding in relation to saliva
Unstimulated saliva flow rate (uSFR) Stimulated saliva flow rate (sSRF)	Brazil[11,12,13,20,23] Israel[18,19] Korea[29,31] Japan[7] Spain[20] Sweden[36] Finland[16] Italy[28] Turkey[24]	< uSFR[7,23,29,36] < sSRF[16,23] = uSFR[12,13,18,19,20,24] = sSRF[11,12,16,20,28,29,31]
Salivary electrolytes levels	Israel[18,19] Brasil[11] Spain[30] Turkey[24]	> K[11,19,24], Cl[11,19,24], P[11], Ca[19,24,] Na[18,19,24] = Na[11,30], K[18,12,30], Ca[30], Mg[30], Fe[30], Cu[22,30], Mn[30], Zn[22,30], B[30], P[30], S[30], Al[30], Pb[30], Cd[30], Cr[30], Ni[30], As[30], Be[30], Bi[30], Co[30], Li[30], Mo[30], Sb[30], Se[30], Sr[30], Ti[30], Tl[30], V[30], Cl[30], P[30] < Mg[24]
Salivary organic biomarkers levels	Brasil[11,12,13,22,23,40] Croatia[21,27,35] Italy[28,37] Israel[18,19,38] Korea[25,29] Iran[34] Turkey[24] France[32] Japan[7] USA[33] UK[17] Finland[16] Serbia[26]	= IL-6[24,25,27]; < IL-6[13]; > IL-6[21] = IL-2[24]; > IL-2[21] = IL-8[25] > IL-18[33] = IL-1 β[25] = Progesterone[29] =TNF-α[25,27]; < TNF-α[13] = Opiorphin[32]; >Opiorphin[35] > α-amylase[18,19,34] > NGF activity[28] >Tryptase activity[28] < SP activity[28] = Cathepsin G[33] > Cystatin SN[37] < BDNF[13] < Cortisol[13]; > Cortisol[12,29, 34] > IgA[18,19]; < IgA[7] > IgG, IgM[18] >17β-estradiol in sSRF[29] > KLK13[33] > α-enolase[33] < CS[22] > Glandular kallikrein[22] < DHEA[23] < Total salivary protein[11] >Total salivary protein[13] > P75NTR activity[38] =Role for major parotid glycoproteins[17] > Thiocyanate activity[40] = CGRP[26] = NKA[28] = Sialic Acid[16]

Supplementary Appendix A2. The search strategy

Database	Keywords used	Number of articles found
PubMed	(((((("Burning Mouth Syndrome"[Mesh]) OR (Burning Mouth Syndromes) OR (Mouth Syndrome, Burning) OR (Mouth Syndromes, Burning) OR (Syndrome, Burning Mouth) OR (Syndromes, Burning Mouth))))) AND ((((("Saliva"[Mesh]) OR (Salivas))))	131
Scopus	("Burning Mouth Syndrome" OR "Burning Mouth Syndromes" OR "Mouth Syndrome, Burning" OR "Mouth Syndromes, Burning" OR "Syndrome, Burning Mouth" OR "Syndromes, Burning Mouth") AND ("Saliva" OR "Salivas")	782
Web of Science	TS=("Burning Mouth Syndrome" OR “Burning Mouth Syndromes” OR “Mouth Syndrome, Burning” OR “Mouth Syndromes, Burning” OR “Syndrome, Burning Mouth” OR “Syndromes, Burning Mouth”) AND TS=("Saliva" OR “Salivas”)	88
Cochrane	Saliva AND Burning Mouth Syndrome	8

Supplementary Appendix A2. Risk of bias of the selected studies by JBI-MAStARI.



7 Artigo 4

Salivary changes may be associated with pathogenesis, systemic health and quality of life in women with burning mouth syndrome?[4]

Running tittle: Salivary changes in women with BMS

Juan Aitken Saavedra[1,2]
Ana Carolina Uchoa Vasconcellos[3]
Wellington Luiz De Oliveira da Rosa[4]
Ana Paula Neutzling Gomes[3]
Adriana Fernandes da Silva[4]
Matheus dos Santos Fernandez[5]
Andresa Goicochea Moreira[1]
Sandra Beatriz Chaves Tarquinio[3*]

[1] Post Graduate program in Dentistry. Federal University of Pelotas. Pelotas, Brazil

[2] Department of Oral Pathology and Medicine, School of Dentistry, University of Chile. Santiago, Chile

[3] Center of Diagnosis of Diseases of the Mouth, Federal University of Pelotas. Pelotas, Brazil

[4] Department of Restorative Dentistry, School of Dentistry, Federal University of Pelotas. Pelotas, Brazil

[5] School of Dentistry, Federal University of Pelotas. Pelotas, Brazil

Keywords: Xerostomia; Burning mouth syndrome; Viscosity

*** Corresponding author.**

Sandra Beatriz Chaves Tarquinio, PhD
Email: sbtarquinio@gmail.com
Gonçalves Chaves, 457 / Rm 702-3
Centro District - Zip code: 96015-560, Pelotas, RS, Brazil
Telephone number: +55 53981266551

[4] Artigo formatado segundo as normas do periódico Oral Disease.

Introduction:

Burning mouth syndrome (BMS) is a chronic condition characterized by a burning sensation in the oral cavity, but no clinical or laboratory findings to justify these symptoms. Some salivary changes may be part of the etiology or reflect the systemic status of these patients. This case-control study aimed to trace the salivary profile of women with BMS.

Study design:

40 women with BMS and 40 control women were recruited. Unstimulated salivary flow rate (uSFR), pH, salivary cortisol levels, salivary viscosity, and oral health impact profile (OHIP-14 questioner) were determined. $P < 0.05$ was considered statistically significant.

Results:

For uSFR, mean values obtained for BMS and for control group respectively were 0.35 and 0.61 mL/min; for pH, 7.23 and 7.34; for cortisol levels, 0.36 and 0.15 μg/dL; for viscosity values, 31.1 and 45.01 mPas and for OHIP-14 scores, 21.7 and 5.7. To uSFR, cortisol levels, viscosity values and OHIP-14 scores, differences were statistically significant. Salivary cortisol levels and OHIP-14 scores were correlated positively (rho = 0.624).

Conclusion:

BMS women have lower uSFR and salivary viscosity and higher salivary cortisol levels compared with the control group that were associated with worse quality of life.

Keywords: Xerostomia; Burning mouth syndrome; Viscosity

Introduction

Burning mouth syndrome (BMS) is a chronic oral condition that can dramatically undermine the quality of life of affected individuals (mainly adult women); it is characterized by burning and uncomfortable sensations (mainly adult women) with no clinical alterations or laboratory findings (Cerchiari et al., 2006; Coculescu, Radu, & Coculescu, 2014; Feller et al., 2017; Neville, Damm, Allen, & Chi, 2015; Ritchie & Kramer, 2018). BMS may be a primary process or be attributed to some locals or systemic pathological processes. Although the main cause of primary BMS has not been fully identified, local, systemic, and psychological factors have been associated with its pathogenesis (Kim, Kim, & Kho, 2018). As such, qualitative and quantitative salivary changes that present patients with BMS have been pointed in this direction (Imura, Shimada, Yamazaki, & Sugimoto, 2016). The evaluation of the salivary characteristics of people with BMS can help the understanding of the pathogenesis of this condition to estimate the systemic state and even to determine the response to the therapies of the affected persons. Moreover, the following advantages of the salivary analysis should be considered; for example, it can be used as a diagnostic fluid that can be easily obtained with a sufficient volume for various analyses; saliva collection involves simple methods of collecting repeated samples for serial analyses and requires noninvasive methods (Segal & Wong, 2008).

Saliva has essential properties to maintain health and homeostasis in the oral cavity (Küstner & Marques, 2002). Some salivary changes can produce neurological disorders of transduction that can produce alterations in the sensory perception of patients with BMS (Küstner & Marques, 2002; Moura et al., 2007). Patients with BMS exhibit significantly more symptoms of depression, anxiety and psychosocial stress levels and (Amenábar et al., 2008; Moura et al., 2007) these characteristics are related to changes in cortisol levels. This hormone is responsible for the regulation of physiological stress and metabolic and immunological functions and can be evaluated in saliva as an indicator of anxiety, stress, and quality of life (Amenábar et al., 2008). Although other salivary characteristics, such as the viscosity and quantification of total proteins, may serve as determinants of BMS (Küstner & Marques, 2002), evidence remain insufficient to establish their association with the etiology of BMS or even if they may be a consequence of the systemic status of BMS in this condition (Imura et al., 2016).

Although some qualitative and quantitative salivary changes have been evaluated in people with BMS (Amenábar et al., 2008; Küstner & Marques, 2002; Moura et al., 2007), findings are still contradictory. This study aimed to trace the salivary profile of women with BMS, compare them with the profile of women without BMS, and associate these results with systemic health, drug use, and effect of BMS symptoms on the quality of life. This study could improve guidance on associated therapies.

Materials and Methods

Study design and sampling

This was a case-control study conducted in Brazil, between August 2016 and March 2019. The study was approved by the Research Ethics Committee of the School of Dentistry of Federal University of Pelotas (UFPel), Brazil, with the approval code of nº 2,078,409. Individuals who agreed to participate in the study signed a free informed consent form. The study was performed under the Declaration of Helsinki.

Determination of the severity of BMS and xerostomia

The International Classification of Headache Disorders criteria were applied to establish the diagnosis of primary BMS: 1) moderate-to-severe, daily, and bilateral burning sensation in the oral mucosa; 2) burning sensation with a duration of at least 4–6 months; 3) burning sensation could remain constant or increase the intensity during the day; 4) burning sensation could improve with food or liquid intake or interfere with sleep; and 5) absence of local and systemic factors that justify burning sensation (Kim et al., 2018; Scala, Checchi, Montevecchi, Marini, & Giamberardino, 2003). A questionnaire was used to determine xerostomia (Fox, Busch, & Baum, 1987). The intensity of the symptoms was estimated using a visual analog scale wherein 1 represented the absence of symptoms, and 10 the maximum symptomatic perception experienced by the patient.

A medical history, including information related to current systemic diseases, ongoing medications and smoking and alcohol habits (CDC, 2003) was obtained for all patients. There were classified as smokers who reported having smoked more than 100 cigarettes in their lifetime and who currently smoke every day or a few days (CDC, 2003). The Eleventh Revision of the International Classification of Diseases (ICD-11)

was used as criteria to define alcohol habits (Saunders, Degenhardt, Reed, & Poznyak, 2019). All volunteers were evaluated by a dentist specializing in oral pathology. Patients also underwent laboratory tests (complete blood cell counts and glycated hemoglobin). Exclusion criteria for cases and control group included the presence of any of the following: previous history of head and neck malignancy, a history of radiation therapy in the head and neck area, chronic thyroid disease, known Sjogren's disease, any alteration in blood cell counts, those who had prior surgery of the salivary glands, rheumatoid arthritis, contact allergies, pregnant women, history of herpes zoster, with signs and symptoms of buccal lichen planus and patients treated symptomatically or that showed relief after the use of corticosteroids or antifungals in relation to the burning sensation. In the case of patients with type 2 diabetes mellitus (DM2), only those with glycated hemoglobin (hemoglobin A1C) of <7%, which is considered adequate glycemic control, were recruited in the study (Hanas & John, 2010).

Determination of unstimulated salivary flow rate (uSFR)

Saliva was collected under resting conditions in a quiet room between 9:00 A.M. and 12:00 P.M. The patients were asked to avoid smoking, brush their teeth, or consume food 1h before saliva collection. After 5 min of relaxation, they were instructed to collect their saliva for 5 min and dispense it in preweighed and labeled centrifuge tubes, in accordance with the protocol described by Navazesh (Navazesh, 2011). During the procedure, the participants were instructed to remain seated, stay quiet, and avoid speaking. After 5 min, the tubes with saliva were kept in a container at 5 °C for transport to the laboratory of Diagnosis Center of Oral Diseases (DCOD) of UFPel. Each tube was later weighed through gravimetry, a specific weight of 1.005 g/mL was assigned to the fluid, and the calculated total volume was expressed in milliliters per minute to determine the uSFR.

Salivary pH measurement

The pH of the saliva samples from each individual was determined using the saliva from the same tube used for SFR measurement. A digital pH meter (PL-600 EZDO-OMEGA model in accordance with ISO-9001 regulation) automatically provided the pH with two decimal ranges (Kitasako et al., 2008).

Salivary viscosity

Salivary samples were analyzed with a dynamic rheology technique by using a HAAKE CaBER-1 extensional rheometer (Thermo-Fisher Scientific, MA, USA). The samples were thawed, vortexed, and loaded between 6 mm-diameter plates set within an initial 2 mm range. All the measurements were performed at 37 °C by using 1 mL of each sample. Viscosity was recorded for 150 s of continuous monitoring. Rheometer plates were cleaned initially with ethanol and subsequently with distilled water; they were then air-dried between the evaluated samples. Three measurements were taken per sample, and the mean of each sample was used for statistical analysis (Christersson, Lindh, & Arnebrant, 2000; Gardner, So, & Carpenter, 2020)

Quality of life assessment

To determine the different aspects of oral function and quality of life in relation to BMS suffering, we assessed the study participants who were asked to answer a questionnaire for assessing their oral health impact profile (OHIP-14), validated in Brazil (de Oliveira & Nadanovsky, 2005), and used it to establish the quality of life-based on the sum of their answers to the 14 questions. The answer options with their respective values were as follows: never (0), rarely (1), sometimes (2), repeatedly (3), and always (4). The higher the score (out of a maximum of 56), the worse the quality of life.

Determination of salivary cortisol

On the day of the test, the saliva samples were thawed completely, vortexed, and centrifuged at 1500 rpm for 15 min at 4 °C. The supernatants were kept at room temperature and analyzed in duplicate using salivary cortisol enzyme immunoassay Enzyme-Linked ImmunoSorbent Assay (ELISA). A spectrophotometers (MFA), whose measuring capacity was 450 nm, were used to determine absorbance. The test protocol was carried out in accordance with the manufacturer's specifications (Salimetrics®).

Statistical analysis

Descriptive and quantitative data analysis was performed using the Statistical Package for the Social Sciences for Windows 22.0 (SPSS, Inc., Chicago, IL, USA). A Mann-Whitney test was performed to compare the uSFR, pH, salivary cortisol and

viscosity between BMS and control group and the average of the intensity of burning sensation among patients with primary and secondary BMS and according to types of drugs used. Spearman test was conducted to associate the levels of salivary cortisol with the impact profile of oral health. $P < 0.05$ was considered statistically significant. According to a pilot study, a sample size of 80 patients (including cases and controls) was determined in order to have an 80% power assuming a 5% significance level.

Results

Baseline characteristics

BMS group consisted of 40 white female patients (age = 62.7 ± 10.8 years; range = 37–84 years). From these, 20 (50%) reported xerostomia. Sixteen patients (40%) were classified as primary BMS and 24 (60%) as secondary BMS. The average intensity of burning sensation in evaluated by visual analog scale was 7.73 (± 2.15); 8.3 (±2.15) for primary BMS and 7.5 (±2.10) for secondary BMS. Ten patients (25%) had no comorbidity. The most frequent additional comorbidity was depression (n=21/52.5%) followed by arterial hypertension (AH) (n=20/50%) and DM2 (n=6/15%). In terms of antihypertensive drugs, the most used were diuretics (n=15/37.5%), followed by drugs of the angiotensin-renin system (ACEI) or blockers or antagonists of angiotensin II receptors (ARAII) (13/32.5%). Six (15%) patients used both types of medication at the same time. For the treatment of depression, 16 (40%) used benzodiazepines and 12 (30%) selective serotonin reuptake inhibitors (SSRIs). For DM2 treatment, 6 (15%) used metformin. Five 5 (12.5%) did not use drugs. No significant differences were observed when compared average burn intensity between patients with primary and secondary BMS, BMS patients with and without any comorbidities neither when compared patients with AH or depression that used different types of drugs to the treatment of these diseases ($p < 0.05$).

The control group was comprised of 40 white women without BMS (age = 48.5 ± 12.35 years; range = 30–66 years). From these, 5 (12.5%) reported xerostomia. Twenty patients (50%) had no comorbidity. The most frequent additional comorbidity was AH (n=15/37.5%) followed by depression (n=8/20%). Ten patients (25%) received diuretics, nine (22.5%) received ACEI or ARA II and 4 (10%) used both types of medication at the same time. For the treatment of depression, 7 (17.5%) used benzodiazepines and 5 (12.5%) SSRIs. For DM2 treatment, 3 (7.5%) used metformin.

Nineteen (47.5%) did not use drugs. The concomitant medical conditions, the most frequent drugs, and habits are summarized in table 1.

Salivary characterization

The results of salivary characterization are presented in figures 1 and 2. The mean and standard deviation for pH to BMS and control group respectively were 7.23 (± 0.52) and 7.34 (± 0.49); for uSFR were 0.35 (± 0.24) and 0.61 (± 0.61) mL/min; for cortisol were 0.361 (± 0.47) and 0.152 (± 0.23) µg/dL and for viscosity were 31.13 (± 0.23) and 45.01 (± 0.65) mPas. The BMS group showed higher levels of cortisol and lower values of uSRF and viscosity compared to the control group with statistically significant differences ($p < 0.05$). The pH values did not differ between both groups ($p > 0.05$).

Quality of life (OHIP-14 scores)

Regarding quality of life, which was measured with the OHIP-14 questionnaire, women with and without BMS revealed an average of 21.7 (DS 7.27) and 5.7 (DS 4.73), respectively. BMS patients showed worse quality of life with statistically significant differences ($p = 0.001$).

Correlation between the quality of life (OHIP-14 scores) and salivary cortisol levels

Salivary cortisol levels were positively correlated with OHIP-14 scores ($r = 0.514$ and $P = 0.0005$). When the groups were evaluated separately, we found that salivary cortisol levels were positively correlated with high OHIP-14 scores in the group of women with BMS ($r = 0.6242$ and $P = 0.0002$) (Figure 3). No correlation was found between these two variables in the control group.

Discussion

BMS is an idiopathic condition characterized by chronic pain and a burning sensation in the oral mucosa (Feller et al., 2017). The prevalence of the syndrome is higher among women, especially after menopause. The mean age of women with BMS observed in our sample agree with the data described in the literature that indicate an average of around 60 years due to biological, sociocultural and psychological factors (Coculescu et al., 2014; Moghadam-Kia & Fazel, 2017; Ritchie & Kramer, 2018). The female predominance of BMS increases with age, which may suggest that hormonal changes, especially in the activity of estrogen and progesterone that produce hot

flashes, interruption of control mechanisms in menopause, increased night sweating, and emotional lability, play an important role in the etiopathogenesis of the syndrome (Ślebioda & Szponar, 2014).

Some evidence suggest that the burning symptom may arise from the direct effect of the drugs used in to treat systemic conditions, such as diuretics (Soares, Chimenos-Küstner, Subira-Pifarre, Rodríguez, & López-López, 2005), IECA or ARAII (Salort-Llorca, Mínguez-Serra, & Silvestre, 2008) and not necessarily due to the presence of comorbidity. No differences were observed between primary and secondary BMS in terms of the intensity of burning or respect to the presence of xerostomia in the present study. In addition, AH, comorbidity not associated with the diagnosis of secondary BMS, was highly frequent in BMS group (Mitsikostas, Ljubisavljevic, & Deligianni, 2017). Our results agree with the current evidence that does not associate AH with BMS in terms of its etiopathogenesis (Moghadam-Kia & Fazel, 2017) and the high frequency of this comorbidity observed in BMS group in our study, can be related with a worldwide trend where more than 66% of people over 60 in the world presents HA (Banegas et al., 2017). Another fact that could explain the absence of differences in burning intensity and the presence of xerostomia between primary and secondary is the presence of adequate glycemic values in BMS group (Moore, Guggenheimer, & Orchard, 2007).

The fact that in our study depression was the common comorbidity in patients with BMS, agrees with many previous findings (Di Stasio et al., 2018; Schiavone et al., 2012). A previous study found that patients with BMS with psychological problems (secondary BMS) have a higher intensity of burning sensation in BMS (Kim et al., 2018). This phenomenon could be the result of a statistically significant decrease in uSRF as a result of the use of antidepressant drugs, such as benzodiazepines, what would exacerbate the intensity of the burning sensation and could induce xerostomia (Grushka, Epstein, & Mott, 1998; Kim et al., 2018). The fact that, in our study, no changes were observed in relation to FSUr, could explain the absence of differences for the intensity of the burning sensation and the presence of xerostomia between primary and secondary SMB. In addition, it is indicated that the percentage of BMS patients with xerostomia varies from 10 to 66% (Thomson, Chalmers, Spencer, & Ketabi, 1999). This variability and the lack of association between this symptom and

BMS in our research, point out that xerostomia in the syndrome may have a multifactorial etiology (Soares et al., 2005).

Hyposalivation or a decrease in uSRF, can promote the lack of chemical and physical protection of the oral mucosa, facilitating the establishment of BMS (Fernandes et al., 2009). According to our results, the uSRF in BMS was statistically lower than that of women without the syndrome. Several factors could be determining this result. The chronic assumption of antihypertensive, anxiolytic and antidepressant medications, on the one hand, and the contextual presence of psychological distress on the other hand, could influence the basal tone of the submandibular, sublingual salivary glands and minor salivary glands, responsible for the non-basal salivary flow unstimulated. These glands are innervated by parasympathetic fibers, which could be affected in BMS in the case of a neuropathic origin of the syndrome. However, an assessment to distinguish the type of salivary secretion would have to be done to confirm this phenomenon (Poon, Su, Ching, Darling, & Grushka, 2014). In our study, 25% of women with BMS had a resting salivary flow of less than 0.2% (compared to none in the control group). It is assumed that in order to have SRF ≤ 0.2 ml/min, it would be necessary that approximately 50% of the glandular parenchyma was affected. In this sense, it has been suggested that glandular hypofunction could be a contributing factor in BMS, a phenomenon that would be more frequent in postmenopausal women (Vaidya, 2012) and could be enhanced due to the anticholinergic effect of medications used to treat comorbidities associated with aging such as antidepressants (Chimenos Küstner & Marques Soares, 2002; Lemay, Hiscock, & Keegan, 1988). The decrease in salivary flow could influence the reception of stimuli and alter the perception in patients with BMS (Chimenos Küstner & Marques Soares, 2002). The association between BMS and the lower salivary flow found in the present study and agreement with the explanation of previous research may be due to the simultaneity of systemic diseases, medication use, aging and even associated glandular damage, which supports the hypothesis of a multifactorial etiology of BMS (Ship et al., 1995; Soares et al., 2005).

According to our research, BMS does not appear to be determined by differences in salivary pH, which has also been described in other research (Soares et al., 2005). Even if there was not enough evidence found in the literature to establish an association between differences in salivary pH and SMB, it is known that even this

salivary characteristic could influence oral sensitivity, it would not necessarily determine the burning sensation (Matsuo, 2000). The viscosity, related to the energy dissipated during salivary flow (Zussman, Yarin, & Nagler, 2007) should be carefully evaluated in relation to the etiology of BMS (Chimenos Küstner & Marques Soares, 2002). In our study, the salivary viscosity of women without the syndrome was significantly higher than that of women with BMS. Even evidence that associates salivary viscosity with the syndrome is scarce, it was described that changes in this salivary characteristic could induce feelings of discomfort associated with BMS (Imura et al., 2016). Another study showed an increase in salivary viscosity associated with a relief of xerostomia (BMS-associated symptom) in patients treated with capsaicin (Gardner et al., 2020), one possible therapy of the syndrome. Even our results suggest that some salivary characteristics founded in BMS patients could determine suffering of the syndrome, which agrees with previous evidence (Chimenos Küstner & Marques Soares, 2002), we must consider that the differences between the frequency of drug consumption and even the difference in age average between our BMS group and the control group, could also influence our results.

Studies have established an association between the poorer quality of life and depression (Demyttenaere, De Fruyt, & Huygens, 2002). According to our results, women with BMS have a worse quality of life than women who do not present the syndrome possibly related to emotional disorders, such as depression (Di Stasio et al., 2018; Grushka et al., 1998; Kim et al., 2018; Merigo et al., 2007). Evidence has shown that these psychological variables are related to changes in cortisol levels and this hormone can be evaluated in saliva as an indicator of anxiety and stress (Amenábar et al., 2008). Our results are consistent with some studies establishing that patients with BMS have higher salivary cortisol levels compared with those individuals without the syndrome (Amenábar et al., 2008). Objective factors such as income, age, weight, and social group, and lifestyle factors such as tobacco and alcohol consumption, drug use, exercise, diet and other aspects associated with quality of life (Ventegodt, Flensborg-Madsen, Andersen, & Merrick, 2008) not evaluated in our research, would have to be considered in future studies.

As the pathogenesis of BMS is apparently multifactorial, it is difficult to rule out all the variables that could determine its suffering. Factors such as age, presence of comorbidities (with or without association with BMS), use of drugs used for its

treatment, and even some undiagnosed disease, are elements that, even trying to isolate and standardize between in cases and controls groups, is not always possible in their wholeness. Another limitation of the present study was that it did not consider the dose of the drug once it's described that drug-induced BMS is dose-dependent (Salort-Llorca et al., 2008). According to our results, lower USFR, lower salivary viscosity and higher levels of salivary cortisol may be a reflection or part of the etiology of BMS. Our study also revealed that the percentage of depression in BMS women was higher than that observed in the control group and that most women with a syndrome used at least one medication to treat their comorbidities. These factors could also influence the differences found in the salivary characteristics in this study. Even so, salivary characteristics can help to better understand both the etiopathogenesis and the consequences of this still enigmatic syndrome.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL AND INFORMED CONSENT

The study was approved by the Research Ethics Committee of the School of Dentistry-Federal University of Pelotas, Pelotas, Brazil (UFPel), with the approval protocol code of nº 2.078.409. Individuals who agreed to participate in the study signed a free informed consent form. The study was performed in accordance with the Declaration of Helsinki.

AUTHORSHIP CONTRIBUTIONS

J.P.A.S designed the study, also being responsible for all stages of development of this research, from the acquisition and analysis of data and writing of the manuscript. A.C.U.V and S.B.C.T, were the teachers responsible for guiding the study, participating in all stages of this study, from conception and design of the study, interpretation of results and writing of the manuscript. W.L.O.R; A.P.N.G and A.F.S were indispensable during the acquisition and interpretation of data and critical review of the manuscript. M.S.F; A.M.G and A.W made contributions related to the acquisition and interpretation of data and critical review of the manuscript. All authors approved the final version of the manuscript.

ORCID

Juan Pablo Aitken Saavedra: http://orcid.org/0000-0002-2525-8433

Ana Carolina Uchoa Vasconcellos: http://orcid.org/0000-0001-6560-9536

Wellington Luiz De Oliveira da Rosa: http://orcid.org/0000-0001-6992-3127

Ana Paula Neutzling Gomes: http://orcid.org/0000-0003-2214-5246

Adriana Fernandes da Silva: http://orcid.org/0000-0002-3942-7138

Matheus dos Santos Fernandez: http://orcid.org/0000-0001-7781-3083

Andresa Moreira Goicochea: http://orcid.org/0000-0001-6784-0571

Sandra Beatriz Chaves Tarquinio: http://orcid.org/0000-0003-1496-4137

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FIGURE LEGENDS

Figure 1. Comparison of the medians of uSFR, pH, and salivary cortisol between the group of women with BMS and the control group. *Statistically significant differences ($p<0.05$).

Figure 2. Viscosity measurements were performed at 37 °C by using 1 mL of each sample and recorded for 150 s of continuous monitoring. Average viscosity after 150 s of continuous monitoring in the control group and in the group of women with BMS showed statistically significant differences ($p = 0.001$).

Figure 3. Scatter diagram showing the association between OHIP-14 scores and salivary cortisol levels in patients with BMS as revealed by Spearman correlation analysis.

Table 1. Baseline characteristics of women with and without burning mouth syndrome.

				Comorbidity (n/%)				Drugs (n/%)					
								Antihypertensives (n)		*Antidepressants (n)*		*Antidiabetic (n)*	
Groups	Age (SD)	Xerostomia (%)	Average of intensity (SD)	No comorbidity	AH	Depression	DM2	Diuretic	ACEIs or ARAII	*Benzodiazepine*	SSRIs	Metformin	Without Drugs
BMS (n=40)	62.7 (10.8)	20 (50)	7.73 (2.15)	10 (25)	20 (50%)	21 (52.5)	6 (15)	15 (37.5)	13 (32.5)	16 (40)	12 (30)	6 (15)	5 (12.5)
CONTROL (n=40)	48.5 (12.35)	5 (12.5)	NA	20 (50)	15 (37.5)	8 (20)	3 (7.5)	10 (25)	9 (22.5)	7 (17.5)	5 (12.5)	3 (7.5)	19 (47.5)

Legend: Diuretic (furosemide, hydrochlorothiazide); ACEIs: angiotensin-renin system (captopril, enalapril and lisinopril); ARAII: angiotensin II receptor blockers or antagonists (eprosartan and candesartan); *Benzodiazepines (*alprazolam clonazepam, diazepam); SSRIs: selective serotonin reuptake inhibitors (fluoxetine, paroxetine, sertraline); Beta-1 selective blockers (atenolol); *NA: Not apply;
SD: standard deviation**.**

FIGURES

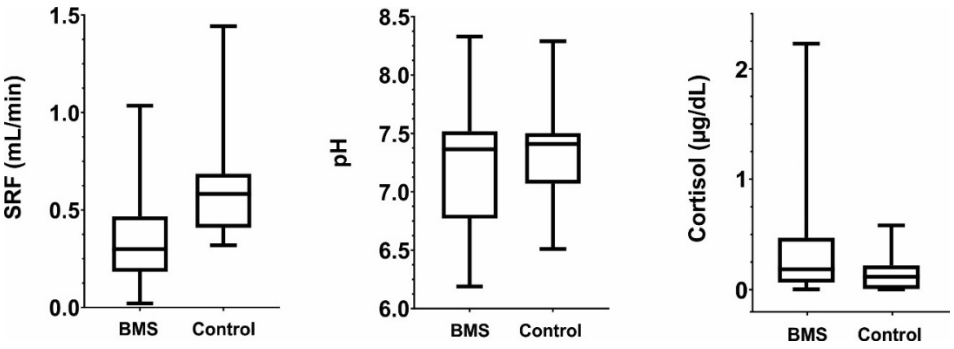

Figure 1. Comparison of unstimulated salivary flow rate (uSFR), pH, and salivary cortisol between women with burning mouth syndrome and women in the control group.

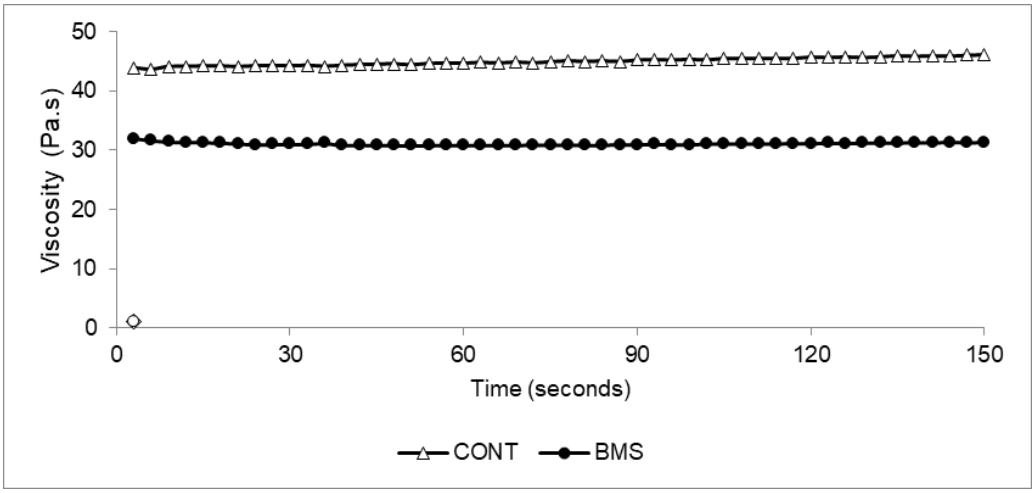

Figure 2. Comparison of the average viscosity of the whole saliva between women with burning mouth syndrome and women in the control group.

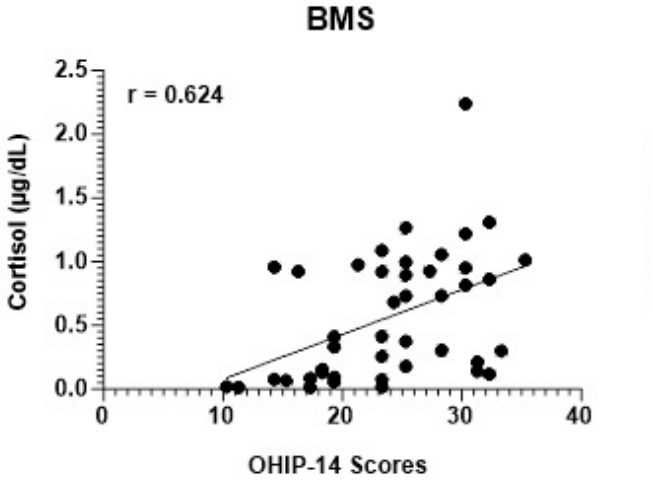

Figure 3. Association between scores ot OHIP-14 (quality of life) and salivary cortisol levels in women with burning mouth syndrome and women in the control group.

8 Considerações finais

Diante dos resultados obtidos dos quatro estudos realizados e que compõem esta tese, pudemos concluir que:

Embora não pudesse ter sido possível o estabelecimento da associação entre o FAT1 salivar e tecidual, devido principalmente à natureza da proteína detectada por cada reagente utilizado para avaliar os seus níveis salivares e teciduais, a maior e mais frequente concentração citoplasmática do FAT1 observada nas células tumorais, poderia sugerir caraterísticas tumorais como agressividade e desdiferenciarão celular e ser útil como marcador tecidual da progressão do CEC.

Em relação à esta neoplasia e à necessidade de estabelecer diagnóstico em estágios iniciais da doença, evitando sua progressão, o presente trabalho de tese também sugere que o Mg pode ser um eletrólito importante na detecção de lesões orais potencialmente malignas e provavelmente no seu desenvolvimento potencial como neoplasia maligna. Um aumento dos níveis deste eletrólito neste tipo de distúrbios, poderia estar relacionado com um aumento da angiogênese e ser um estímulo inicial para a proliferação endotelial na progressão do CEC. Tal angiogênese provavelmente necessitaria da atuação de outros fatores, do que níveis de Mg, na manutenção da neovascularização observada nesta neoplasia. Mais estudos são necessários para confirmar esta teoria e para fornecer informações adicionais sobre o papel dos eletrólitos na progressão do câncer e o seu potencial uso como biomarcadores nesta doença.

Em relação à síndrome de ardência bucal, menor fluxo salivar não estimulado, menor viscosidade salivar e níveis mais altos de cortisol salivar podem ser um reflexo ou parte da etiologia da SAB. Nosso estudo também revelou que o percentual de depressão em mulheres com SAB é maior do que o observado no grupo controle e que a maioria das mulheres com a síndrome usava pelo menos um medicamento para tratar suas comorbidades. Esses

fatores também podem influenciar as diferenças encontradas nas características salivares neste estudo. Mesmo assim, estas características podem ajudar a entender melhor a etiopatogenia e as consequências dessa síndrome, ainda enigmática.

Por outro lado, a análise salivar qualitativa, segundo a revisão sistemática realizada, aponta para uma origem multidisciplinar da síndrome. Os biomarcadores orgânicos avaliados sugerem que a SAB possa ser determinada ou refletir a ação de fatores neuropáticos (também sugeridos por alguns eletrólitos salivares), fatores inflamatórios, emocionais, imunológicos e hormonais. A falta de padronização, tanto para o estabelecimento do diagnóstico da síndrome, quanto para o uso de medicamentos nos pacientes afetados (que podem ter consequências principalmente nas variáveis quantitativas salivares avaliadas), deve ser um fator a ser aprimorado em pesquisas futuras. Nesse sentido, e embora sejam necessários mais estudos com variáveis mais padronizadas, características salivares qualitativas podem ser úteis na avaliação da etiologia e do estado sistêmico, na progressão e até na orientação de terapias mais eficazes dessa síndrome ainda enigmática.

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Apêndices

Apêndice A – Nota da Tese

Avaliação quantitativa e qualitativa das alterações salivares na síndrome de ardência bucal (condição sistêmica) e no carcinoma espinocelular oral (progressão e resposta às terapias)

Quantitative and qualitative assessment of salivary changes in burning mouth syndrome (systemic condition) and oral squamous cell carcinoma (progression and response to therapies).

A saliva como meio de diagnóstico de doenças bucais e sistêmicas tem sido objeto de estudo com o intuito de acrescentar sua utilização como exame complementar, considerando as vantagens do seu uso. O carcinoma espinocelular (CEC) é a neoplasia maligna mais comum da cavidade oral e apresenta um comportamento agressivo e invasivo. A detecção precoce de lesões orais potencialmente malignas (LOPM) é essencial para evitar a progressão para o CEC e a detecção de mudanças na composição eletrolítica salivar poderia ser de utilidade neste sentido. Além disso, um recente sequenciamento massivo mostrou que dos genes mutados mais associados com o desenvolvimento de CEC, o *FAT1* é o único que codifica uma proteína secretória, potencialmente detectável na saliva e utilizável como biomarcador. A síndrome de ardência bucal (SAB) é uma condição crônica caracterizada por uma sensação de queimação na boca sem achados clínicos ou laboratoriais que justifiquem esse sintoma e pode afetar dramaticamente a qualidade de vida dos afetados. Alterações salivares poderiam determinar a sua etiologia ou refletir o status sistêmico na síndrome. Segundo os nossos resultados, 1) Maiores níveis de Mg salivar estão associados às alterações displásicas vistas nas LOPMs. 2) O uso de FAT1 nativo como marcador salivar da progressão ou da resposta à terapia no CEC não é recomendado, de acordo com a metodologia utilizada no presente estudo. A imunomarcação citoplasmática de FAT1, amplamente mais frequente em CEC sugerem seu fenótipo aberrante e poderia estar associado com caraterísticas neoplásicas como agressividade. 3) A análise salivar qualitativa aponta para uma origem multidisciplinar na SAB. 4) Alterações salivares presentes em mulheres com SAB como menor uSRF e menor viscosidade poderiam estar associadas com a patogênese da síndrome o ser reflexo do status sistêmico das afetadas. Maiores níveis de cortisol salivar refletem a pior qualidade de vida que mulheres com SAB relatam ter.

Campo da pesquisa: Clinica Odontologica Clínica Odontológica, ênfase Diagnóstico Bucal

Candidato: Juan Pablo Aitken Saavedra, mestre em *Ciências Odontológicas ênfase em Patologia e Medicina Bucal e especialista em Patologia Buco Maxilo Facial pela Universidade do Chile.*

Data da defesa e horário: 30/04/2020

Local: Emissão de parecer. Programa de Pós-graduação em Odontologia da Universidade Federal de Pelotas.

Membros da banca: Profª. Drª. Sandra Beatriz Chaves Tarquinio (Presidente). Doutora em Odontologia (Área de Concentração em Patologia Bucal) pela Universidade de São Paulo. Profª. Drª. Maria Cássia Ferreira Aguiar. Doutora em Odontologia (Área de Concentração em Patologia Bucal) pela Universidade de São Paulo. Prof. Dr. Gonzalo Rojas Alcayaga. Doutor em Odontologia (Área de Concentração em Psicologia e Patologia Bucal) pela Universidade do Chile. Profª. Drª. Adriana Etges. Doutora em Odontologia (Área de Concentração em Patologia Bucal) pela Universidade de São Paulo. Profª. Drª. Ana Paula Neutzling Gomes (suplente). Doutora em Odontologia (Área de Concentração em Patologia Bucal) pela Universidade de São Paulo. Profª. Drª. Karine Duarte da Silva (suplente). Doutora em Odontologia (Área de Concentração em Patologia Bucal) pela Universidade Federal de Pelotas.

Orientadora: Profa. Dra. Sandra Beatriz Chaves Tarquinio. Doutora em Odontologia (Área de Concentração em Patologia Bucal) pela Universidade de São Paulo.

Coorientadores: Profa. Dra. Ana Carolina Uchoa Vasconcellos, Prof. Dr. Ricardo Ramires-Fernandez.

Informação de contato: Juan Pablo Aitken Saavedra. Gonçalves Chaves, 457- Centro de Diagnóstico de Doenças da Boca

Apêndice B – Súmula do currículo do candidato

Juan Pablo Aitken Saavedra nasceu em 1979 em Santiago do Chile. Completou o ensino fundamental no Instituto Miguel Leon Prado. No ano 1998, ingressou na *Faculdade de Odontologia da Universidade do Chile* tendo sido graduado de cirurgião-dentista em 2005. Em 2011, ingressou no Mestrado em *Ciências Odontológicas ênfase em Patologia e Medicina Oral,* sob orientação do Professor Gonzalo Rojas Alcayaga, onde trabalhou na área de biomarcadores salivares em diabete melito tipo 2. Dissertação defendida e aprovada em 2014, ano que ingressou como Professor Assistente do Departamento de Patologia e Medicina Oral na Universidade do Chile. Entre 2013 e 2015 realizou a especialização em Patologia Buco Maxilo Facial na mesma instituição. Em 2015 iniciou o Doutorado na Universidade Federal de Pelotas (UFPel) na área de Clínica Odontológica ênfase em Diagnóstico Bucal sob orientação da Profa. Dra Sandra Beatriz Chaves Tarquinio. Durante o período 2016/1 – 2020/1 de doutorado foi bolsista do programa OAS-Grupo Coimbra de Universidades Brasileiras (GCUB) Scholarship.

Publicações:

Escobar, Alejandro, **Aitken-Saavedra, Juan**. Xerostomia: An Update of Causes and Treatments in: Salivary Glands - New Approaches in Diagnostics and Treatment.2 ed.: IntechOpen, 2019

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Anexos

Anexo A – Parecer do Comitê de Ética em Pesquisa da Faculdade de Odontologia. UFPel

UFPEL - FACULDADE DE ODONTOLOGIA DA UNIVERSIDADE FEDERAL DE

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito de um substituto salivar caseiro à base de camomila (Matricaria chamomilla) e linhaça (Linum usitatissimum) no alívio dos sintomas da Síndrome da Ardência Bucal

Pesquisador: ANA PAULA NEUTZLING GOMES

Área Temática:

Versão: 1

CAAE: 67305317.2.0000.5318

Instituição Proponente: Faculdade de Odontologia da Universidade Federal de Pelotas/ FO-UFPel

Patrocinador Principal: Universidade Federal de Pelotas

DADOS DO PARECER

Número do Parecer: 2.078.409

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PELOTAS, 23 de Maio de 2017

Assinado por:
Fernanda G Pappen
(Coordenador)

Endereço: Rua Gonçalves Chaves, 457
Bairro: Centro **CEP:** 96.015-560
UF: RS **Município:** PELOTAS
Telefone: (53)3222-4439 **Fax:** (53)3222-4439 **E-mail:** cep.fop@gmail.com

Anexo B – Parecer da Comissão Nacional de Ética em Pesquisa

COMISSÃO NACIONAL DE
ÉTICA EM PESQUISA

PARECER CONSUBSTANCIADO DA CONEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Detecção de FAT1 (Atypical Cadherin 1) tecidual e salivar e sua avaliação como um preditor da progressão das displasias de baixo e alto risco para o carcinoma espinocelular oral

Pesquisador: Sandra Beatriz Chaves Tarquinio

Área Temática: Pesquisas com coordenação e/ou patrocínio originados fora do Brasil, excetuadas aquelas com copatrocínio do Governo Brasileiro;

Versão: 5

CAAE: 67302017.0.0000.5318

Instituição Proponente: Faculdade de Odontologia da Universidade Federal de Pelotas/ FO-UFPel

Patrocinador Principal: Faculdade de Odontologia

DADOS DO PARECER

Número do Parecer: 2.476.399

Situação do Parecer:
Aprovado

BRASILIA, 30 de Janeiro de 2018

Assinado por:
Jorge Alves de Almeida Venancio
(Coordenador)

Endereço: SEPN 510 NORTE, BLOCO A 3º ANDAR, Edifício Ex-INAN - Unidade II - Ministério da Saúde
Bairro: Asa Norte **CEP:** 70.750-521
UF: DF **Município:** BRASILIA
Telefone: (61)3315-5878 **E-mail:** conep@saude.gov.br