UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

NATÁLIA RANGEL PALMIER

NOVAS PERSPECTIVAS RELACIONADAS ÀS CARACTERÍSTICAS CLÍNICAS E BIOMARCADORES PROTEÔMICOS SALIVARES DAS TOXICIDADES ORAIS INDUZIDAS PELA RADIOTERAPIA

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Estomatopatologia, na Área de Estomatologia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Stomatopathology, in Stomatology area.

Orientador Prof. Dr. Alan Roger dos Santos Silva
Coorientadora: Profa. Dra. Ana Carolina Prado Ribeiro

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA NATÁLIA RANGEL PALMIER, E ORIENTADA PELO PROF. DR. ALAN ROGER DOS SANTOS SILVA E COORIENTADA PELA PROFA. DRA. ANA CAROLINA PRADO RIBEIRO.

Piracicaba
2021

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

P185n

Palmier, Natália Rangel, 1992-
Novas perspectivas relacionadas às características clínicas e biomarcadores proteômicos salivares das toxicidades orais induzidas pela radioterapia / Natália Rangel Palmier. – Piracicaba, SP : [s.n.], 2021.

Orientador: Alan Roger dos Santos Silva.
Coorientador: Ana Carolina Prado Ribeiro.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Neoplasias bucais. 2. Marcadores biológicos. 3. Proteínas e peptídeos salivares. I. Santos-Silva, Alan Roger, 1981-. II. Ribeiro, Ana Carolina Prado, 1981-. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: New perspectives of clinical features and salivary protein biomarkers of radiation induced oral toxicities
Palavras-chave em inglês:
Mouth neoplasms
Biological markers
Salivary proteins and peptides
Área de concentração: Estomatologia
Titulação: Doutora em Estomatopatologia
Banca examinadora:
Alan Roger dos Santos Silva [Orientador]
Cesar Augusto Migliorati
Wilfredo Alejandro Gonzalez Arriagada
Luiz Alcino Monteiro Gueiros
Márcio Ajudarte Lopes
Data de defesa: 20-08-2021
Programa de Pós-Graduação: Estomatopatologia

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0001-9588-6227
- Currículo Lattes do autor: http://lattes.cnpq.br/6810029388361979

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Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 20 de agosto de 2021, considerou a candidata NATÁLIA RANGEL PALMIER aprovada.

PROF. DR. ALAN ROGER DOS SANTOS SILVA

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A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

“Thus, the task is not so much to see what no one yet has seen, but to think what nobody yet has thought about that which everybody sees.”

(J Arthur Schopenhauer)

DEDICATORIA

Aos meus amados pais, Gilberto e Monica, meu irmão Gustavo, meu noivo Alexandre e a minha afilhada Valentina, vocês são minha força e meu maior incentivo, esta vitória é de todos nós, sem vocês nada disso seria possível.

Obrigada de todo meu coração, eu amo vocês.

AGRADECIMENTOS

À Deus, por todas as bênçãos concedidas diariamente.

À Universidade Estadual de Campinas, na pessoa do Magnífico Reitor, Prof. Dr. Antonio José de Almeida Meirelles.

À Faculdade de Odontologia de Piracicaba, na pessoa de seu Diretor, Prof. Dr. Francisco Haiter Neto e seu Diretor Associado, Prof. Dr. Flávio Henrique Baggio Aguiar.

À Profa. Dra. Karina Ruiz, Coordenadora Geral da Pós-Graduação da Faculdade de Odontologia de Piracicaba.

Ao Coordenador do Programa de Pós-Graduação em Estomatopatologia, Prof. Dr. Pablo Agustin Vargas.

O presente trabalho foi realizado com apoio da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), processo nº 2018/04657-8.

Ao meu orientador, Prof. Dr. Alan Roger dos Santos Silva, não há palavras que possam expressar minha gratidão por você. Só tenho a agradecer por todos os ensinamentos, suporte e oportunidades. Alan muito obrigada por ter sido tão presente não só como orientador, mas também como amigo. Obrigada por todo trabalho, por toda paciência, por todo carinho e atenção não só comigo, mas também com minha família. Obrigada por acreditar em mim e no meu potencial e por me demonstrar isso. Obrigada por se preocupar não somente com os assuntos de pesquisa, mas também por entender e se preocupar se estava tudo bem comigo como pessoa, se eu estava feliz na pós-graduação e sempre disponível para me ajudar no que fosse necessário. As conquistas são nossas e tenho certeza de que muitas mais estão por vir. *Thanks a lot for everything!*

À Dra. Ana Carolina Prado Ribeiro, minha querida co-orientadora. Esse termo com certeza não faz jus a todo trabalho e suporte que você me deu ao longo desses 4 anos que temos trabalhado juntas de forma mais direta. Desde o primeiro dia em que cheguei em São Paulo você me recebeu de braços abertos, me incentivou, me ajudou e batalhou comigo de forma incansável desde a seleção de cada paciente, coletas de saliva, acompanhamento, compra de materiais, parcerias, você esteve e está presente em cada uma dessas vitórias que compartilhamos e tenho certeza de que teremos muitas mais para comemorar. Eu te admiro imensamente como a pessoa e profissional que você é, e sou muito grata por ter a

oportunidade de aprender tanto com você.

À Dra. Thaís Bianca Brandão, você é um exemplo sem igual de profissionalismo, cuidado e carinho com os pacientes. Agradeço toda confiança e oportunidades que me reservou desde o começo da nossa jornada quando iniciei minhas idas ao ICESP. Obrigada por incentivar e acreditar nas pesquisas e por comemorar junto cada vitória. Obrigada por confiar em mim para fazer parte da equipe do Serviço de Odontologia Oncológica do ICESP, sou e serei eternamente grata por isso. Ter a oportunidade de trabalhar de forma direta com você me faz admirá-la cada vez mais como profissional e como pessoa. Ver a forma humana como você trata todos os pacientes, todo o cuidado e carinho me inspira todos os dias a me tornar uma profissional melhor. Muito obrigada por tudo!

À minha querida orientadora da graduação Profa. Dra. Aline Corrêa Abrahão. Obrigada por me apoiar e me fazer crescer. Obrigada por me iniciar nesse caminho. Por me escolher para trabalhar com você e por todas as oportunidades que você me proporcionou. Obrigada por vibrar tanto com as minhas conquistas, você também faz parte delas. Uma vez orientadora sempre orientadora. Muito obrigada por tudo!

Aos Profs. Drs. das Áreas de Semiologia e Patologia da Faculdade de Odontologia de Piracicaba: Márcio Ajudarte Lopes, Pablo Agustin Vargas, Oslei Paes de Almeida, Ricardo Della Coletta, Edgard Graner e Jacks Jorge Júnior pelos ensinamentos durante esses seis anos de pós-graduação.

Ao Orocentro (FOP-UNICAMP) em nome dos Profs. Alan Roger dos Santos Silva e Márcio Ajudarte Lopes, por ter sido um local de imenso aprendizado durante o mestrado que me auxiliaram a me transformar na profissional que sou hoje.

A todos os membros do Serviço de Odontologia Oncológica do Instituto do Câncer do Estado de São Paulo (ICESP-FMUSP) em especial Dra. Karina Morais Faria Barreto, Dra. Maria Cecília Querido de Oliveira; Dr. Aljomar José Vechiato Filho, vocês tornaram toda essa jornada possível. Obrigada por todo carinho, por me ajudar durante todas as etapas das pesquisas, por me cederem seus ambulatórios. Ninguém chega a lugar nenhum sozinho, e hoje com certeza eu estou aqui com todos esses trabalhos graças a todo suporte que vocês me proporcionaram. Obrigada por acima de tudo serem meus amigos, minha família em São Paulo, sou muito grata de todo coração.

A Profa. Dra. Adriana Franco Paes Leme coordenadora do Laboratório Nacional de Biociências (LNBio), assim como todas as técnicas que auxiliaram no processo de análise e

geração de dados da presente tese.

Aos meus colegas do setor de Medicina Bucal do Hospital Sírio Libanês, Claudia Joffily e Wagner Gomes por me darem suporte nos momentos necessários, principalmente nos momentos em que tive que me afastar fisicamente do ambulatório para realização das análises. Obrigada por todo o carinho, apoio e torcida para que esse momento fosse possível.

A todos os meus colegas de pós-graduação: Débora, Carolina, Jéssica, Mariana, Renata, Isabel, Marisol, Juliana de Souza, Iara, Luan, Raissa de Sá, João, Erison, Maurício por terem tornado meus dias na semiologia mais legais, pelo companheirismo, pelas risadas pelas brincadeiras, por me ajudarem sempre nem que fosse com um simples abraço e uma palavra de incentivo sou muito feliz por ter cultivado a amizade de vocês.

Agradeço em particular à Iara Aquino e Erison Santos por toda ajuda durante o preparo das amostras, todo cuidado e disponibilidade. Este trabalho não seria possível sem vocês.

Agradeço as minhas companheiras de ICESP Carol, Elisa e Letícia. Obrigada por todo apoio, pela ajuda nas coletas, nos agendamentos, na fotos, vocês participaram ativamente de todo esse processo e tornaram esses anos mais tranquilos, leves e alegres.

A minha queria amiga Patrícia Fernandes, você é uma das pessoas de melhor coração que eu conheço. Obrigada por compartilhar tantos momentos comigo. Obrigada pelos 6 meses que moramos juntas e por tudo que você fez por mim, sempre me estendendo a mão quando precisei. Obrigada por nossa amizade, obrigada pelo seu carinho e companheirismo. Te amo!

Ao meu querido Bruno Augusto Mariz, é até difícil escrever um agradecimento para você. Você é meu parceiro desde o seu *day one*! Tanto que todos achavam que nós éramos da mesma turma. Você é um irmão para mim, um amigo que sei que posso contar de olhos fechados seja para me ajudar em momentos difíceis seja para fazer estatística em 1 hora porque eu estou desesperada. Você é esse cara! Tenho certeza de que você terá um caminho brilhante pela frente e eu estarei em todos os momentos de pé aplaudindo você! Obrigada por tudo! Te amo!

A minha melhor amiga Barbara Grisolia, não tenho palavras para descrever a importância que você tem na minha vida. Obrigada por cada "tá aí", "amiga preciso desabafar", "amiga me ajuda". Obrigada por sempre acreditar em mim e comemorar todas as minhas conquistas como se fossem suas. Obrigada por todo amor que você demonstra, por me acolher como família. Você é minha irmã de outra mãe e faz uma falta imensa no

meu dia a dia. Te amo!

Ao meu noivo Alexandre Camargo, a admiração que eu tenho por você é fora de série. Te admiro como homem, como pessoa, como amigo e como profissional. Você é a melhor coisa que já me aconteceu. Obrigada por me apoiar em cada momento, obrigada por ser meu parceiro em todas as dificuldades e celebrar de forma tão intensa cada vitória, cada passo que conquistamos juntos. Obrigada por topar minhas loucuras, por cuidar de mim e da minha família, por apoiar minhas decisões e fazer de tudo para que eu alcance todos os meus sonhos. Você me faz feliz de mais formas do que eu achava ser possível, você me inspira, me incentiva e me fortalece todos os dias. Obrigada por toda paciência, especialmente nos últimos meses com toda essa jornada de finalização da tese, obrigada por compreender minhas dificuldades e fazer de tudo para que tudo acontecesse de forma mais tranquila. Na falta de um verbo melhor só tenho a dizer que te amo cada dia mais e amo a família que estamos construindo com a chegada do nosso Simba.

Ao meu irmão Gustavo Palmier, você é meu primeiro e melhor amigo, meu confidente, *my little bro*. Tenho muito orgulho da pessoa maravilhosa que você é, do coração enorme que você tem e do pai incrível que você é para nossa querida Valentina. Obrigada por sempre cuidar de mim eu te amo muito.

À minha mãe Monica Palmier, minha rocha, meu exemplo, sinônimo de todo meu amor. Você é a mulher mais incrível que eu conheço e eu tenho muito orgulho de ser sua filha. Não existem palavras que possam expressar a minha alegria de ser sua filha, de ter aprendido tanto com você e de receber sempre todo o seu amor. Obrigada por sempre nos colocar em primeiro lugar, por fazer o impossível tanto por mim quanto pelo Gustavo. Obrigada por ser simplesmente a melhor mãe do mundo! Eu amo você.

Ao meu pai Gilberto Palmier, você é o espelho que eu sempre tento seguir. Obrigada por fazer todos os meus sonhos possíveis, obrigada por nunca medir esforços para me ajudar. Obrigada por me guiar e me apoiar mesmo quando discordou das minhas opiniões e vontades. Você é um exemplo de integridade, perseverança, respeito e amor. Eu tenho muito orgulho de te chamar de meu pai. Eu te amo.

RESUMO

Descobertas recentes acerca do efeito sinérgico entre as diversas toxicidades orais induzidas pela radioterapia (RT) no tratamento do câncer de cabeça e pescoço (CCP) renovaram a importância de uma melhor compreensão fisiopatológica deste agrupamento de sintomas orais e do modo como eles impactam desfechos odontológicos e médicos de pacientes oncológicos. Nesse contexto, atualmente, existe uma grande busca pelo conhecimento do perfil molecular das doenças com potencial diagnóstico ou balizador de tratamentos personalizados mais eficientes e menos tóxicos. Neste campo, a presente tese de doutoramento se propôs a categorizar padrões clínicos e moleculares salivares de toxicidades orais induzidas pela RT de pacientes com carcinoma espinocelular (CEC) de cavidade oral e orofaringe. Os resultados desta tese estão apresentados por meio dos manuscritos de dois estudos clínicos tipo coorte, uma revisão sistemática, uma revisão narrativa e dois estudos de análise de perfil molecular. Os estudos clínicos incluíram pacientes com CCP submetidos à RT com intuito curativo associados ou não a cirurgia prévia ou concomitância com protocolos de quimioterapia. Resultados clínicos demonstram evidente impacto da introdução da quimioterapia concomitante no desenvolvimento precoce de disgeusia intensa, bem como correlação estatisticamente significante entre xerostomia e mucosite oral (MO) no desenvolvimento de disgeusia ($r=0.29$ e $r=0.42$, respectivamente - $p<0.001$, para ambos). Essas associações foram validadas pela revisão sistemática, que revelou um padrão específico de agrupamento de sintomas orais e gastrointestinais que se correlacionam e sobrepõe levando tanto ao agravamento de toxicidades agudas como ao favorecimento do desenvolvimento de toxicidades crônicas como cárie de radiação (CR) e osteorradionecrose (ORN). A partir dos resultados da análise proteômica da saliva, foi possível identificar potenciais biomarcadores salivares, principalmente relacionados a processos biológicos, como respostas imunes inatas, resposta inflamatória, migração celular, atividade de inibidor de peptidase e coordenação de ferro, que podem ser considerados preditores de toxicidades agudas da RT. De forma interessante, oito biomarcadores foram associados a gravidade clínica de xerostomia e candidose oral e um biomarcador associado a disgeusia e candidose oral, trazendo evidências originais, em termos biológicos, para a existência de um agrupamento de sintomas e toxicidades orais resultantes da RT. Originalmente, os resultados da presente tese também sugerem o potencial impacto do agrupamento de sintomas orais no desenvolvimento da CR, que, por sua vez, apresentou impacto importante na morbidade dos pacientes incluídos neste estudo os quais apresentaram maior necessidade de consultas odontológicas especializadas, maior incidência de ORN e

consequentemente maior necessidade de procedimentos cirúrgicos invasivos pós-RT. Os resultados da presente tese demonstram novas perspectivas em relação ao padrão de desenvolvimento assim como o impacto que as toxicidades agudas induzidas pela RT podem representar no desenvolvimento e agravamento uma das outras na forma do recém descrito agrupamento de sintomas orais. A presente tese também apresenta de forma original preditores proteômicos salivares de toxicidades orais agudas debilitantes induzidas por RT em pacientes com CEC oral e de orofaringe em estadio avançado, apresentando potencial de aprimoramento dos protocolos clínicos de suporte odontológico personalizados em Oncologia. Nesse contexto, descobertas quanto aos padrões de agrupamento de sintomas orais suportam a teoria de que o principal fator etiológico da CR seja relacionado aos efeitos indiretos da RT, e através dos resultados observados foi possível propor uma nova metodologia para guiar os dentistas no diagnóstico precoce e tratamento adequado da CR.

Palavras-chave: Câncer bucal, radioterapia de cabeça e pescoço, toxicidades bucais, biomarcadores, proteínas salivares, mucosite, disfagia, disgeusia, cárie relacionada à radiação, xerostomia, osteorradionecrose

ABSTRACT

Recent discoveries about the synergistic effect between the several oral toxicities induced by radiotherapy (RT) in the treatment of head and neck cancer (HNC) have renewed the importance of a better understanding on the pathophysiological features of the cluster of oral symptoms and how they impact dental and medical outcomes of cancer patients. In this context, lately, there is a great search for knowledge of the molecular profile of diseases with diagnostic potential for more efficient and less toxic personalized treatments. Considering this, the present doctoral thesis aimed to categorize clinical and molecular salivary patterns of oral toxicities induced by RT in patients with squamous cell carcinoma (SCC) of the oral cavity and oropharynx. The results of this thesis are presented through two clinical cohort studies, one systematic review, one narrative review and two studies of salivary molecular analysis. Clinical studies included patients with HNC undergoing RT with curative intent, associated or not with previous surgery or concomitant with chemotherapy protocols. Clinical results demonstrate a clear impact of the introduction of concomitant chemotherapy on the early development of severe dysgeusia, as well as a statistically significant correlation between xerostomia and oral mucositis (OM) on the development of dysgeusia ($r=0.29$ and $r=0.42$, respectively - $p<0.001$ for both). These associations were validated by the systematic review, which revealed a specific pattern of a cluster of oral and gastrointestinal symptoms that correlate and overlap, leading both to worsening acute toxicities and favoring the development of chronic toxicities such as radiation caries (RC) and osteoradionecrosis (ORN). From the results of the proteomic analysis of saliva, it was possible to identify potential salivary biomarkers, mainly related to biological processes, such as innate immune responses, inflammatory response, cell migration, peptidase inhibitor activity and iron coordination, which can be considered predictors of acute toxicities of RT. Interestingly, eight biomarkers were associated with clinical severity of xerostomia and oral candidiasis and one biomarker associated with dysgeusia and oral candidiasis, bringing original evidence, in biological terms, for the existence of a cluster of symptoms and oral toxicities resulting from RT. Originally, the results of this thesis also suggest the potential impact of the cluster of oral symptoms on the development of RC, which, in turn, had an important impact on the morbidity of patients included in this study, who had a greater need for specialized dental appointments, higher incidence of ORN and consequently a greater need for invasive surgical procedures after RT. The results of the present thesis demonstrate new perspectives regarding the pattern of development as well as the impact that acute toxicities induced by RT can represent on the development and aggravation of each other in the form of the just described

cluster of oral symptoms. The present thesis also originally presents salivary proteomic predictors of acute debilitating oral toxicities induced by RT in patients with advanced oral and oropharyngeal SCC, with potential to improve clinical protocols for personalized dental support in Oncology. In this context, findings regarding the patterns of cluster of oral symptoms support the theory that the main etiological factor of RC is related to the indirect effects of RT, and through the observed results it was possible to propose a new methodology to guide dentists in early diagnosis and proper treatment of RC.

Keywords: Oral cancer, head and neck radiotherapy, oral toxicities, biomarkers, salivary proteins, mucositis, dysphagia, dysgeusia, radiation-related caries, osteoradionecrosis

SUMÁRIO

1. INTRODUÇÃO

O câncer de cabeça e pescoço (CCPs) inclui doenças malignas originadas na cavidade oral, na orofaringe, na nasofaringe, na hipofaringe, na laringe e nas glândulas salivares, entre outras topografias anatômicas (Conway et al., 2009; Sloan et al., 2017). Tumores malignos da cavidade oral são um dos tipos de neoplasia mais prevalentes no mundo, sendo 90% diagnosticados como carcinoma espinocelular (CEC). Segundo dados do GLOBOCAN, no ano de 2020, foram diagnosticados mais de 800.000 novos casos de CCP e mais de 400.000 óbitos em função destas doenças, no mundo (Sung et al., 2021).

O tratamento para o CCP é usualmente multimodal incluindo ressecção cirúrgica, radioterapia (RT), quimioterapia (QT), terapia alvo molecular e imunoterapia (Kowalski et al., 2005; Brener et al., 2007), especialmente devido ao fato destes tumores serem diagnosticados de forma tardia com presença de tumores avançados associados a metástases e, consequentemente, apresentando um prognóstico ruim e a necessidade de tratamentos mais intensos (Scully et al., 2006; Gunieri et al., 2014). Nesse contexto, a RT é uma das principais modalidades de tratamento utilizadas e estima-se que, no mundo, 75% dos pacientes diagnosticados com CCP serão submetidos a essa modalidade de tratamento seja de forma isolada, de forma adjuvante à cirurgia de ressecção ou concomitante na forma de protocolos de quimiorradioterapia (QRT), (Kowalski et al., 2005; Brener et al., 2007; Grégoire et al., 2014).

Os benefícios da RT no controle locoregional do CCP são atingidos às expensas de uma série de efeitos colaterais aos tecidos sadios incluídos no campo de radiação que podem ser agudos, tais como a mucosite oral (MO), disgeusia, disfagia, trismo, radiodermite e infecções bucais recorrentes, ou crônicos como a hipossalivação, cárie de radiação (CR) e a osteorradionecrose (ORN). Apesar da hipossalivação ser considerada como toxicidade crônica, ela pode ter início durante as primeiras semanas de RT e se perpetuar por toda a vida dos

pacientes devido ao dano radiogênico permanente às glândulas salivares (Silva et al., 2009; Huber e Terezhalmy, 2003; Kielbassa et al., 2006; Faria et al., 2014). Nesse contexto, publicações recentes apresentaram o conceito de agrupamento de sintomas orais ou "*cluster of oral symptoms"* composto por OM, alterações do paladar, infecções orais, dor em cavidade oral, trismo, alterações qualitativas e quantitativas no perfil salivar e na composição da microbiota oral (Xiao et al., 2013; Xiao et al., 2014; Madrid et al., 2017). Estudos relatam um sinergismo entre este agrupamento de sintomas agudos da RT que promove um agravamento recíproco e que contribui para mudanças na dieta assim como dificuldades de realizar higiene oral promovendo, também, um ambiente oral altamente cariogênico o que por sua vez impacta o risco para o desenvolvimento e a progressão clínica da CR (Bressan et al., 2016; Gouvêa Vasconcellos et al., 2020).

Atualmente, há uma busca pelo tratamento personalizado para o CCP considerando o perfil molecular dos tumores, de forma a aprimorar a resposta ao tratamento oncológico e reduzir as taxas de toxicidade e morbidade a longo prazo (Cohen et al., 2016). Nesse âmbito, o conhecimento do perfil proteômico salivar desses pacientes tem potencial para identificar preditores diagnósticos das toxicidades orais induzidas pela RT para pacientes com CCP (Ventura et al., 2021) buscando a elaboração de estratégias mais individualizadas de suporte odontológico dos pacientes oncológicos.

Considerando o exposto, a presente tese de doutoramento se propôs a categorizar o perfil proteômico salivar de toxicidades orais agudas induzidas pela RT como modalidade de tratamento de CEC de cavidade oral e orofaringe, assim como avaliar o padrão de progressão e correlação clínica das toxicidades orais agudas e crônicas da RT.

2. ARTIGOS

2.1 Artigo: The impact of clustering of oral symptoms in the pathogenesis of radiation caries: A systematic review.

Adriele Ferreira Gouvêa Vasconcellos, Gouvêa AF[1*]; Natália Rangel Palmier, Palmier NR[1*]; Ana Carolina Prado Ribeiro, Ribeiro ACP[1,2]; Ana Gabriela Costa Normando[1], Normando AGC; Karina Morais Faria, Morais-Faria K[2]; Wagner Gomes-Silva, Gomes-Silva W[2,3]; Aljomar José Vechiato Filho, Vechiato Filho, J[2]; Mario Fernando de Goes[1], Goes, MF; Adriana Franco Paes Leme, Paes Leme AF[4]; Thaís Bianca Brandão, Brandão TB[2]; Marcio Ajudarte Lopes, Lopes MA[1]; Philip D. Marsh, Marsh PD[5*]; Alan Roger Santos-Silva, Santos-Silva AR[1]*. Impact of Clustering Oral Symptoms in the Pathogenesis of Radiation Caries: A Systematic Review. Caries Res. 2020;54(2):113-126. doi: 10.1159/000504878.

[*]These authors contributed equally to this work.

Short title: Clustering of oral symptoms in radiation caries

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Key words: caries; cancer; radiotherapy; chemotherapy; xerostomia.

Declaration of Interests: The authors declare that there are no conflicts of interest.

Abstract

Radiation-related caries (RRC) is a disease with a high potential for destruction of the dentition, which impairs quality of life in head and neck cancer (HNC) patients who undergo radiotherapy. In light of the recently described "clustering of oral symptoms theory", the present systematic review (PROSPERO CRD42019132709) aimed to assess the Head and Neck (HN) and Gastrointestinal (GI) symptom clusters among HNC patients and discusses how these indirect effects of cancer therapy have a pivotal role in the pathophysiology of RRC. The search was performed at Pubmed, Scopus and Embase and resulted in 11 studies that met the inclusion criteria. Data extraction was performed regarding the presence of HN/GI symptom clusters among HNC patients. The methodological data of the included studies was assessed using the MAStARI and GRADE instruments. The most prevalent reported HN symptoms were dysphagia, xerostomia and pain. Taste alterations and fatigue were also commonly reported by the patients. Loss of appetite and weight loss was regularly reported by the studies, as well as nausea and vomiting. The results of the present study suggest that HNC treatment generates clusters of oral symptoms, leading to dietary changes, deficient oral hygiene, enamel fragility and a highly cariogenic oral environment, which may impact the risk for RRC. A better understanding of the clustering of oral symptoms could be of considerable clinical significance for the oral health and quality of life of HNC patients. Therefore, RRC contemporary protocols of prevention must take into account this broader treatment scenario of cluster of oral side effects.

Introduction

Radiation-related caries (RRC) is a chronic side effect of head and neck radiotherapy (HNRT), and has a high potential for tooth destruction. Its causes are still not fully understood and the ability of HNRT to cause direct radiogenic damage to the dentition leading to RRC is a major topic for discussion in oral oncology [Lieshout & Bots, 2014; Morais-Faria et al., 2014].

Recent publications have linked the elevated risk of the clinically aggressive RRC in head and neck cancer (HNC) patients to the indirect effects of cancer therapies [Santos-Silva et al., 2015; Sroussi et al., 2017], which were reinforced by increasing evidence that "symptoms clusters" may have a pivotal role in several head and neck chemoradiotherapy (CRT) toxicities [Xiao et al., 2013; Xiao et al., 2014]. The so-called "clustering of oral symptoms" has been previously described and is composed of concurrent mucositis, taste changes, oral infections, oral pain, trismus, hyposalivation, altered saliva composition and shifts in the composition of the oral microbiota, which lead to significant dietary changes, deficient oral hygiene and the development of a highly cariogenic oral environment, working in synergy to increase the risk for RRC development and progression [Ribeiro et al., 2013; Xiao et al, 2013; Xiao et al. 2014; Santos-Silva, et al., 2015; Madrid et al., 2017; Gomes-Silva et al., 2017].

Therefore, the aim of this article is to present a systematic review of the recently described "clustering of oral symptoms" [Xiao et al., 2013; Xiao et al., 2014] associated with HNC treatment toxicities in an attempt to emphasize that RRC pathophysiology may be inserted into a broader and multifactorial setting than has been previously suggested.

Material and Methods

Study design

The present systematic review was conducted following the Guidelines of Preferred

Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (Supplementary Table 1) [Moher et al., 2009] and was registered at the PROSPERO platform CRD42019132709 (Palmier et al., 2019). The research question was: Is there a specific clustering of oral symptoms associated with HNC treatment that could impact the pathogenesis of radiation caries?

Studies that assessed the presence of treatment-related symptom clusters among HNC patients were selected. The inclusion criteria followed the PICOS strategy: Patients – HNC patients; Intervention – HNRT or CRT; Comparison – Head and neck specific toxicities (HN) and gastrointestinal toxicities (GI); Outcomes – Presence and cluster of symptoms from HNC treatment; Study design - clinical trials, descriptive and observational studies.

Studies were excluded for one of the following reasons: (1) Non-HNC symptoms; (2) Psychological/psychiatric disorders symptoms; (3) Respiratory system symptoms (4) Cardiovascular symptoms, and (5) Other reasons such as studies assessing molecular features of toxicities, studies assessing symptoms of other disorders such as fibromyalgia, among others.

Electronic and systematic searches of scientific studies that assessed the presence and cluster of symptoms from HNC treatment were conducted in April 2019 (Last update June 2019). English language restriction was applied, and there was no restriction to publication year. Medline/PubMed (https://www.ncbi.nlm.nih.gov/pubmed), EMBASE (https://www.embase.com/login) and Scopus (https://www.scopus.com) databases were screened. Related MeSH (Medical subjects headings) as well as free-terms were combined on different search strategies to find the articles. The process was repeated in each database to ensure that any relevant result would not be missed during the identification phase. Two combinations were performed at each database. Complete searching strategies are presented in Supplementary Table 2. Additional searches were conducted by reading reference lists from all selected studies to detect other potentially eligible reports that could meet the inclusion criteria.

Study Selection and data collection

All titles were systematically organized in Microsoft Office Excel 2016 (Microsoft Corporation, Redmond, Washington, USA). They were verified and counted to exclude duplicated items. The articles were selected in two phases. In phase 1, 2 authors independently reviewed the titles and abstracts and selected those that apparently met the inclusion criteria. In phase 2, the same authors read the full texts of the selected articles at phase 1 and excluded those that did not meet the inclusion criteria (Supplementary Table 3). Any disagreements in the first or second phases were resolved by discussion and mutual agreement between the two authors. Studies were classified into the following categories: duplicated, excluded by title, excluded by abstract, excluded by methodology and included studies. In the end, reports assessed for eligibility were downloaded from databases in full text version and they were read in detail in PDF formatted files. Studies that omitted relevant methodological information were also excluded from the current review.

The process for methodological data collection involved two investigators (AFGV and NRP). Data were independently extracted by each investigator and then compared; any disagreements were solved by discussion between the two investigators. Methodological data extracted from selected studies were related to first author name, year, country and journal of publication, type of study, number of patients, tumour topography, stage of disease, cancer treatment, mean radiation dose, type of radiotherapy, chemotherapy medications, chemotherapy cycles, treatment-related toxicities, time of assessment, HN specific symptoms, GI and general symptoms, toxicities assessment criteria and criteria for inclusion of toxicities in the Results section. The presence of the reported symptoms per included manuscript was assessed.

Risk of bias within studies

Methodologically, the authors appraised all included studies according to a checklist

based in Meta-Analysis of Statistics Assessment and Review Instrument (MAStARI) [The Joanna Briggs Institute, 2014]. The reviewers (AFGV and NRP) independently answered nine questions for descriptive studies and eight questions for Cross-sectional studies as Y for "yes," N for "no," U for "unclear," and NA for "not applicable" (Supplementary Table 4).

After that, the risk of bias was categorized as high when the study reached up to 49% of a "yes" score, moderate when the study reached 50–69% of a "yes" score, and low when the study reached more than 70% of a "yes" score. Disagreements were solved by discussion between the two authors.

Risk of Bias Across Studies

Quality of evidence and grading of recommendation was assessed by the Grading of Recommendation, Assessment, Development and Evaluation (GRADE) instrument. The assessment was based on radiation-related symptoms clusters evaluated by different study designs. The criteria included the number of studies, study design, risk of bias, inconsistency, indirectness, imprecision, and other considerations such as publication bias and confounding factors. Impact, certainty and importance were graded based on the assessed criteria and the quality of evidence was characterized as high, moderate, low, or very low for each outcome. The GRADE was assessed using tools from the following website http://gradepro.org.

Data analysis

Primary outcome was to assess the presence of HN specific symptoms cluster. Secondary outcome was to assess the presence of GI symptoms cluster. Tertiary outcome was to assess the possible impact of symptoms cluster in the pathogenesis of RRC. There was homogeneity in the research purpose among the studies but a great variability in time of assessment of toxicities and criteria used for the assessment of treatment-related toxicities. A

detailed qualitative synthesis of the results was performed considering the presence of patient-reported symptoms among the included studies.

Results

Study selection and characteristics

A flow diagram summarizing the selection process is shown in Figure 1. A total of 4,611 studies were identified through the search strategies on three databases (PubMed, Embase and Scopus). After the first review process, 1,682 studies were excluded due to inter-database duplication. One study was added from the search on the reference list of the included studies. The total of 2,919 studies were excluded because they did not meet the inclusion criteria, resulting in 11 studies being eligible for the review. **Table 1** shows the main methodological aspects from the 11 included studies.

Seven studies (63.6%) assessed patients with heterogeneous HN topographies [Murphy et al., 2010; Xiao et al., 2013; Rosenthal et al., 2014; Kirka and Kutluturkan, 2016; Barnhart et al., 2018; Chiang et al., 2018; Ridner et al., 2018], two studies (18.2%) assessed patients with oropharynx/larynx tumours [Haisfield-Wolfe et al., 2012; Eraj et al., 2017] and two studies (18.2%) assessed patients with nasopharynx tumours [Xiao et al., 2017; McDowell et al., 2018]. Eight studies (72.2%) reported clarified information on patients' stage of disease, from which six (54.5%) assessed patients with clinical stage of disease I to IV [Haisfield-Wolfe et al., 2012; Rosenthal et al., 2014; Kirka and Kutluturkan, 2016; Xiao et al., 2017; Eraj et al., 2017; McDowell et al., 2018] and two (18.2%) assessed patients with advanced clinical stage of disease III/IV [Xiao et al., 2013; Chiang et al., 2018].

Information on treatment modalities were also retrieved from the included studies: seven studies (63.6%) assessed patients treated with either RT or CRT protocols [Haisfield-Wolfe et al., 2012; Rosenthal et al., 2014; Xiao et al., 2017; Eraj et al., 2017; Barnhart et al., 2018;

McDowell et al., 2018; Ridner et al., 2018], two studies (18.2%) assessed patients submitted to RT [Kirka and Kutluturkan, 2016; Chiang et al., 2018] and two studies (18.2%) assessed patients submitted to CRT protocols [Murphy et al., 2010; Xiao et al., 2013]. Four studies (36.3%) reported the use of the Intensity Modulated Radiation Therapy (IMRT) technique for radiation delivery [Rosenthal et al., 2014; Xiao et al., 2017; Eraj et al., 2017; McDowell et al., 2018], one study (7.1%) reported the use of IMRT and the 3D Conformational Radiotherapy (3DRT) [Barnhart et al., 2018] and one study (7.1%) compared the outcomes of the Accelerated Fractionation Radiotherapy (AFR) and Standard Fractionation Radiotherapy (SFR) [Xiao et al., 2013]. For the studies that assessed CRT protocols as treatment modality, cisplatin was the main medication used [Haisfield-Wolfe et al., 2012; Xiao et al., 2013; Rosenthal et al., 2014; Xiao et al., 2017; Eraj et al., 2017; McDowell et al., 2018].

Considering the treatment-related toxicity assessment, five studies (45.4%) assessed patients both during RT and after RT completion [Murphy et al., 2010; Haisfield-Wolfe et al., 2012; Xiao et al., 2013; Kirka and Kutluturkan, 2016; Barnhart et al., 2018], three studies (27.3%) assessed patients after the conclusion of RT [Eraj et al., 2017; McDowell et al., 2018; Ridner et al., 2018] and three studies (27.3%) assessed patients during the course of RT [Rosenthal et al., 2014; Xiao et al., 2017; Chiang et al., 2018]. For the classification of the observed toxicities, five studies (45.4%) used the M. D. Anderson Symptom Inventory [Rosenthal et al., 2014; Xiao et al., 2017; Eraj et al., 2017; McDowell et al., 2018; Chiang et al., 2018], two studies (28.2%) used The Memorial Symptom Assessment Scale [Haisfield-Wolfe et al., 2012; Kirka and Kutluturkan, 2016], one (9.1%) used the NCI Common Toxicity Criteria (CTC) 2.0 [Xiao et al., 2013], one (9.1%) used the Vanderbilt Head and Neck Symptom Survey [Murphy et al., 2010], one (9.1%) used the Vanderbilt Head and Neck Symptom Survey version 2.0 [Ridner et al., 2018] and one (7.1%) characterized the toxicities as present or absent [Barnhart et al., 2018]

Results of the risk of bias assessment are shown in Figure 2. Six studies (54.5%) were classified as moderate risk of bias [Murphy et al., 2010; Haisfield-Wolfe et al., 2012; Rosenthal et al., 2014; Kirka and Kutluturkan, 2016; Barnhart et al., 2018; Ridner et al., 2018] and five studies (45.4%) were classified as low risk of bias [Xiao et al., 2013; Eraj et al., 2017; Xiao et al., 2017; McDowell et al., 2018; Chiang et al., 2018].

Since meta-analysis was not feasible due to the heterogeneity across studies, the quality of evidence was reported in a narrative summary of findings of GRADE and based on study design of included papers (**Supplementary Table 5**). The nine descriptive studies provided weaker scientific evidence and had heterogeneous methodologies, resulting in a serious level of inconsistency. Also, moderate risk of bias in most studies downgraded it to a serious rate, leading to a low quality of evidence. The second outcome included only two studies and had fewer patients; however, they represented stronger level of evidence (cross-sectional), had minor inconsistency across them and had low risk of bias, leading to a moderate quality of evidence. Based on these results, further research may have an important impact on the estimate of these effects.

Synthesis of Results

From the selected studies, all 11 (100%) reported the symptoms of difficult swallowing/dysphagia, dry mouth/xerostomia and pain [Murphy et al., 2010; Haisfield-Wolfe et al., 2012; Xiao et al., 2013; Rosenthal et al., 2014; Kirca and Kutluturkan, 2016; Xiao et al., 2017; Eraj et al., 2017; Barnhart et al., 2018; McDowell et al., 2018; Chiang et al., 2018; Ridner et al., 2018], eight studies (72.7%) reported taste alterations [Murphy et al., 2010; Xiao et al., 2013; Rosenthal et al., 2014; Kirca and Kutluturkan, 2016; Xiao et al., 2017; Eraj et al., 2017; Barnhart et al., 2018; Ridner et al., 2018], seven studies (63.6%) reported fatigue [Xiao et al., 2013; Rosenthal et al., 2014; Kirca and Kutluturkan, 2016; Xiao et al., 2017; Barnhart et al.,

2018; McDowell et al., 2018; Chiang et al., 2018], five studies (45.4%) reported sore mouth [Murphy et al., 2010; Haisfield-Wolfe et al., 2012; Rosenthal et al., 2014; Kirca and Kutluturkan, 2016; Xiao et al., 2017], six studies (54.5%) reported problems with the presence of mucous on the mouth/throat [Murphy et al., 2010; Rosenthal et al., 2014; Xiao et al., 2017; Eraj et al., 2017; McDowell et al., 2018; Ridner et al., 2018], four studies (36.3%) reported chewing problems [Rosenthal et al., 2014; Xiao et al., 2017; Eraj et al., 2017; McDowell et al., 2018], three studies (27.3%) reported teeth/gum problems - dental caries [Barnhart et al., 2018 McDowell et al., 2018; Ridner et al., 2018], three (27.3%) with radiodermatitis [Haisfiel-Wolfe et al., 2012; Xiao et al., 2013; Xiao et al., 2017], two studies (18.2%) reported problems related to oral mucositis [Xiao et al., 2013; Ridner et al., 2018], , two studies (18.2%) reported trismus [Barnhart et al., 2018; Ridner et al., 2018] and finally, one study (9.1%) reported smell alterations [Ridner et al., 2018]. Results of the distribution of HN specific symptoms among the studies are shown in Supplementary Figure 1.

Results of the analysis of the presence of GI symptoms are shown in Supplementary Figure 2. Eight studies (72.7%) reported loss of appetite [Murphy et al., 2010; Haisfield-Wolfe et al., 2012; Rosenthal et al., 2014; Kirca and Kutluturkan, 2016; Xiao et al., 2017; Barnhart et al., 2018; Chiang et al., 2018; Ridner et al., 2018], five studies (45.4%) reported weight loss [Murphy et al., 2010;; Haisfield-Wolfe et al., 2012; Xiao et al., 2013; Kirca and Kutluturkan, 2016; Ridner et al., 2018], four studies (36.3%) reported nausea and vomiting [Xiao et al., 2013; Rosenthal et al., 2014; Xiao et al., 2017; Chiang et al., 2018] and one study (9.1%) reported dehydration [Xiao et al., 2013].

The high heterogeneity in reporting the results observed in the included studies made it impossible to assess frequency and prevalence of treatment-related toxicities among HN cancer patients. Nevertheless, four studies (36.3%) reported frequency values for HN and GI symptoms (Figures 3 and 4) [Haisfield-Wolfe et al., 2012; Xiao et al., 2017; Barnhart et al., 2018; Chiang

et al., 2018]. Swallowing problems/dysphagia were reported by three studies with a mean frequency of 97.7% for 243 patients [Haisfield-Wolfe et al., 2012; Xiao et al., 2017; Barnhart et al., 2018]. Dry mouth/Xerostomia was reported by all studies with a mean frequency of 94.75% for 343 patients [Haisfield-Wolfe et al., 2012; Xiao et al., 2017; Barnhart et al., 2018; Chiang et al., 2018]. Pain was reported by three studies with a mean frequency of 91.3% for 151 patients [Haisfield-Wolfe et al., 2012; Xiao et al., 2017; Chiang et al., 2018]. Taste alterations were reported by three studies with a mean frequency of 89.6% for 243 patients [Haisfield-Wolfe et al., 2012; Xiao et al., 2017; Barnhart et al., 2018]. Fatigue was reported by three studies with a mean frequency of 92.2% for 322 patients [Xiao et al., 2017; Barnhart et al., 2018; Chiang et al., 2018]. Mucous was reported by one study with a frequency of 99.2% for 130 patients [Xiao et al., 2017]. Sore mouth was reported by two studies with a mean frequency of 83.5% for 151 patients [Haisfield-Wolfe et al., 2012; Xiao et al., 2017]. Chewing problems were reported by one study with a frequency of 98.5% for 130 patients [Xiao et al., 2017]. Teeth/gum problems - dental caries were reported by two studies with a mean frequency of 48.8% for 222 patients [Xiao et al., 2017; Barnhart et al., 2018]. Radiodermatitis was reported by two studies with a mean frequency of 73.9% for 151 patients [Haisfield-Wolfe et al., 2012; Xiao et al., 2017]. Trismus was reported by one study with a frequency of 14.1% for 92 patients [Barnhart et al., 2018]. Four studies reported lack of appetite with a mean frequency of 90.9% for 343 patients [Haisfield-Wolfe et al., 2012; Xiao et al., 2017; Barnhart et al., 2018; Chiang et al., 2018]. One study reported weight loss with a frequency of 91% for 21 patients [Haisfield-Wolfe et al., 2012]. Two studies reported nausea and vomiting with a mean frequency of 87.8% and 74.3%, respectively, for 230 patients [Xiao et al., 2017; Chiang et al., 2018]. No studies reported frequency values for OM, smell alterations and dehydration. Detailed information of reported results from included studies are available in Supplementary Table 6.

Symptom clusters in patients with head and neck cancer

Results from the present systematic review described several clusters of symptoms following HNC treatment, which include specific HN conditions, such as dry mouth, dysphagia, pain, taste disturbances, fatigue, oral mucositis, radiodermatitis, and GI manifestations, such as nausea, vomiting, and dehydration [Murphy et al., 2010; Haisfield-Wolfe et al., 2012; Xiao et al., 2013; Rosenthal et al., 2014; Kirca and Kutluturkan, 2016; Xiao et al., 2017; Eraj et al., 2017; Barnhart et al., 2018; McDowell et al., 2018; Chiang et al., 2018; Ridner et al., 2018]. These clustering of oral symptoms using contemporary concepts brought new ideas for the analysis of RRC pathogenesis and the impact of dietary changes, deficient oral hygiene, and the highly cariogenic oral environment on the dentition of HNC survivors **(Figure 5)**.

Discussion

HNRT is known to cause several acute and chronic toxicities to the oral cavity. Within the first 3 weeks, patients undergoing HNRT experience a series of symptoms that burden, evolve and overlap. They often develop oral mucositis (OM), radiation dermatitis, edema, dysgeusia and a shift in the oral microbiota composition [Murphy et al., 2010; Xiao et al., 2013; Chiang et al., 2018; Ridner et al., 2018]. Additionally, these patients may develop associated pain, copious mucous production, hyposalivation, xerostomia, and acute tissue swelling, which contribute to acute dysphagia [Murphy et al., 2010; Haisfield-Wolfe et al., 2012; Xiao et al., 2013; Xiao et al., 2017; Eraj et al., 2017; Barnhart et al., 2018; McDowell et al., 2018; Chiang et al., 2018; Ridner et al., 2018]. Late effects include skin and salivary gland fibrosis, lymphedema and damage to neural structures, hyposalivation, trismus, dysphagia, RRC and osteoradionecrosis [Kielbassa et al., 2006; Eraj et al., 2017; Barnhart et al., 2018; McDowell et al., 2018]. Adverse effects of cancer treatment represent profound and long-lasting alterations on function and diminished quality of life, which is composed of a complex network of inter-

related factors that include functional, biological, psychological and social components [Murphy et al., 2007; Murphy and Gilbert, 2009; Vanderbilt et al., 2018].

The symptoms experienced by HNC patients are broad in scope and encompass both local and systemic symptoms. Furthermore, instead of occurring in isolation, results observed in the present systematic review indicate that they occur in clusters, exacerbating the overall symptom experience. 'Symptom clusters' are defined as groups of at least two or three concurrent symptoms that are synergistically interrelated [Murphy et al., 2007; Xiao et al., 2013; Dong et al, 2014]. Two main distinct and stable clusters were described for HNC patients, identified through factor modelling among 10 identified treatment-related symptoms: HN specific symptoms cluster (encompassing mucositis; radiodermatitis; pain; dysphagia; taste disturbances; dry mouth and fatigue) and GI cluster (nausea, vomiting and dehydration) [Aguiar et al, 2009; Silva et al., 2009; Murphy et al., 2010; Haisfield-Wolfe et al., 2012; Xiao et al., 2013; Xiao et al., 2017; Eraj et al., 2017; Barnhart et al., 2018; McDowell et al., 2018; Chiang et al., 2018; Ridner et al., 2018]. These clustered symptoms may be associated with the development of a highly cariogenic oral environment and the lack of proper oral hygiene leading to onset and development of RRC [Cohen et al., 2016].

Dysphagia is defined as difficulty in swallowing and can be an acute or late result of HNRT. Acute dysphagia is associated with mucosa and soft tissue damage within the treatment field particularly because of OM, radiation dermatitis, and edema of the soft tissues. Pain, hyposalivation associated with thickened and more viscous mucous production, and tissue swelling contribute to acute dysphagia. Late dysphagia is the result of tissue fibrosis and stiffness due to the ongoing inflammatory cytokine cascade effects, as well as to lymphedema and radiation-induced damage to neural structures. Patients suffer aspiration, choking, and may consciously or unconsciously alter the type and consistency of food that they eat, resulting in nutritional deficiencies and an oral environment favourable for RRC onset and progression

[Murphy et al., 2007; Nevens et al., 2017; Santa Cruz et al., 2018; Ridner et al., 2018].

Dry mouth, or xerostomia, observed in HNC patients is caused by hyposalivation due to radiogenic effects on salivary glands. It has a rapid onset and it is the most common persistent oral side effect for patients receiving HNRT [Sciubba and Goldenberg, 2006]. Saliva becomes scant and thicker causing difficulties in speaking; and induces taste alteration, as well as distress in chewing and swallowing. This scenario has an influence on dietary alterations, leading to the intake of softer and more carbohydrate-rich food [Aguiar et al., 2009]. Besides the quantitative effects, qualitative changes to saliva also occur unleashing an imbalance in its ionic composition. In this way, its buffering and tooth remineralization capacity are reduced, leading to loss of the demineralization/remineralization equilibrium and facilitating the more rapid loss of minerals from dentin and enamel following RT [Marsh, 2003; Murphy and Gilbert, 2000; Barnhart et al., 2018; Ridner et al., 2018].

In addition, an imbalance in both salivary organic components (glycoproteins and proteins) and in adaptive and innate immunity occurs following HNRT, altering the establishment and selection of the oral microbiota present on oral hard and soft tissues. Also, the frequent sugar and carbohydrate-rich food intake creates regular conditions of low pH within the dental biofilm and selects for acidogenic and aciduric bacteria such as mutans streptococci and lactobacilli, predisposing the enamel – which is known for being highly porous and permeable after HNC treatment [Madrid et al., 2017] – to the rapid onset and progression of RRC. In other words, a real “ecological catastrophe” occurs in the oral cavity of cancer patients following HNRT, due to the disruption of the natural balance that normally exists in the mouth between the microbiota and the host, and which drives dysbiotic changes in the composition of the biofilm, thereby creating a favourable environment for RRC [Marsh, 2003].

Pain is a ubiquitous problem faced by all HNC patients both due to the tumour before therapy begins and up to 76% of patients suffer severe pain related to acute therapy toxicities

such as OM and radiodermatitis, despite the use of opioids [Murphy et al, 2007]. After treatment completion, they experience pain when doing several basic physical functions due to fibrosis, muscular loss, neck dissection and neural impairment. Pain significantly impacts on function, with high percentages of patients reporting difficulties in swallowing, eating, drinking, talking, sleeping and maintaining basic self-day-care such as oral hygiene [Murphy and Gilbert, 2000; Xiao et al., 2017; Ridner et al., 2018; Chiang et al., 2018; Vanderbilt et al., 2018].

All HNC patients undergoing cancer therapy experience taste disturbances. It is caused by a multitude of other toxicities including OM, deficient oral hygiene, a shift in their oral microbiota, taste buds and oral neural structure impairment, medications or chemotherapies intake and especially salivary flow decrease [Sciubba and Goldenberg, 2006; Murphy et al. 2007; Barnhart et al., 2018; Ridner et al., 2018]. It importantly impairs a patient's quality of life, leading to decreased food intake and a switch to sweeter foods (the most maintained flavour, reported by the patients). Unfortunately, intake of carbohydrate-rich foods and sweeter foods provide a highly cariogenic environment and fosters RRC development and rapidly progression [Aguiar et al., 2009].

Fatigue is another well-documented side-effect observed in patients undergoing radiation therapy. The lack of appetite, mainly due to the presence of chemosensory dysfunctions such as taste and smell dysfunctions, can result in patients general deconditioning which may lead to profound weight loss, with a decrease in lean and fat body mass, and individuals experiencing weakness and fatigue [Murphy et al., 2010; Haisfield-Wolfe et al., 2012; Xiao et al., 2013; Kirca and Kutluturkan, 2016; Ridner et al., 2018]. This occurs due to chemotherapy and radiation metabolic changes; impaired food intake caused by pain, tumour-related factors dysphagia, socio-economic difficulties impairing the purchase of nutritional supplements and even depression [Murphy et al., 2007; Murphy et al. 2009]. All of these events compound a complex network leading to a decrease in physical functioning and loss of the

ability to conduct daily activities such as proper oral hygiene, further propitiating RRC.

HNC patients that undergo radiotherapy will develop OM, especially when radiation treatment is associated with concurrent chemotherapy. The site of OM development depends on the tumour site, size and treatment planning, but in any case it produces mucosal pain and swelling, leading to bleeding, difficulty in speaking; sleeping; mouth opening; dysphagia and anorexia. In addition, it leads to dietary adaptations with a switch to softer and carbohydrate-rich foods, with their intake at an increased frequency. This fact, associated with an impaired or absent oral hygiene, produces an environment conducive to RRC onset [Murphy and Gilbert, 2000; Aguiar et al., 2009; Xiao et al., 2013; Ridner et al., 2018].

Radiodermatitis causes wounds, pain and a burning sensation on the skin included in the treatment field. The radiogenic soft tissue damage may also affect the local lymphatic structures and muscles, being associated in the long-term with lymphedema, cutaneous and muscular fibrosis and consequent trismus. In this way, besides the swallowing difficulties, patients present distress on opening the mouth and must change their dietary habits to softer and more cariogenic food, which combined with the additional impairment of proper oral hygiene due to pain and trismus, increases their risk of RRC [Murphy and Gilbert, 2009; Nevens et al., 2017; Santa Cruz et al., 2018; Ridner et al., 2018].

Systemic symptoms cluster associated with HNC treatment toxicities were described by Xiao et al, in 2013, as a stable identified GI cluster involving nausea, vomiting and dehydration, often induced by CT or CRT. We go further and suggest that this “GI cluster” may have a significant impact on RCC pathophysiology, especially due to recurrent vomiting, which may result in dehydration and intensifies hyposalivation, lowering the protective salivary effects against caries. In addition, vomiting may produce a lower oral pH, leading to elevated risk of enamel and dentin dissolution. All of the side effects associated with nausea create an additional obstacle for proper oral hygiene in HNC patients, and represent a favourable environment for

the onset and development of RRC.

Lastly, it is relevant to mention that most of the oral cancer patients are poorly educated, low-income individuals, with minimal oral hygiene and level of dental awareness. Many of these patients had never undergone dental treatment and previous studies have demonstrated that nearly all the HNC patients examined just before HNRT need extensive dental care due to advanced periodontal disease, residual roots, and caries **(Figure 6)** (Jham et al., 2008). These complex psychosocial and behavioural features of HNC patients create a poor oral health scenario even before HNRT (Jham et al., 2008), which might be considered another pillar to the development and rapidly progression of RRC.-

Strengths and Limitations

Main strengths of this systematic review were rigorous searching and assessment methods and homogeneity in study objectives. Nonetheless, we found limitations such as heterogeneity of studies that met inclusion criteria regarding the methodology and criteria for toxicity assessment and report of observed results. In addition, frequency values were possible to be obtained for only 4 from the 11 included studies and, unfortunately, none of the included

manuscripts correlated the presence of symptoms or symptoms clusters with the onset and progression of RRC, which made it impossible to perform a meta-analysis of the correlation of the cluster of oral symptoms and RRC.

Conclusions

This review is the first to explore symptom clusters in HNC patients and their possible impact on RRC development and progression. HNC patients seldom present with a single oral symptom; thus the understanding and managing of the specific conditions of the HN and GI

manifestations symptoms clusters may be paramount for the preservation of cancer survivor's quality of life. Remarkably, there is evidence that the observed HN and GI symptom clusters may indirectly contribute to RRC onset and progression. This scenario composes a much more complex panorama than what has been previously suggested in terms of RRC pathogenesis, and should be considered pivotal for RRC progression. Therefore, contemporary protocols of RRC prevention and treatment must take into account this broader HNRT-associated clustering of toxicities scenario.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors declare that there are no conflicts of interest.

Funding Sources

The authors would like to gratefully acknowledge the financial support of the Coordination for the Improvement of Higher Education Personnel through the National Post Doctoral Program (CAPES/PNPD, Brazil), process number 1724203; São Paulo Research Foundation (FAPESP, Brazil), processes number 2013/18402-8, 2016/22059-5, 2018/02233-6 and 2018/04657-8; and The National Council for Scientific and Technological Development (CNPq, Brazil).

Author Contributions

Adriele Ferreira Gouvêa Vasconcellos, Natália Rangel Palmier and Alan Roger Santos-Silva performed the systematic review methodology process and wrote the manuscript in

consultation with Adriana Franco Paes Leme and Philip Marsh. Ana Gabriela Costa Normando and Mario Fernando de Goes performed risk of bias analysis within and across studies (GRADE). Thaís Bianca Brandão, Marcio Ajudarte Lopes, and Ana Carolina Prado Ribeiro designed the study. Karina Morais Faria, Wagner Gomes-Silva and Aljomar José Vechiato Filho drafted the manuscript and designed the figures. All authors discussed the results and commented on the manuscript.

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Table 1. Main methodological data extracted from the included studies about the presence of radiation-related symptoms clusters

Studies features	Study 1	Study 2	Study 3	Study 4
Author	Murphy et al.,	Haisfield-Wolfe et al.,	Xiao et al.,	Rosenthal et al.,
Year	2010	2012	2013	2014
Country	US	US	US/Canada	US
Journal	Head & Neck	Supportive Care in Cancer	Oral Oncology	Cancer
Type of Study	Questionnaire based	Prospective descriptive study	Secondary data analysis of previous study	Prospective, questionnaire-based study
Number of Patients	235	21	684	149
Tumor topography	Head and Neck	Oropharynx and larynx	Oral cavity, oropharynx, hypopharynx, or larynx	Oral cavity, oropharynx, nasopharynx, hypopharynx, thyroid/trachea, major salivary glands, nasal cavity/paranasal sinus, skin, unknown
Stage of Disease	NI	I, II, III, IV	III, IV	I, II, III or IV
Cancer Treatment	CRT	RT or CRT	CRT	RT or CRT
Mean Radiation dose	NI	NI	71.24Gy	66Gy
Type of RT	NI	NI	AFR or SFR	IMRT
CT medication	NI	Cisplatin, taxol, taxotere or erbitrux	Cisplatin	Cisplatin, cetuximab, multiagent regimens and carboplatin
CT cycles	NI	NI	2 or 3	NI
Toxicities time of Assessment	Patients receiving CRT up to 10-14 months post-treatment	1st week of RT, middle of RT, end of RT, 6 weeks post-RT	End of 1st CT, End of 2nd CT, 3 Months after RT beginning	Pre-RT and Weekly during the 6-7 week RT course
HNC Cluster	swallowing problems, altered taste, mucous/dry mouth, pain/sore mouth	Dysphagia, change in skin, dry mouth, difficult swallowing, mouth sores, pain	Radiodermatite, Dysphagia, Pain, Taste disturbance, Fatigue, Radiomucositis, Dry mouth	Dry mouth, mouth/throat mucous, difficult chewing/swallowing, mouth/throat sores, fatigue, problems with tasting food
GI/General Cluster	Weight loss, lost of appetite	Feeling irritable, lack of appetite, lack of energy, weight loss, worrying	Nausea, Vomiting, Dehydration, Esophagitis, Weight Loss	Nausea, vomiting, Pain, distress, sleep disturbance, drowsiness, lack of appetite, problem remembering, constipation
Toxicities Criteria	Vanderbilt Head and Neck Symptom Survey	The Memorial Symptom Assessment Scale	NCI Common Toxicity Criteria (CTC) 2.0	MD Anderson Symptom Inventory-Head and Neck Module
Criteria for Toxicity inclusion	Results from a survey of the most experienced symptoms by 26 HNC patients submitted to CRT	Eleven specific symptoms most problematic reported by the patients	Symptoms with more than 10% average prevalence across the three time points	Hierarchical cluster analysis of symptoms at the end of therapy

NI- Not informed; CRT -Chemoradiotherapy; HNC- Head and Neck Cancer; RT- Radiotherapy; IMRT- Intensity modulated radiotherapy; Gy- Grays; AFR- Accelerated fractionation radiotherapy; SFR- Standard fractionation radiotherapy; NA- Not applied

Table 1 (continued). Main methodological data extracted from the included studies about the presence of radiation-related symptoms clusters

Studies features	Study 5	Study 6	Study 7	Study 8
Author	Kirca and Kutluturkan	Xiao et al.,	Eraj et al.,	Barnhart et al.,
Year	2016	2017	2017	2018
Country	Turkey	China	US	Australia
Journal	European Journal of Cancer Care	European Journal of Oncology Nursing	Radiation Oncology	Supportive Care in Cancer
Type of Study	Descriptive work	Cross-sectional study	Cross sectional assessment of a prospective symptom survey	Longitudinal study
Number of Patients	47	130	79	92
Tumor topography	Nasal cavity, Larynx, Lips and oral cavity, thyroid, salivary gland, oropharynx	Nasopharyngeal	Oropharynx (base of tongue, tonsil, soft palate or pharyngeal wall)	Nasopharynx, Oropharynx, Larynx, Hypopharynx
Stage of Disease	II, III, IV	I, II, III, IV	I,II,III,IV	Not clear
Cancer Treatment	RT	RT, CRT, Neoadjuvant CT	RT or CRT, Induction CT	RT or CRT
Mean Radiation dose	NI	NI	68.4Gy	NI
Type of RT	NI	IMRT	IMRT	IMRT or 3DRT
CT medication	NA	Cisplatin	cisplatin, carboplatin, cetuximab, combination of platinum and taxane-based therapies	NI
CT cycles	NA	2 or 3	NI	NI
Toxicities time of Assessment	Middle of RT, end of RT, 1 month post-RT	Patients between week 4-6/7 of RT	6 months to 2 years after the conclusion of the treatment	End of treatment, 3,6,12,24 and 36 months after treatment
HNC Cluster	Changes in taste of food, dry mouth, pain, difficult swallowing, fatigue, mouth sores	Mucus, difficult swallowing/chewing, dry mouth, problems tasting food, mouth/throat sores, pain, problems with voice/speech, fatigue, skin problems	dry mouth, problems tasting food, difficult swallowing/chewing, mucous in mouth/throat	Odynophagia, xerostomia, dysgeusia, fatigue, trismus, dentition
GI/General Cluster	Difficult sleeping, worrying, difficult concentrating, not resembling oneself, weight loss, loss of appetite	Lack of appetite, nausea, vomiting, sleep disturbance, distress, drowsiness, constipation, chocking/coughing, numbness, memory problems, shortness of breath, feeling sad, constipation	Choking/coughing	Appetite, swallowing problems
Toxicities Criteria	The Memorial Symptom Assessment Scale	M.D. Anderson Symptom Inventory Head and Neck Module and the Functional Assessment of Cancer Therapy - Head and Neck Scale	M.D. Anderson Symptom Inventory - Head and Neck Module	Present/Abscent
Criteria for Toxicity inclusion	Descriptive analysis of the most common reported symptoms at each time of assessment	Descriptive analysis of the most prevalent symptoms reported by the patients	Long-term five most highly rated symptoms	Based on literature review

NI- Not informed; CRT -Chemoradiotherapy; HNC- Head and Neck Cancer; RT- Radiotherapy; IMRT- Intensity modulated radiotherapy; Gy- Grays; AFR- Accelerated fractionation radiotherapy; SFR- Standard fractionation radiotherapy; NA- Not applied

Table 1 (continued). Main methodological data extracted from the included studies about the presence of radiation-related symptoms clusters

Studies features	Study 9	Study 10	Study 11
Author	McDowell et al.,	Chiang et al.,	Ridner et al.,
Year	2018	2018	2018
Country	Canada	Taiwan	US
Journal	International Journal of Radiation Oncology Biology Physics	European Journal of Oncology Nursing	Oral Oncology
Type of Study	Questionnaire -based cross-sectional cohort study	Longitudinal study	Questionnaire based among 3 individual studies
Number of Patients	107	100	150
Tumor topography	Nasopharynx	Buccal, hypopharynx, laryngeal, tongue, gingiva	Pharynx, oral cavity, larynx, nasal, paranasal sinus, other
Stage of Disease	I, II, III, IV	III,IV	Not clear
Cancer Treatment	RT or CRT	RT	RT or CRT
Mean Radiation dose	70Gy	62.41Gy	NI
Type of RT	IMRT	NI	NI
CT medication	Cisplatin or Cisplatin/5-fluoracil	NA	NI
CT cycles	1, 2 or 3	NA	NI
Toxicities time of Assessment	4 years after treatment	Before RT, 1,2,3,4,5,6 weeks after starting RT	Study 1- baseline, end of therapy, every 6 weeks during first year post-treatment; Study 2- baseline, weekly during CRT, 1,2,3 and 6 months post-treatment; Study 3- Dental health visit post-treatment
HNC Cluster	Dry mouth, mucus, problems swallowing/chewing, teeth or gums problems, fatigue, pain	Dry mouth, pain, Lack of appetite, sleep disturbance, fatigue, drowsiness, distress and sadness	Swallowing problems, mucous, dry mouth, mouth pain, mucositis, dysphagia, taste, smell, trismus, teeth problems, voice problems
GI/General Cluster	Memory	nausea, vomiting, numbness, shortness of breath, difficult remembering	Loss of weight, loss of appetite
Toxicities Criteria	M.D. Anderson Symptom Inventory-Head and Neck, Functional Assessment of Chronic Illness Therapy-Fatigue	M.D. Anderson Symptom Inventory	Vanderbilt head and neck symptom survey version 2.0
Criteria for Toxicity inclusion	Highest scoring symptoms reported by the patients	HNC specific and GI clusters observed	Clusters observed on the questionnaire answers

NI- Not informed; CRT -Chemoradiotherapy; HNC- Head and Neck Cancer; RT- Radiotherapy; IMRT- Intensity modulated radiotherapy; Gy- Grays; AFR- Accelerated fractionation radiotherapy; SFR- Standard fractionation radiotherapy; NA- Not applied

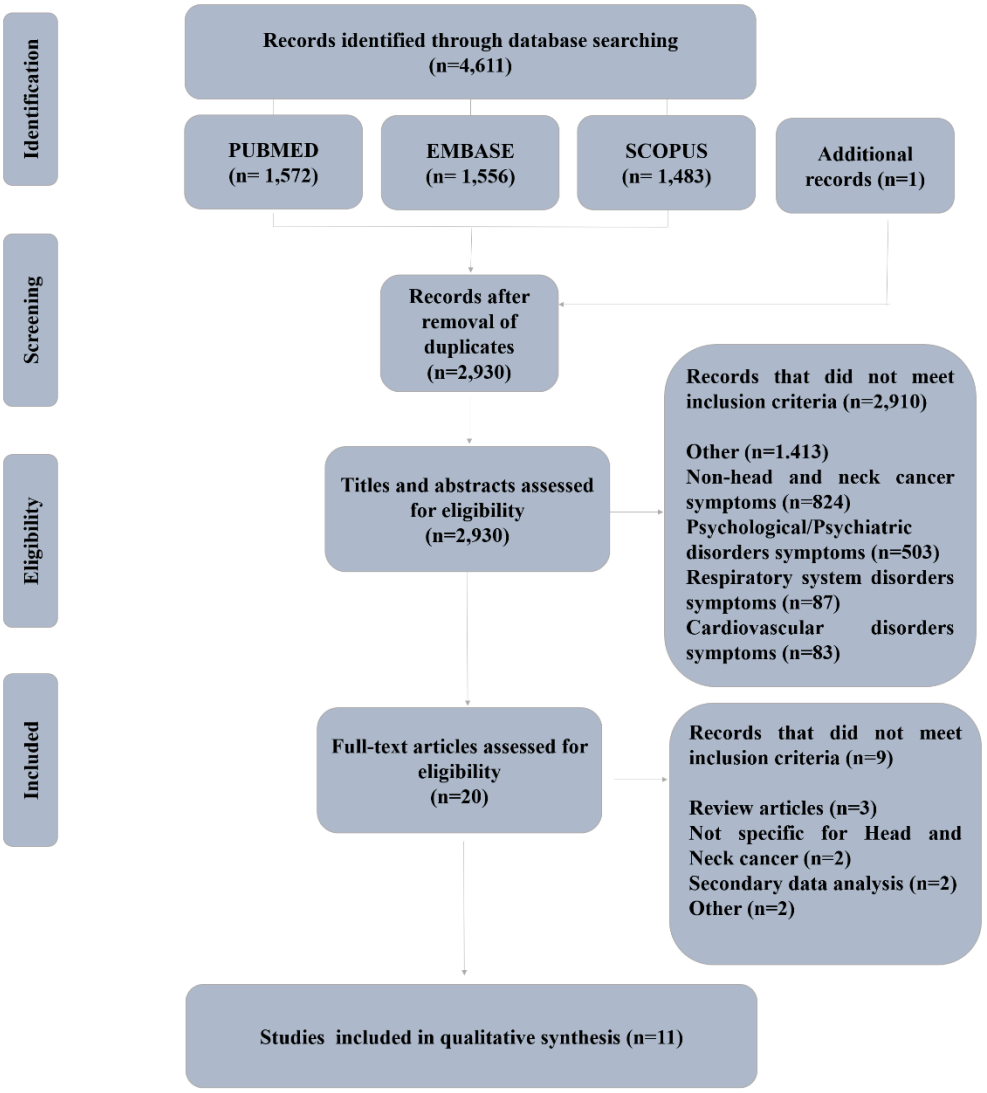

Figure 1. Flow diagram that summarizes selection process (PRISMA format).

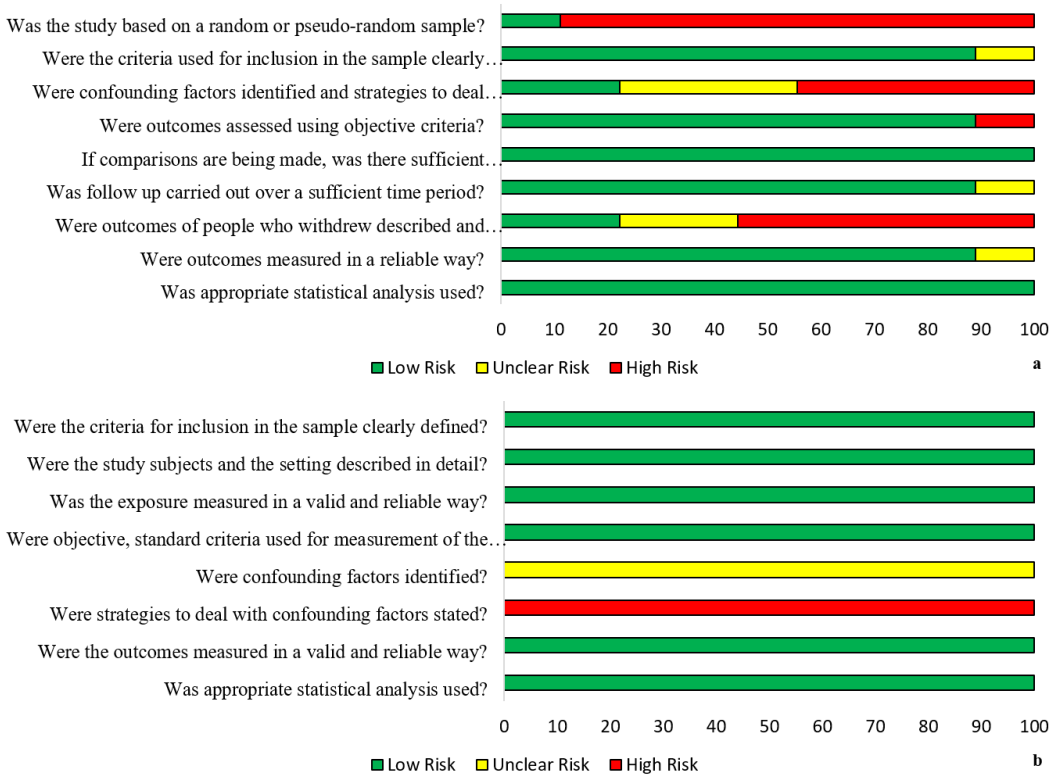

Figure 2. Risk of bias in included studies about the symptoms cluster among Head and Neck Cancer patients.

a: MAStARI critical appraisal tools for Descriptive/Case series

b: Joanna Briggs Institute Critical Appraisal Checklist for Analytical Cross Sectional Studies

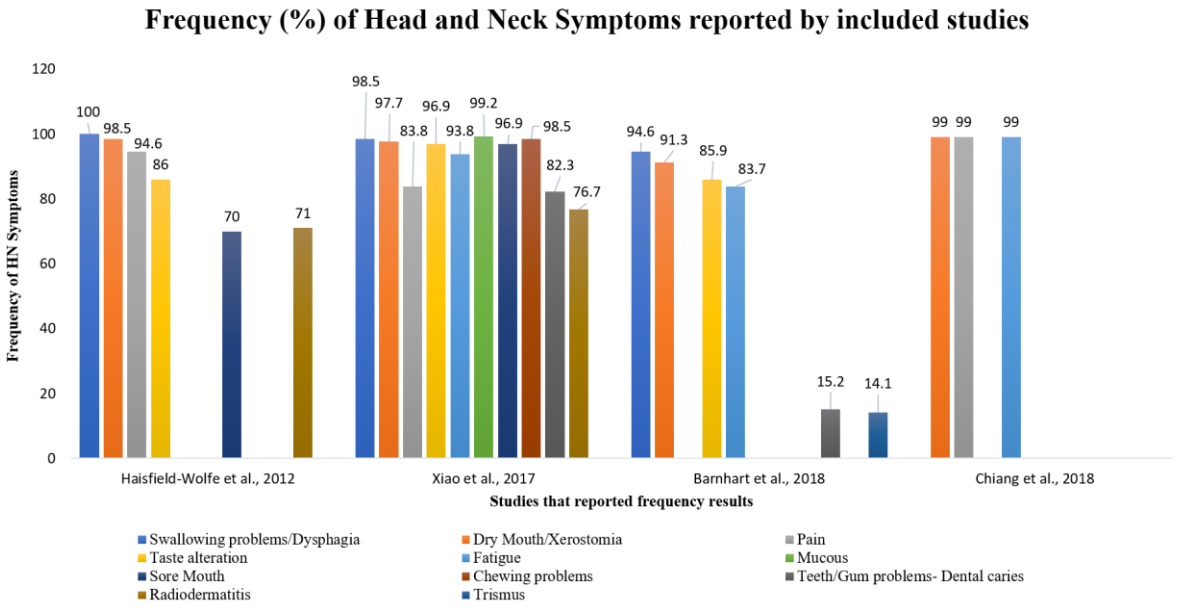

Figure 3. Frequency (%) of Head and Neck specific symptoms reported included studies.

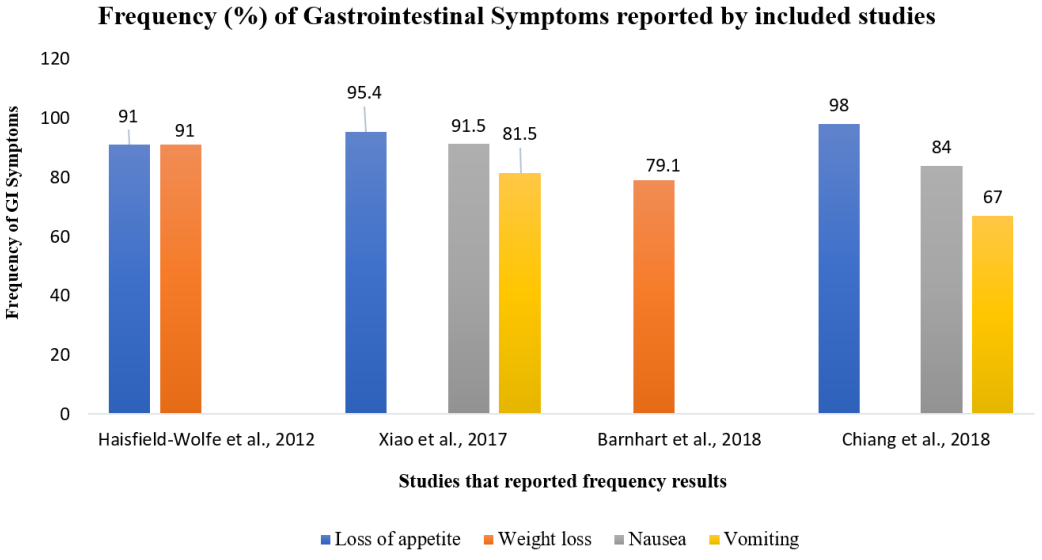

Figure 4. Frequency (%) of Gastrointestinal symptoms reported included studies.

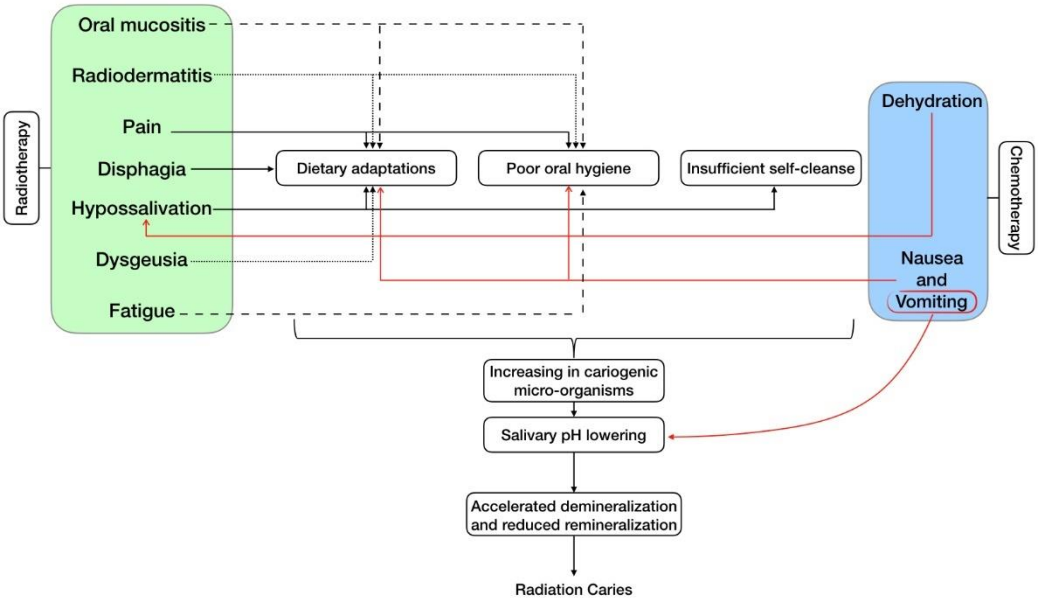

Figure 5: Flow chart presenting the interactions between the head and neck and the gastrointestinal symptoms clusters in RRC pathogenesis. Green: head and neck specific symptoms cluster. Blue: gastrointestinal symptoms cluster.

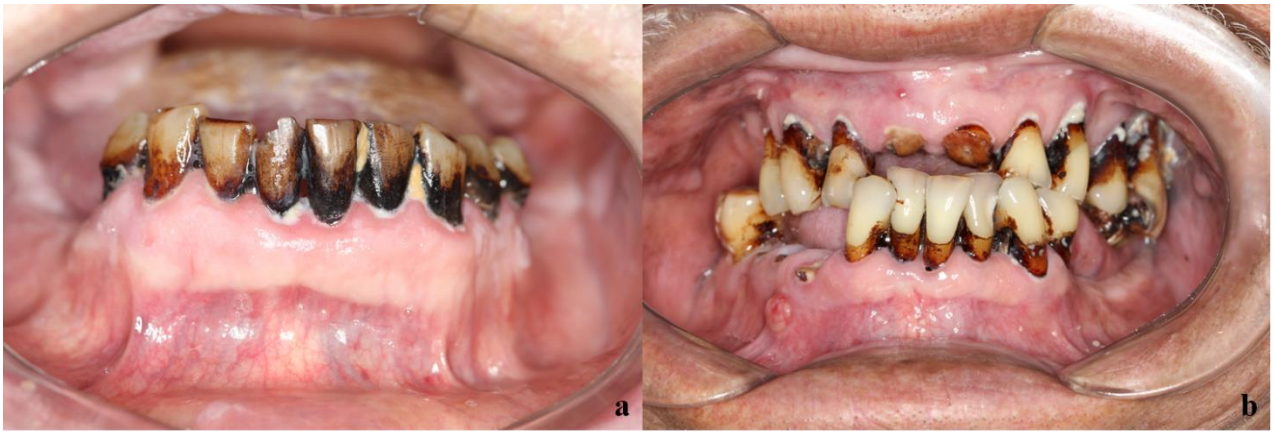

Figure 6: Oral health status in two head and neck cancer patients examined before radiotherapy resembling radiation-related caries patients. a. Note the poor oral hygiene, extensive carious lesions, brown-blackish colour pigmentation due to smoking habit and extensive teeth loss. b. Presence of extensive periodontal disease, teeth loss, several caries and multiple residual roots – one of them (in the lower right mandibular area) presenting sign of apical periodontitis.

Supplementary Table 1: PRISMA Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	3
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	3
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	4
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	4
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	4/5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	4/5
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	NA

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	NA
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	5
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	6/7
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	7
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	7
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	NA
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	8
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	NA
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	9
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	12
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	12
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	13

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097 For more information, visit: **www.prisma-statement.org**.

Supplementary Table 2. Search strategy in the databases.

Database	Search
PubMed (April, 2019)	Search #1 –("Head and neck cancer" OR "Head and neck neoplasm") AND "Head and neck cancer treatment" AND "Head and neck cancer chemotherapy" AND "Head and neck cancer radiotherapy" AND ("Head and neck symptoms" OR "Multiple symptoms" OR "Symptom cluster"); Search #2 - "Head and neck cancer" AND "Treatment related symptoms" AND "Cluster".
Scopus (April, 2019)	Search #1 –("Head and neck cancer" OR "Head and neck neoplasm") AND "Head and neck cancer treatment" AND "Head and neck cancer chemotherapy" AND "Head and neck cancer radiotherapy" AND ("Head and neck symptoms" OR "Multiple symptoms" OR "Symptom cluster"); Search #2 - "Head and neck cancer" AND "Treatment related symptoms" AND "Cluster".
Embase (April, 2019)	Search #1 –('Head and neck cancer' OR 'Head and neck neoplasm') AND 'Head and neck cancer treatment' AND 'Head and neck cancer chemotherapy' AND 'Head and neck cancer radiotherapy' AND ('Head and neck symptoms' OR 'Multiple symptoms' OR 'Symptom cluster'); Search #2 – 'Head and neck cancer' AND 'Treatment related symptoms' AND 'Cluster'.

Supplementary Table 3. Phase 2 excluded manuscripts and reasons for exclusion

Author	Year	Reasons for Exclusion
Ferreira, et al	2008	Not focused on head and neck cancer patients; Not focused on head and neck specific treatment-related symptoms
Logan, RM	2009	Review of the most common chemoradiotherapy and target therapy toxicities
Kubrak et al.,	2012	Reports the impact of toxicities on weight loss but no mention to toxicities frequency
Xiao et al.,	2014	Secondary data analysis from previously published Xiao et al., 2013
Lopez et al.,	2015	Not focused on head and neck cancer patients;
Murphy and Deng	2015	Review article - Exclusion due to repeated data
Haisfield-Wolfe et al.,	2015	Secondary data analysis from previously published Haisfield-Wolfe et al., 2012
Kjaer et al.,	2016	Reports the impact of toxicities on Quality of Life but no mention to toxicities frequency
Bressan et al.,	2017	Systematic review and Meta-analysis of nutritional impact of toxicities

Supplementary Table 4. Risk of bias assessed by Meta Analysis of Statistics Assessment and Review Instrument (MAStARI)[1] critical appraisal tools. Risk of bias was categorized as **High** when the study reaches up to 49% score "yes", **Moderate** when the study reached 50% to 69% score "yes", and **Low** when the study reached more than 70% score "yes".

MAStARI critical appraisal tools for Descriptive/Case series

Question	Answer*								
	Murphy et al., 2010	Haisfield-Wolfe et al., 2012	Xiao et al., 2013	Rosenthal et al., 2014	Kirca and Kutluturkan 2016	Barnhart et al., 2018	McDowell et al., 2018	Chiang et al., 2018	Ridner et al., 2018
1. Was the study based on a random or pseudo-random sample?	N	N	Y	N	N	N	N	N	N
2. Were the criteria used for inclusion in the sample clearly defined?	Y	Y	Y	Y	Y	Y	Y	Y	U
3.Were confounding factors identified and strategies to deal with them stated?	N	U	Y	U	N	N	U	Y	N
4. Were outcomes assessed using objective criteria?	Y	Y	Y	Y	Y	N	Y	Y	Y
5. If comparisons are being made, was there sufficient description of the groups?	Y	Y	Y	Y	Y	Y	Y	Y	Y
6. Was follow up carried out over a sufficient time period?	U	Y	Y	Y	Y	Y	Y	Y	Y
7. Were outcomes of people who withdrew described and included in the analysis?	N	U	N	N	N	Y	Y	U	N
8. Were outcomes measured in a reliable way?	Y	Y	Y	Y	Y	U	Y	Y	Y
9. Was appropriate statistical analysis used?	Y	Y	Y	Y	Y	Y	Y	Y	Y
% yes/risk	55.5 % M	66.6% M	88.8 % L	66.6 % M	66.6% M	55.5% M	77.7 % L	77.7 % L	55.5 % M

*Y=Yes, N=No, U=Unclear, M=Moderate, H=High, L=Low.

[1]Meta Analysis of Statistics Assessment and Review Instrument (MAStARI). Joanna Briggs Institute Reviewers Manual. Australia: The Joanna Briggs Institute, 2014.

Supplementary Table 4 (continued). Risk of bias assessed by the Meta Analysis of Statistics Assessment and Review Instrument (MAStARI)[1] critical appraisal tools. Risk of bias was categorized as **High** when the study reaches up to 49% score "yes", **Moderate** when the study reached 50% to 69% score "yes", and **Low** when the study reached more than 70% score "yes".

Joanna Briggs Institute Critical Appraisal Checklist for Analytical Cross Sectional Studies

Question	Answer*	
	Eraj et al., 2017	Xiao et al., 2017
1. Were the criteria for inclusion in the sample clearly defined?	Y	Y
2. Were the study subjects and the setting described in detail?	Y	Y
3. Was the exposure measured in a valid and reliable way?	Y	Y
4. Were objective, standard criteria used for measurement of the condition?	Y	Y
5. Were confounding factors identified?	U	U
6. Were strategies to deal with confounding factors stated?	N	N
7. Were the outcomes measured in a valid and reliable way?	Y	Y
8. Was appropriate statistical analysis used?	Y	Y
% yes/risk	75% L	75% L

*Y=Yes, N=No, U=Unclear, M=Moderate, H=High, L=Low.

[1]Meta Analysis of Statistics Assessment and Review Instrument (MAStARI). Joanna Briggs Institute Reviewers Manual. Australia: The Joanna Briggs Institute, 2014

Supplementary Table 5: Question: Is there a specific clustering of oral symptoms associated with HNC treatment that could impact the pathogenesis of radiation caries?

Certainty assessment							Impact	Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations			
Radiation-related symptoms clusters in descriptive studies (follow up: mean 6 months; assessed with: Assessment Scales or Questionnaires)									
9	observational studies	serious [a]	serious [b]	not serious	not serious	very strong association	Patients usually experience severe symptoms at the end of radiotherapy and there is an impact of individual patterns. During the treatment process, determining the symptoms clusters may contribute on control and consequent improvement of quality of life.	⊕⊕◯◯ LOW	IMPORTANT
Radiation-related symptoms clusters in cross-sectional studies (follow up: mean 1 years; assessed with: Questionnaire)									
2	observational studies	not serious	not serious	not serious	not serious	strong association	Despite improvements in radiotherapy, HNC patients still suffer from numerous distressing acute and chronic side effects. Understanding the underlying relationship among symptoms may lead to a more efficient and effective approach to manage the symptom cluster as a whole.	⊕⊕⊕◯ MODERATE	IMPORTANT

Explanations
a. Most studies were categorized as having a moderate risk of bias.
b. Symptoms were measured, analyzed and reported heterogeneously across studies.

Supplementary Table 6. Results reported from the included studies, total number of assessed patients and criteria for assessment and results report

Study	Swalling problems/Dysphagia	Dry mouth/Xerostomia	Pain	Taste alterations	Fatigue	Mucous	Sore mouth	Chewing problems	Teeth/Gum problems - Dental Caries	Radiodermatitis	Oral Mucositis	Trismus	Smell alterations
Murphy et al., 2010	0.940	0.939	0.941	0.942	x	0.941	0.944	0.943	0.943	x	x	x	x
Haisfield-Wolfe et al., 2012	21 (100%)	19 (91%)	19 (91%)	16 (86%)	x	x	14 (70%)	x	x	17 (71%)	x	x	x
Xiao et al., 2013	0.65/0.62	0.43/0.51	0.50/0.51	0.47	0.43	x	x	x	x	0.65/0.62	0.46/0.46	x	x
Rosenthal et al., 2014	5.98	5.55	4.77	6.99	5.66	6.31	5.24	5.98	x	x	x	x	x
Kirka and Kutluturkan, 2016	NI	NI	NI	NI	NI	x	NI	x	x	x	x	x	x
Xiao et al., 2017	98.5%	97.7%	83.8%	96.9%	93.8%	99.2%	96.9%	98.5%	82.3%	76.7%	x	x	x
Eraj et al., 2017	2.59	3.48	0.75	2.81	1.08	2.04	0.54	2.59	0.78	0.13	x	x	x
Barnhart et al., 2018	87 (94.6%)	84 (91.3%)	x	79 (85.9%)	77 (83.7%)	x	x	x	14 (15.2%)	x	x	13 (14.1%)	x
McDowell et al., 2018	3.8	5.9	1.4	2.7	2.6	4.1	1.4	3.8	3	1	x	x	x
Chiang et al., 2018	x	99%	99%	x	99%	x	x	x	x	x	x	x	x
Ridner et al., 2018	0.92	0.92	0.94	0.89	x	0.95	x	x	0.75	x	0.87	x	0.91

NI: Not informed; X: Not assessed.

Supplementary Table 6 (continuation). Results reported from the included studies, total number of assessed patients and criteria for assessment and results report

Study	Loss of Appetite	Weight Loss	Nausea	Vomiting	Dehydration	Total of patients	Method of Assessment
Murphy et al., 2010	0.942	0.943	x	x	x	235	Vanderbilt*
Haisfield-Wofe et al., 2012	19 (91%)	19 (91%)	x	x	x	21	N/%
Xiao et al., 2013	x	x	0.85/0.92	0.78/0.78	0.50/0.50	684	CTC**
Rosenthal et al., 2014	5.02	x	3.42	2.12	x	149	MDASI***
Kirka and Kutluturkan, 2016	NI	NI	x	x	x	47	MSAS****
Xiao et al., 2017	95.4%	x	91.5%	81.5%	x	130	N/%
Eraj et al., 2017	1.10	x	0.15	0.00	x	79	MDASI
Barnhart et al., 2018	72 (79.1%)	x	x	x	x	92	N/%
McDowell et al., 2018	1.5	x	0.8	0.4	x	102	MDASI
Chiang et al., 2018	98%	x	84%	67%	x	100	N/%
Ridner et al., 2018	x	x	x	x	x	150	Vanderbilt

NI: Not informed; X: Not assessed. *Vanderbilt: Vanderbilt Head and Neck Symptom Survey; **CTC: NCI Common Toxicity Criteria (CTC) 2.0; ***MDASI: M. D. Anderson Symptom Inventory; ****MSAS: Memorial Symptom Assessment Scale. Except from the 4 studies that reported frequency values as percentage numbers, all other included studies reported mean values of response to Questionnaire-based assessment.

2.2 Artigo: Natural history of radiotherapy-induced dysgeusia among oral and oropharyngeal cancer patients undergoing different treatment modalities

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Abstract word count: 248
Word count: 4,317
References: 43

Figures: 4

Tables: 1

Supplementary Tables: 1

Key Words: Head and neck cancer, head and neck radiotherapy, chemotherapy, head and neck surgery, dysgeusia, xerostomia, oral mucositis

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ABSTRACT

Aims: To assess the patterns of radiotherapy-induced dysgeusia among oral and oropharyngeal squamous cell carcinoma (OOPSCC) patients subjected to different treatment modalities.

Patients and Methods: OOPSCC patients who underwent radiotherapy (RT) or chemoradiotherapy (CRT). Patients were paired in 8 groups divided by primary treatment (surgery or induction CT) followed by CRT or RT and definitive treatment with CRT or exclusive RT. Dysgeusia, xerostomia, oral mucositis (OM) grades and tube feeding information were retrieved from electronic medical charts. Statistical analysis was performed regarding the correlation of treatment modalities with dysgeusia, and of xerostomia and OM with dysgeusia.

Results: Over 90% of 150 patients developed dysgeusia during RT. Groups that included CRT presented early severe dysgeusia. Tongue surgery followed by CRT presented grade 2 dysgeusia on the first week when compared to RT ($p=0.04$), groups with other surgical strategies or induction CT followed by CRT presented grade 2 dysgeusia more frequently on the fourth week of RT ($p=0.04$). Xerostomia and dysgeusia grades and OM and dysgeusia grades presented a positive correlation ($p<0.001$, each). Malnutrition associated with appetite loss and dysgeusia was the indication for tube feeding in 26.8% of patients.

Conclusions: Results of the presented study show a natural increase in dysgeusia grades throughout RT weeks and that several factors related to the choice of treatment modalities and presence of xerostomia or OM may represent an important impact on how dysgeusia develops and early incidence of severe grade 2 dysgeusia. CRT protocols may be predictors of severe dysgeusia early onset. High incidence of tube feeding due to malnutrition associated to appetite loss and dysgeusia reinforces the importance of working on strategies for dysgeusia prevention and treatment.

INTRODUCTION

First described in 1959 by McCarthy Leventhal as “post-radiation mouth blindness”, dysgeusia is one of the most common acute oral toxicities of head and neck radiotherapy (HNRT) protocols, affecting 70% up to 95% of head and neck cancer patients (HNC). It is defined as an abnormal or impaired sense of taste, unpleasant alteration of taste sensation or taste loss (Baharvand et al., 2013; Cohen et al., 2016; Desphande et al., 2018; Jin et al., 2018; Ridner et al., 2018; Epstein et al., 2019).

HNC treatment is known to be multimodal including surgery, radiotherapy (RT) and chemotherapy (CT) which can be applied as induction CT, neoadjuvant or concomitant to radiotherapy (CRT) (Kowalski et al., 2005; Brener et al., 2007). All the above-mentioned treatment modalities present the ability of, either individually or combined, directly impair taste buds in tongue tissue and consequently decrease taste perception (Baharvand et al., 2013; Sapir et al., 2016; Amezaga et al., 2018).

In this context, several factors have been reported to have an impact on dysgeusia onset, such as tongue tissue surgical resection, direct radiogenic effect inducing the loss of taste buds, irradiated tongue volume, percentage of anterior mouth/tongue apex in the irradiated field, impairment in oral neural structures, direct effect of CT regimens such as cisplatin, 5-fluorouracil (5FU) and taxanes on taste buds cell proliferation, in addition to reported indirect factors, such as the presence of OM in the tongue, a shift in oral microflora and decreased salivary flow rates that also play an important role on taste dysfunction (Sciubba and Goldenberg, 2006; Irune et al., 2014; Cohen et al., 2016; Sapir et al., 2016; Zecha et al., 2016; Amezaga et al., 2018 Ridner et al., 2018; Gouvêa Vasconcellos et al., 2020).

In terms of the direct impact of RT on the development of dysgeusia, studies report that RT presents with cytotoxic and anti-proliferative effect on tissues in the irradiated field. This can lead to cell cycle arrest and apoptosis of basal taste progenitor cells, decrease replacement

of taste buds within the papilla and alter their structure which results in disrupted transduction of flavor molecules (Mossman et al., 1986; Hovan et al., 2010; Irune et al., 2014; Negi et al., 2017; Epstein et al., 2019). RT may also promote neural damage on the afferent nerves that supply taste bud cells and consequently impair the detection of taste through the diffusion of substances to the taste receptors, chemical interaction with taste substances and changes in the sensitivity of the taste receptors (Hovan et al., 2010; Irune et al., 2014; Sapir et la., 2016; Negi et al., 2017; Epstein et al., 2019).

Radiotherapy-induced dysgeusia usually starts on the third or fourth week of RT and extends to 4 to 5 weeks post-HNRT with most of the patients recovering full function in 6 to 12 months post-HNRT; nevertheless, there are reports of patients with recover period of over 5 years post-HNRT (Germano et al., 2015; Sapir et al., 2016; Negi et al., 2017; Desphande et al., 2018). Patients commonly describe the alteration in taste perception as a bitter, metallic, salty and/or unpleasant taste which can implicate in appetite loss, decreased food intake, increased ingestion of sweets (the least affected flavor), induction of nausea, impairment of nutritional status and weight loss (over 10% of weight loss implicates in a poorer treatment prognosis), decreased compliance to treatment, inefficient response to oncologic treatment, emotional distress (Negi et al., 2017; Amezaga et al., 2018; Jin et al., 2018; Barbosa da Silva et al., 2019; Epstein et al., 2019; Martini et al.,2019), and consequently negatively impact the quality of life (QoL) of the patients (Baharvand et al., 2013).

Therefore, this study aimed to assess the patterns of radiotherapy-induced dysgeusia among oral and oropharyngeal patients submitted to different HNC treatment modalities. As secondary aims, the correlation of xerostomia and oral mucositis (OM) with dysgeusia and the main reasons for tube feeding implementation were also assessed.

MATERIALS AND METHODS

Study Design

This was a single-center cohort study designed to evaluate the patterns of dysgeusia development in oral and oropharyngeal squamous cell carcinoma (OOPSCC) patients submitted to RT or CRT protocols at the Instituto do Câncer do Estado de São Paulo (ICESP, Brazil) from January 2009 to July 2019. This study was approved by the Ethics Committee of the School of Medicine, University of São Paulo, São Paulo, Brazil (Protocol# 1.897.352). Written informed consent was obtained from all participants and the study was conducted in accordance with the Declaration of Helsinki. Data collection followed the guideline for reporting observational studies as per Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (von Elm et al., 2007).

Inclusion criteria

This study included OOPSCC who underwent RT or CRT protocols (with or without previous surgery) using a 6MV linear accelerator and 3-Dimensional conformal or intensity-modulated radiation therapy technique (IMRT) (Synergy Platform, Elekta AB, Stockholm, Sweden). The target radiation volumes encompassed the primary site and areas of regional lymph nodes at risk and received cumulative doses that ranged from 60 to 70 Gy. We applied the recommendations for treatment planning and constraints for organs at risk as previously reported (Grégoire et al., 2014; Mendez et al, 2016; Grégoire et al., 2018).

All included patients completed the institutional dental conditioning protocol before the RT onset. Besides routine oral care, all patients were submitted to the standard-of-care daily photobiomodulation (PBM) protocol for prevention of OM as per our institutional (Dental Oncology Service, ICESP, Brazil) protocol (Brandão et al., 2018). Finally, all patients needed to have complete demographic and clinicopathological data available on electronic medical charts including sex, age, tumor location, clinical cancer stage [according to the American Joint

Committee on Cancer Staging System, 7th edition (Edge and Comptom, 2010)], cancer treatment modalities and information regarding weekly dental follow-up during RT and dysgeusia outcomes.

Exclusion criteria

Patients who missed one or more RT, CT or PBM sessions were considered to have received incomplete treatment and were excluded from the study. Patients that started RT feeding exclusively through nasogastric tubes were also excluded due to the impossibility of dysgeusia assessment from the beginning of treatment.

Dysgeusia assessment

Patients were divided according to treatment modality as described below. Group 1: patients submitted to partial glossectomy or hemiglossectomy followed by RT (N=23); Group 2: partial glossectomy or hemiglossectomy followed by CRT (N=18); Group 3: patients submitted to other HNC surgery protocols not including tongue tissue, such as maxillectomy or mandibulectomy, followed by RT (N=16); Group 4: other HNC surgery protocols not including tongue tissue, such as maxillectomy or mandibulectomy, followed by CRT (N=13); Group 5: induction CT followed by RT (N=7); Group 6: induction CT followed by CRT (N=27); Group 7: concomitant CRT (N=35), and Group 8: exclusive RT (N=11).

A trained dental surgeon conducted dysgeusia and OM grading using the National Cancer Institute Common Terminology Criteria for Adverse Events [NCI CTCAE, Version 4.0, 2010 (National Cancer Institute, 2009)] and xerostomia using the criteria by Eisbruch et al., 2003, on the last day of each week of treatment (Day 5, 10, 15, 20, 25, 30 and 35) as part of the Service standard of care. Additionally, information on the need and reasons for the implementation of nasogastric tube feeding throughout RT was also collected.

Statistical analysis

Demographic data, clinicopathological features and clinical outcomes results were grouped into a spreadsheet for descriptive statistical analyzes based on mean, median, standard deviation and proportion values. For the dysgeusia analysis, statistical analysis was performed by pair-matching the groups according to treatment modality as follows: Group 1 vs. Group 2; Group 3 vs. Group 4; Group 5 vs. Group 6 and Group 7 vs. Group 8 and correlated with RT weeks. Additionally, the statistical analysis of the correlation of dysgeusia and xerostomia and OM was performed. All statistical analyses were performed using SPSS 20.0 (IBM, Armonk, New York, USA). Level of statistical significance was set at 5% ($\alpha = 0.05$). Friedman test with post-hoc analyses using a Wilcoxon with Bonferroni correction was used to assess the distribution of dysgeusia, nasogastric tube feed, xerostomia and OM through RT weeks. Spearman correlation test (r=0,70 for strong correlation)was used to assess the distribution of dysgeusia among the groups of oncologic treatment and the correlation of dysgeusia with xerostomia and OM grades.

RESULTS

Patient characteristics

A total of 150 OOPSCC patients were included. The major clinicopathological features including age, sex, tumor site, clinical staging, type of treatment, RT, CT and information on nasogastric tube feeding were summarized in **Table 1**. The mean age was 58.9 years (range 20-86 years), and most patients (78%) were males. The most frequent primary tumor site was the lateral border of the tongue (30%) followed by the base of the tongue with oral extension (20.6%) and oropharynx (13.3%). Patients were mostly diagnosed with advanced stage of disease III or IV (94.7%). All included patients received complete and uninterrupted RT. Forty-

one (27.3%) patients were submitted to partial or hemiglossectomy and 34 (22.7%) were submitted to induction CT protocols based on cisplatin and paclitaxel (21, 61.8%), carboplatin and paclitaxel (12, 35.3%) and cisplatin and 5-Fluoracil (1, 2.9%). A total of 93 (62%) patients were submitted to concomitant CRT protocols, from which 90 (96.8%) received cisplatin, 2 (2.1%) received a protocol of cisplatin and carboplatin and 1 (1.1%) patient received a protocol of carboplatin and paclitaxel. None of the patients submitted to induction CT were submitted to surgical procedures for tumor resection.

Regarding the RT technique, 132 (88%) patients were treated by 3-Dimensional conformal RT; and 18 (12%) with IMRT, with an overall mean dose of 66.6 Gy (ranging from 64.1Gy to 67Gy).

Dysgeusia assessment

The great majority of patients (94.67%) developed dysgeusia during oncologic treatment, with only 8 (5.33%) patients not reporting any grade of dysgeusia throughout RT **(Figure 1A)**. The distribution of patients according to the grade of dysgeusia, excluding the ones on a nasogastric feeding tube is showed in **Figure 1B**. Our analysis showed a statistically significant difference in dysgeusia grades among the different weeks of RT ($p<0.0001$, Friedman test). Post-hoc analyses using a Bonferroni correction showed a statistically significant increase in dysgeusia grade until the fifth week of RT ($p<0.017$, Wilcoxon test), as shown in **Figure 1B**.

The incidence of dysgeusia and incidence of severe grade 2 dysgeusia per group of analysis are shown in **Figure 2**. All groups that included CRT protocols presented patients with grade 2 dysgeusia from the first week of RT while from the groups with no concomitant CT protocols only the exclusive RT (group 8) presented 1 (9.1%) patient with grade 2 dysgeusia in the first week of treatment. On the second week of RT, 69.3% of the sample presented variable

levels of dysgeusia and all groups presented a higher percentage of grade 1 dysgeusia when compared to grade 2. By the third week of RT, most of the patients (83.4%) presented different grades of dysgeusia and all groups that included CRT protocols presented a higher percentage of grade 2 dysgeusia when compared to the RT only counterpart, except for group 1 patients which tongue surgery followed by RT patients presented a higher percentage of grade 2 dysgeusia when compared to patients submitted to CRT protocols. From the fourth week over half the sample presented grade 2 dysgeusia with the highest percentage found in patients submitted to tongue surgery and for the groups of patients submitted to CRT protocols. On the fifth and sixth weeks, all groups presented a higher percentage of patients with grade 2 dysgeusia except for the patients submitted to induction CT followed by RT (Group 5). Finally, on the seventh week only group 1 presented with a similar percentage of grade 2 dysgeusia when compared to grade 1 while all other groups presented a higher incidence of grade 2 dysgeusia, it is worth to mention that group 1 patients presented with 56.6% of the patients finishing the RT with 6 weeks (30 RT sessions, 60Gy). Patients submitted to induction CT followed by CRT protocols (Group 6) presented the highest overall incidence and severe grade 2 dysgeusia incidence from the third week of RT until the end of cancer treatment.

Distribution of dysgeusia grades during RT according to the pair-matched analysis following the type of oncologic treatment is presented in **Figure 3.** The comparison between group 1 and group 2 revealed that patients submitted to CRT protocols presented more dysgeusia ($p=0.04$) in the first week of RT. However, there was no significant difference in terms of dysgeusia outcomes for the other RT weeks **(Figure 3A)**. Group 3 and 4 comparisons revealed that dysgeusia grades were similar on the first 3 weeks; however, after the fourth and fifth weeks (D20/D25), patients receiving CRT had statistically higher levels of dysgeusia than observed in RT ($p=0.04$ for both weeks), with all patients presenting grade 2 dysgeusia **(Figure 3B)**. Comparing groups 5 and 6, patients submitted to CRT protocols presented significantly

higher dysgeusia levels after the fourth week (p=0.04), with almost all patients presenting grade 2 dysgeusia after the fifth week **(Figure 3C)**. Finally, the comparison of group 7 and 8 revealed that, although not statistically different, patients submitted to CRT protocols presented higher dysgeusia levels on the first half of oncologic treatment when compared to patients submitted to exclusive RT (**Figure 3D)**.

Results show a statistically significant difference in xerostomia grades among the weeks of RT ($p<0.0001$, Friedman test). Post-hoc analyses using a Bonferroni correction showed a statistically significant increase in dysgeusia grade until the fifth week of RT ($p<0.017$, Wilcoxon test), as shown in **Figure 3A**. Analyzing the relation among dysgeusia and xerostomia, a positive correlation was observed between the grade of xerostomia and grade of dysgeusia ($r=0.29$, $p<0.001$). Additionally, a significant difference in OM grades among the weeks of RT ($p<0.0001$, Friedman test) was observed. Post-hoc analyses using a Bonferroni correction showed a statistically significant increase in OM grade as RT progressed ($p<0.017$, Wilcoxon test), as shown in **Figure 4B.** Analyzing the relationship between OM and dysgeusia, a positive correlation was observed between the grade of OM and grade of dysgeusia ($r=0.42$, $p<0.001$).

A total of 47 (31.3%) patients required exclusive nasogastric tube feeding throughout RT. On the first two weeks 1 (0.6%) and 2 (1.4%) patients, respectively, started exclusive tube feeding. On the third week, this number increased to 11 (7.3%), followed by 17 (11.4%) on the fourth week, 29 (19,4%) on the fifth week, 36 (24%) on the sixth and finally 27 (18%) on the seventh week of RT. The most reported reason for tube feeding implementation was dysphagia and swallowing problems (17, 41.5%) followed by malnutrition due to appetite loss associated with dysgeusia (as reported in medical charts) (11, 26.8%), severe OM (7, 17.1%) and odynophagia (6, 14.6%).

DISCUSSION

This study evaluated the patterns of dysgeusia development among oral and oropharyngeal patients submitted to HNRT subjected or not to tumor resection surgery, induction CT or CRT protocols. From the present results, it was possible to observe that patients submitted to concomitant CRT protocols presented an early onset of grade 2 dysgeusia which increased throughout RT weeks when compared to patients treated with RT alone or surgery/induction CT followed by RT. This finding is important especially considering that most of HNC patients are diagnosed at advanced stage of disease requiring CRT regimens, hence increasing the incidence of severe dysgeusia and its possible impact on patient's nutritional status and QoL (Matzinger et al., 2009; Baharvand et al.,2013; Amezaga et al., 2018).

In terms of treatment modality, over 90% of the sample was submitted to multimodality treatment protocols, from which 27.3% involved partial or hemiglossectomy as primary surgery protocol, and 62% were submitted to concomitant CRT protocols, which is compatible with literature reports for the management strategies for advanced stage HNC (Kowalski et al., 2005; Scully et al., 2005; Marta et al., 2015; Stewart et a., 2015; Sloan et al., 2017).

Usually, normal human taste bud cells present a turnover rate of 10 days, which is severely impaired with the cumulative effect of RT dose (Hovan et al., 2010). Literature reports that taste becomes measurably impaired on the first week of RT and that doses of 20Gy (2 weeks) are the starting point for the report of taste loss, with over 90% of patients submitted to 60Gy reporting relative taste loss (Irune et al., 2014; Negi et al., 2017). In the present study, over 90% of the patients across all treatment modalities, tumor topography, and clinical stages, developed some degree of dysgeusia starting from the first week and with progressive increase throughout RT, which is in accordance with literature reports for the incidence of dysgeusia among HNC patients (Hovan et al., 2010; Baharvand et al., 2013; Negi et al., 2017; Desphande et al., 2018; Epstein et al., 2019; Martini et al., 2019).

In terms of dysgeusia progression, observed results are similar to previously reported literature results of approximately 40% of patients presenting grade 1 dysgeusia on the second week of RT, with an increase in the severity on weeks 3 and 4, as observed for groups 3 to 6, with approximately 60% of patients presenting with severe grade 2 dysgeusia at the final weeks of treatment (Hovan et al., 2010; Germano et al., 2015; Zecha et al., 2016; Palmieri et al., 2019).

From an anatomic perspective, the early onset of dysgeusia in the tongue surgery group and the lack of difference from the second week to the end of RT between patients submitted to CRT protocols and the ones submitted to RT may be explained by the fact that the removal of tongue tissue, hence taste buds, from the surgical procedure reduces the threshold for taste perception. Additionally, impairment of the taste signal transduction pathway due to damage to the chorda tympani and/or glossopharyngeal nerve regions, which are the main innervations for taste transduction signal, could negatively alter patients subjective taste perceptions, hence justifying the observed results (Epstein and Barasch., 2010; Tomita et al., 2014; Sapir et al., 2016).

All groups submitted to CRT protocols presented with early severe grade 2 dysgeusia starting from the first week of RT. In this context, studies report that CT-induced dysgeusia may start within 3 to 5 days of CT infusion and that taste impairment may increase in patients submitted to induction CT or CRT protocols (Berteretche et al., 2004; Irune et al., 2014; Tomita et al., 2014; Ponticelli et al., 2017). Additionally, virtually all patients submitted to induction CT were submitted to taxane-based CT regimens, such as paclitaxel and carboplatin, which are known to be significant predictors of taste loss and correlated to more severe taste disorders; while patients submitted to CRT protocols were based on cisplatin regimens, which are correlated to high rates of metallic taste reported by the patients (Wickham et al., 1999; Irune et al., 2014; Amezaga et al., 2018).

CT regimens present important cytotoxicity and neurotoxicity targeting rapidly dividing

cells in a non-selective way. Additionally, these medications may be secreted in saliva or through crevicular fluid from plasma and, therefore, present the ability of damaging taste buds and their receptor cells. Also, CT may induce thickening of the epithelia leading to smaller areas of taste pores, corroborating with the findings of the present study that all groups submitted to CRT protocols and the group submitted to induction CT followed by CRT presented a worse prognosis of dysgeusia when compared to their RT only counterpart (Hovan et al., 2010; Irune et al., 2014; Tomita et al., 2014; Epstein et al., 2019; Martini et al., 2019).

In the present study, there was no statistical difference between dysgeusia grades among group 7 (CRT) and group 8 (exclusive RT) patients and although this was probably due to the small number of RT exclusive patients, it was possible to observe an increased incidence of severe grade 2 for the CRT group in the first weeks of treatment, corroborating with above-mentioned information that CT regimens can potentialize the effects of RT. Moreover, by the fourth week of RT, cumulative doses of 40Gy are above the threshold for radiation impact on taste bud cells (usually 20Gy to 30Gy), leading to the peak of dysgeusia incidence and severity (Hovan et al., 2010; Irune et al., 2014; Zecha et al., 2016; Palmieri et al., 2019).

The positive correlation between xerostomia and severe dysgeusia in the first three weeks of RT observed in the present study is in accordance with literature reports on this matter (Sapir et al., 2016; Barnhart et al., 2018). To stimulate taste receptor cells within the taste buds, food particles need to be solubilized, therefore saliva represents an important role in both the transport of flavor molecules and protection of taste receptors by the action of salivary water, electrolytes and mucins that have the ability of modulating the sensitivity of the chorda tympani innervation of taste buds. Therefore, patients that report xerostomia or effective hyposalivation usually present with a decreased secretion and increased viscosity of saliva, interfering with the transportation of flavor process, culminating in the impairment of taste perception (Irune et al., 2014; Bressan et al., 2017; Amezaga et al., 2018; Barnhart et al., 2018). The presence of

combined xerostomia and dysgeusia may synergistically impact patients desire to eat due to altered/restricted food choices, nausea, decreased food intake and ability to enjoy food, consequently leading to malnutrition, weight loss, need for nasogastric tube and a consequent negative impact on patients QoL (Ogama et al., 2010; Bressan et al., 2016; Sapir et al., 2016; Bressan et al., 2017; Barnhart et al., 2018).

In addition to the correlation of xerostomia and dysgeusia, the present study also observed a positive correlation of OM and dysgeusia during RT weeks. With the onset of OM, one of the most affected topographies is the lateral border of the tongue, which with the progression of ulcers represents a reduction of taste buds and could be one of the factors associated with impaired taste perception among post-RT patients (de Pauli Paglioni et al., 2019). Furthermore, these results are in accordance with the concept of the cluster of oral symptoms which is based on the theory that radiation-induced oral toxicities usually combine and overlap in a synergic way (Gouvêa Vasconcelos et al., 2020). Studies report that there may be an interconnection between dysgeusia, xerostomia and OM and a positive correlation where one complication could directly affect the others and when combined dysgeusia, OM and xerostomia could impact patients' appetite and consequently oral intake and nutritional status (Ogama et al., 2010; Ogama et al., 2012; Bressan et al., 2016).

In this context, appetite loss due to dysgeusia was the second most common reason for exclusive nasogastric tube feeding in 26.8% of the patients included in the present study, who required tube feeding implementation to control weight loss and reduce the negative impact on oncologic treatment. This is in accordance with Brown et al., 2017, which reported that appetite loss due to taste alterations was the primary reason for tube feeding implementation on weeks 2 and 3 of RT for over half of the patients with overall values for the indication for tube feeding ranging from 16% to 22%.

Taste represents an important role in patients QoL because it allows to sense and enjoy

food. As observed in the present study, the high rates of incidence and severity of dysgeusia throughout RT may negatively influence patient's nutritional intake, lead to nausea and vomiting, weight loss and consequently need for exclusive nasogastric tube feeding. Moreover, literature reports also correlate it to patients' mood swings, irritation, sadness and, disappointment, limiting patients daily normal activities, hence promoting an important impairment of QoL (Sapir et al., 2016; Ponticelli et al., 2017; Desphande et al., 2018; Martini et al., 2019).

Although dysgeusia is a well-recognized acute toxicity of oncologic treatment, it is still often ignored and overlooked by patients, clinicians and also researchers in the supportive care field, being an under-investigated problem when compared to other toxicities such as dysphagia and xerostomia, regarding its impact on patients' nutritional status and QoL. Hence, more studies are necessary to consolidate dysgeusia and its consequences on patients' outcomes throughout oncologic treatment (Irune et al., 2014; Negi et al., 2017; Jin et al., 2018).

The major strengths of the current study were the rigorous clinical inclusion and exclusion criteria that enabled a relatively homogeneous cohort of subjects and the possibility of dividing the patients with a considerable number per group of oncologic treatment modality.

LIMITATIONS

Limitations of the present work include a single institution study design, availability of only subjective assessment of taste alterations and impossibility to correlate HNRT dosimetry features on tongue tissue and dysgeusia development. These limitations justify the need for a larger and independent cohort and long-term prospective studies with the assessment of both quantitative and patients' perception of taste alterations throughout HNC oncologic treatment.

CONCLUSIONS

Results of the presented study show a natural increase in dysgeusia grades throughout RT weeks and that several factors related to the choice of treatment modalities and presence of xerostomia or OM may represent an important impact on how dysgeusia develops and early incidence of severe grade 2 dysgeusia. CRT protocols represent an important factor on severe grade 2 dysgeusia early onset, which could represent a negative impact on patients' nutritional status and treatment prognosis. Additionally, the fourth week of RT seems to be the turning point for a higher incidence of severe grade 2 dysgeusia. A positive correlation of xerostomia and OM with dysgeusia grades may lead to a decrease in appetite, restricted textures of food and food avoidance, also representing an important impact on the nutritional status of patients. Finally, the observed incidence of tube feeding due malnutrition associated to appetite loss secondary to dysgeusia reinforces the importance of working on strategies for the prevention and management of this debilitating toxicity that affects an important percentage of patients undergoing oncologic treatment for OOSCC.

ACKNOWLEDGEMENTS

The authors thank São Paulo Research Foundation (FAPESP) for financial support (process numbers 2018/04657-8; 2018/02233-6; 2016/22862-2; 2013/18402-8 and 2012/06138-1) as well as the National Council for Scientific and Technological Development (CNPq), Brazil. The authors also state that this study was financed in part by the Coordination for the Improvement of Higher Education Personnel -Brazil (CAPES)- Finance Code 001.

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Table 1. Clinicopathological features of 150 patients included in the study

Parameter	Total (n)	Percentage (%)
Age (mean):	58.9 years (range 20-86)	
Sex		
Male	117	78%
Female	33	22%
Clinical Stage		
Stage I	0	0%
Stage II	8	5.3%
Stage III	24	16%
Stage IV	118	78.7%
Primary tumor site		
Tongue (lateral border)	45	30%
Base of tongue with oral extension	31	20.6%
Oropharynx	20	13.3%
Palate	14	9.3%
Floor of the mouth	14	9.3%
Retromolar area	10	6.7%
Gingiva	6	4%
Buccal mucosa	3	2%
Soft palate	3	2%
Lip mucosa	2	1.4%
Tonsil	2	1.4%
Treatment		
Partial or hemi glossectomy + RT	23	15.3%
Partial or hemi glossectomy + CRT	18	12%
Other HNC surgery + RT	16	10.7%
Other HNC surgery + CRT	13	8.7%
Induction CT + RT	7	4.7%
Induction CT + CRT	27	18%
Exclusive CRT	35	23.3%
Exclusive RT	11	7.3%
RT Modality		
3DRT	132	88%
IMRT	18	12%
Mean radiation dose:	66.6Gy (range 64.1-67Gy)	
Cause of tube feeding*		
Dysphagia	17	41.5%
Malnutrition associated to appetite loss and dysgeusia	11	26.8%
Oral mucositis	7	17.1%
Odynophagia	6	14.6%

Abbreviations used: RT-Radiotherapy; CRT-Chemoradiotherapy; HNC: Head and neck cancer; CT-Chemotherapy; 3DRT-3-dimensional conformal radiotherapy; IMRT-Intensity modulated radiotherapy. *Patients feeding exclusively through nasogastric tube at some point (N=41).

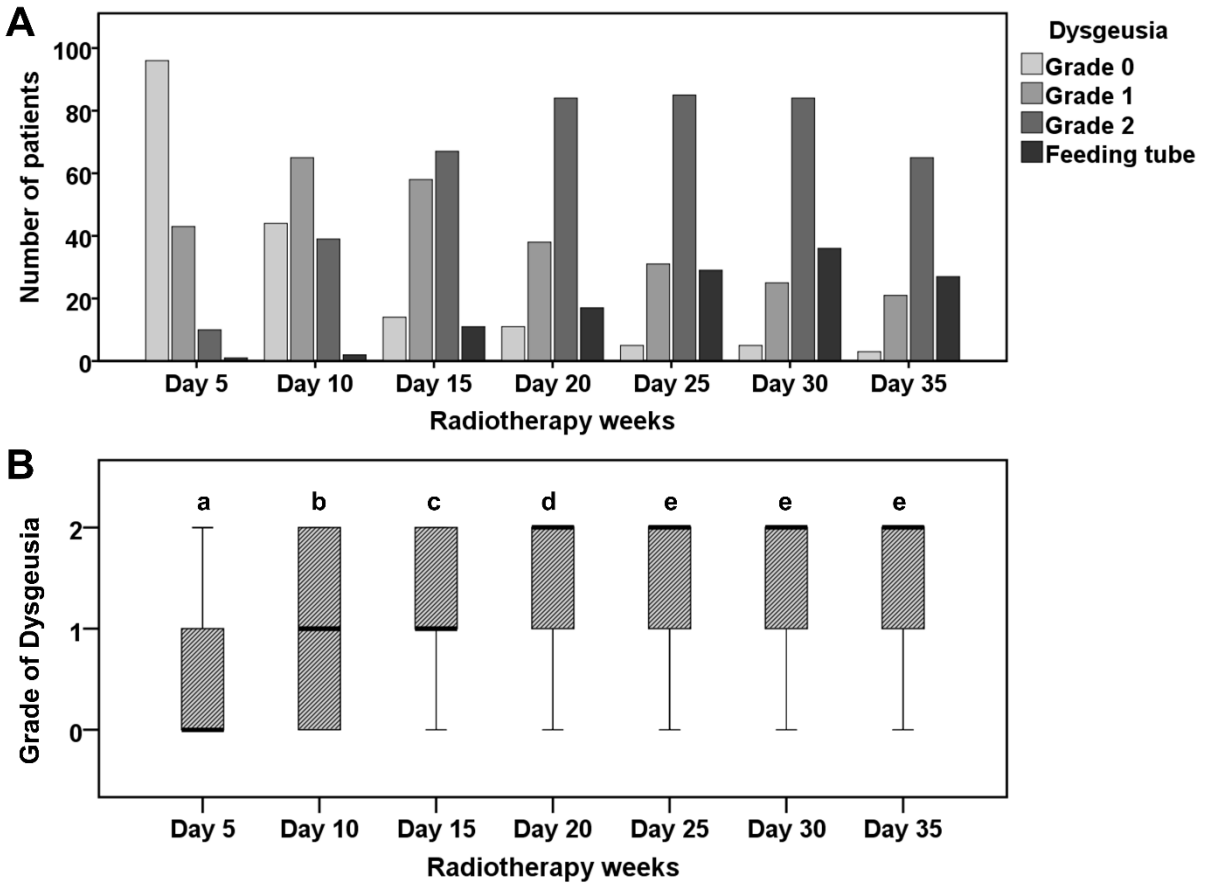

Figure 1: Distribution of dysgeusia grades and exclusive nasogastric tube feeding during radiotherapy weeks in HNC patients. **A:** Number of patients presenting dysgeusia or feeding tube; note the increase of grade 2 dysgeusia after the third week of radiotherapy, and an increase nasogastric feeding tube in the second half of the radiotherapy course. **B:** Distribution of patients according to the grade of dysgeusia (patients on nasogastric feeding tube were excluded). Results show a statistically significant difference in dysgeusia grades among the weeks of RT ($p<0.0001$, Friedman test). Post-hoc analyses using a Bonferroni correction showed a statistically significant increase in dysgeusia grade until the fifth week of RT ($p<0.017$, Wilcoxon test). Different letters mean statistically significant differences.

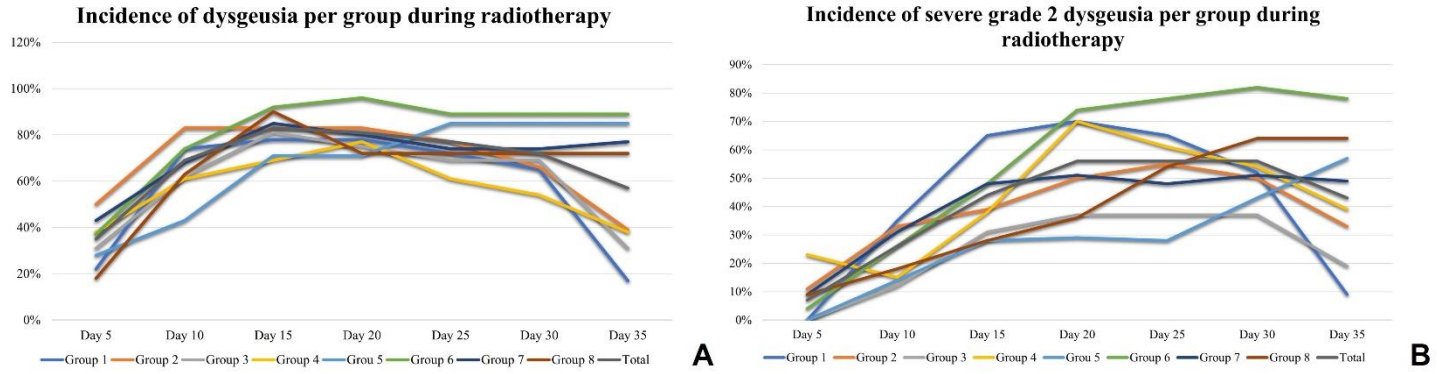

Figure 2: Incidence of dysgeusia and incidence of severe grade 2 dysgeusia per group of analysis during radiotherapy (RT) weeks. Group 1: Patients submitted to partial or hemi glossectomy followed by RT; Group 2: Partial or hemi glossectomy followed by CRT; Group 3: Patients submitted to other HNC surgery protocols not including tongue tissue followed by RT; Group 4: Other HNC surgery protocols not including tongue tissue followed by CRT; Group 5: Induction CT followed by RT; Group 6: Induction CT followed by CRT; Group 7: Concomitant CRT and Group 8: Exclusive RT. **A:** Linear graphic representation of the incidence of dysgeusia per group of study during RT weeks. Note that almost all groups (except from group 1) presented dysgeusia rates of approximately 60% from week 2 of RT which remained stable or increased according to each group until week 5 of RT. **B:** Linear graphic representation of the incidence of severe grade 2 dysgeusia per group of study during RT weeks. Note that most groups that included chemoradiotherapy protocols (Groups 4, 6 and 7) presented earlier higher incidence of severe dysgeusia when compared to the radiotherapy only counterpart.

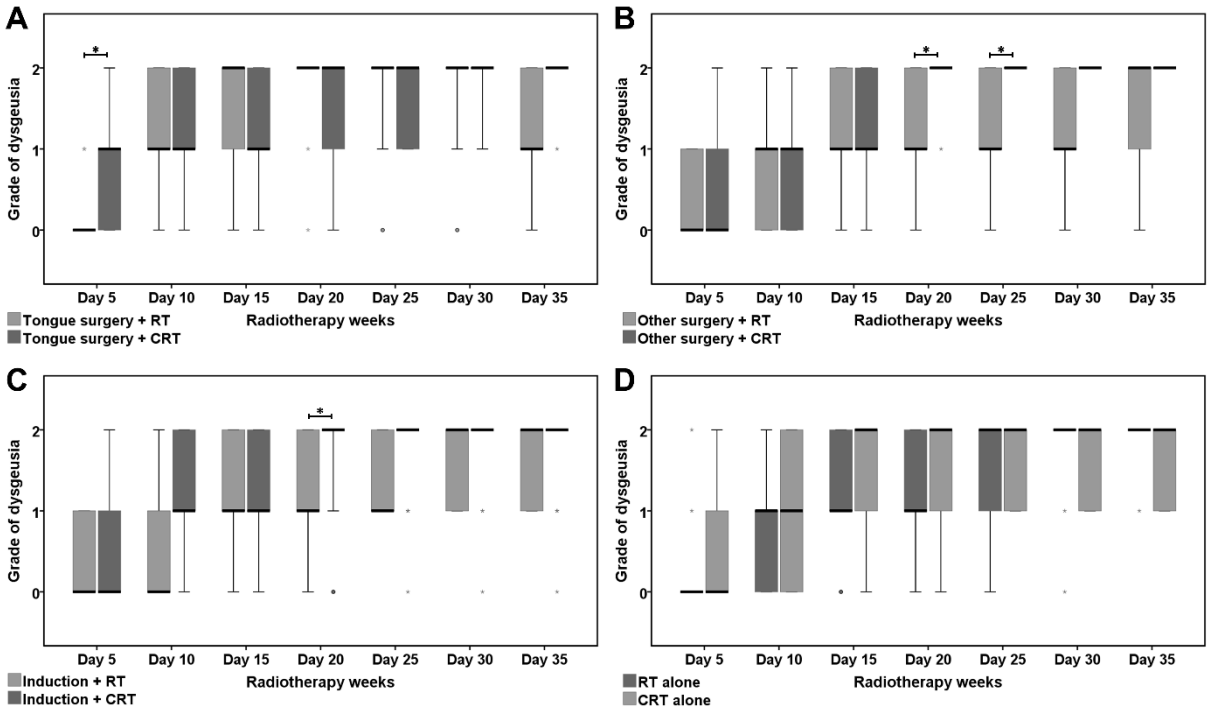

Figure 3: Distribution of dysgeusia grades during radiotherapy treatment according to the type of oncologic treatment in HNC patients. **A:** Group 1 vs Group 2: patients who underwent CRT after tongue surgery had increased dysgeusia levels after the first week of treatment in comparison to patients who received only radiotherapy (p=0.04, Spearman test). However, no differences were observed in the subsequent weeks. **B:** Group 3 vs Group 4: patients who underwent CRT after surgery (other than tongue) had increased dysgeusia levels after the fourth and fifth weeks of treatment in comparison to patients who received only radiotherapy (p=0.04 for both weeks, Mann-Whitney test), with all patients receiving CRT presenting grade 2 dysgeusia after the fourth week. **C:** Group 5 vs Group 6: similar results were observed, with a statistically significantly increased dysgeusia levels in patients submitted to CRT after the fourth week (p=0.04). No differences were observed in the last three weeks of radiotherapy. **D:** Group 7 vs Group 8: patients who underwent CRT protocols presented higher grades of dysgeusia in the first three weeks of treatment, although with no statistical differences.

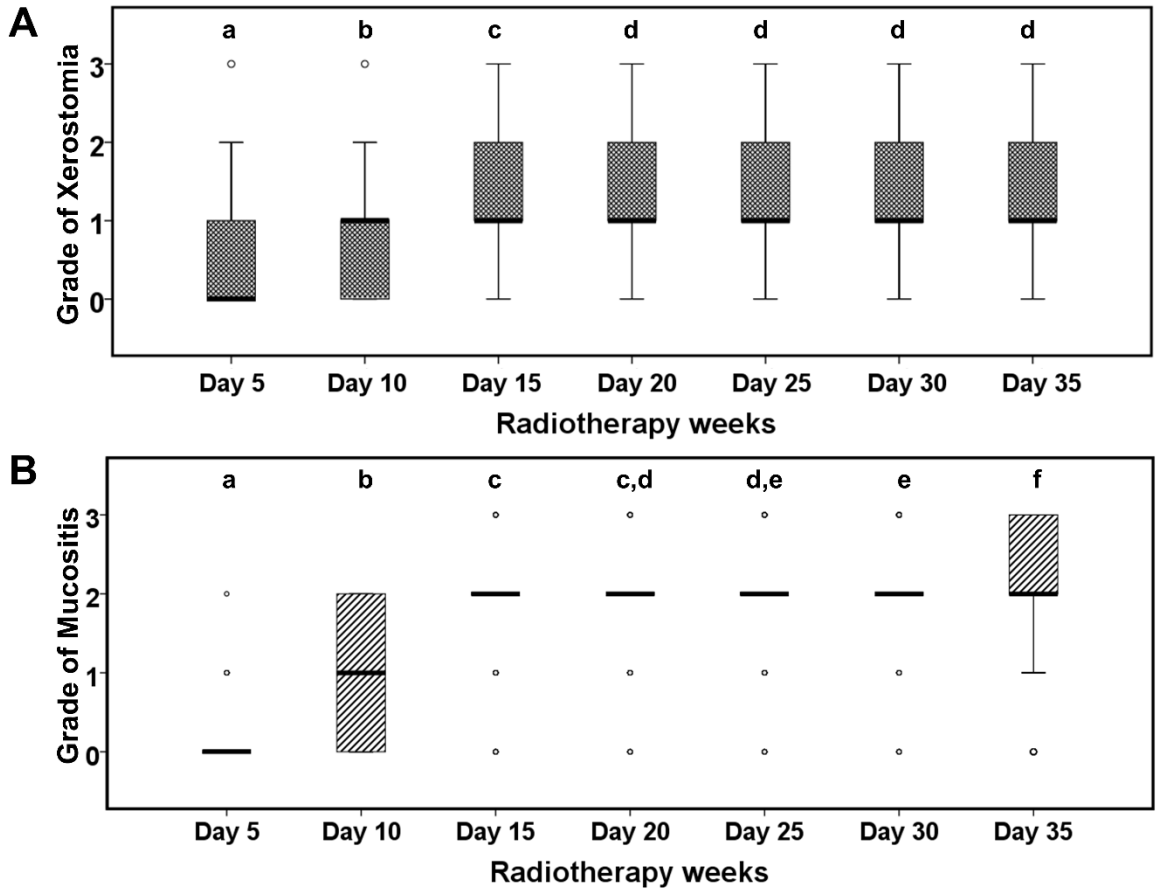

Figure 4: Distribution of xerostomia and oral mucositis grades (OM) during radiotherapy course in HNC patients. **A:** Results show a statistically significant difference in xerostomia grades among the weeks of RT ($p<0.0001$, Friedman test). Post-hoc analyses using a Bonferroni correction showed a statistically significant increase in dysgeusia grade until the fifth week of RT ($p<0.017$, Wilcoxon test). Different letters mean statistically significant differences. **B:** Results show a statistically significant difference in OM grades among the weeks of RT ($p<0.0001$, Friedman test). Post-hoc analyses using a Bonferroni correction showed a statistically significant increase in OM grade as RT progressed ($p<0.017$, Wilcoxon test). Different letters mean statistically significant differences.

Supplementary Table 1: Dysgeusia grades at the end of each week of radiotherapy divided by group of oncologic treatment.

Dysgeusia	Group 1 (N=23) N(%)	Group 2 (N=18) N(%)	Group 3 (N= 16) N(%)	Group 4 (N=13) N(%)	Group 5 (N=7) N(%)	Group 6 (N=27) N(%)	Group 7 (N=35) N(%)	Group 8 (N=11) N(%)	Total (N=150) N(%)
Week 1 (D5)									
Grade 0	18 (78.3%)	9 (50%)	11 (68.7%)	8 (61.5%)	5 (71.4%)	16 (59.3%)	20 (57.1%)	9 (81.8%)	96 (64%)
Grade 1	**5 (21.7%)**	**7 (38.9%)**	**5 (31.3%)**	2 (15.4%)	**2 (28.6%)**	**9 (33.3%)**	**12 (34.3%)**	1 (9.1%)	43 (28.7%)
Grade 2	0 (0%)	2 (11.1%)	0 (0%)	**3 (23.1%)**	0 (0%)	1 (3.7%)	3 (8.6%)	1 (9.1%)	10 (6.7%)
Tube feeding*	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1(3.7%)	0 (0%)	0 (0%)	1 (0.6%)
Not Applicable**	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Week 2 (D10)									
Grade 0	6 (26.1%)	3 (16.7%)	5 (31.3%)	5 (38.5%)	4 (57.1%)	6 (22.2%)	11 (31.4%)	4 (36.4%)	44 (29.3%)
Grade 1	**9 (39.1%)**	**9 (50%)**	**8 (50%)**	**6 (46.1%)**	**2 (28.6%)**	**13 (48.2%)**	**13 (37.2%)**	**5 (45.4%)**	65 (43.3%)
Grade 2	8 (34.8%)	6 (33.3%)	2 (12.5%)	2 (15.4%)	1 (14.3%)	7 (25.9%)	11 (31.4%)	2 (18.2%)	39 (26%)
Tube feeding*	0 (0%)	0 (0%)	1 (6.2%)	0 (0%)	0 (0%)	1 (3.7%)	0 (0%)	0 (0%)	2 (1.4%)
Not Applicable**	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Week 3 (D15)									
Grade 0	3 (13.1%)	1 (5.6%)	1 (6.2%)	2 (15.4%)	2 (28.6%)	2 (7.4%)	2 (5.7%)	1 (9.1%)	14 (9.3%)
Grade 1	3 (13.1%)	**8 (44.4%)**	**8 (50%)**	4 (30.7%)	**3 (42.8%)**	12 (44.4%)	13 (37.2%)	**7 (63.6%)**	58 (38.7%)
Grade 2	**15 (65.1%)**	7 (38.9%)	5 (31.3%)	**5 (38.5%)**	2 (28.6%)	**13 (48.2%)**	**17 (48.5%)**	3 (27.3%)	67 (44.7%)
Tube feeding*	2 (8.7%)	2 (11.1%)	2 (12.5%)	2 (15.4%)	0 (0%)	0 (0%)	3 (8.6%)	0 (0%)	11 (7.3%)
Not Applicable**	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Dysgeusia	Group 1 (N=23) N(%)	Group 2 (N=18) N(%)	Group 3 (N= 16) N(%)	Group 4 (N=13) N(%)	Group 5 (N=7) N(%)	Group 6 (N=27) N(%)	Group 7 (N=35) N(%)	Group 8 (N=11) N(%)	Total (N=150) N(%)
Week 4 (D20)									
Grade 0	2 (8.7%)	1 (5.6%)	2 (12.5%)	0 (0%)	2 (28.6%)	1 (3.7%)	2 (5.7%)	1 (9.1%)	11 (7.3%)
Grade 1	2 (8.7%)	6 (33.3%)	6 (37.5%)	1 (7.7%)	**3 (42.8%)**	6 (22.2%)	10 (28.6%)	4 (36.35%)	38 (25.3%)
Grade 2	**16 (69.5%)**	**9 (50%)**	6 (37.5%)	**9 (69.2%)**	2 (28.6%)	**20 (74.1%)**	**18 (51.4%)**	4 (36.35%)	84 (56%)
Tube feeding*	3 (13.1%)	2 (11.1%)	2 (12.5%)	3 (23.1%)	0 (0%)	0 (0%)	5 (14.3%)	2 (18.2%)	17 (11.4%)
Not Applicable**	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Week 5 (D25)									
Grade 0	1 (4.3%)	0 (0%)	2 (12.5%)	0 (0%)	0 (0%)	1 (3.7%)	0 (0%)	1 (9.1%)	5 (3.3%)
Grade 1	4 (17.4%)	4 (22.2%)	5 (31.3%)	0 (0%)	4 (57.1%)	3 (11.1%)	9 (25.7%)	2 (18.2%)	31 (20.6%)
Grade 2	**15 (65.2%)**	**10 (55.6%)**	**6 (37.5%)**	**8 (61.5%)**	2 (28.6%)	**21 (77.8%)**	**17 (48.6%)**	**6 (54.5%)**	85 (56.7%)
Tube feeding*	3 (13.1%)	4 (22.2%)	3 (18.7%)	5 (38.5%)	1 (14.3%)	2 (7.4%)	9 (25.7%)	2 (18.2%)	29 (19.4%)
Not Applicable**	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Week 6 (D30)									
Grade 0	1 (4.3%)	0 (0%)	2 (12.5%)	0 (0%)	0 (0%)	1 (3.7%)	0 (0%)	1 (9.1%)	5 (3.3%)
Grade 1	3 (13.1%)	3 (16.7%)	5 (31.3%)	0 (0%)	3 (42.8%)	2 (7.4%)	8 (22.8%)	1 (9.1%)	25 (16.7%)
Grade 2	**12 (52.2%)**	**9 (50%)**	**6 (37.5%)**	**7 (53.9%)**	3 (42.8%)	**22 (81.5%)**	**18 (51.5%)**	**7 (63.6%)**	84 (56%)
Tube feeding*	7 (30.4%)	6 (33.3%)	3 (18.7%)	6 (46.1%)	1 (14.3%)	2 (7.4%)	9 (25.7%)	2 (18.2%)	36 (24%)
Not Applicable**	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Dysgeusia	Group 1 (N=23) N(%)	Group 2 (N=18) N(%)	Group 3 (N= 16) N(%)	Group 4 (N=13) N(%)	Group 5 (N=7) N(%)	Group 6 (N=27) N(%)	Group 7 (N=35) N(%)	Group 8 (N=11) N(%)	Total (N=150) N(%)
Week 7 (D35)									
Grade 0	1 (4.3%)	0 (0%)	1 (6.2%)	0 (0%)	0 (0%)	1 (3.7%)	0 (0%)	0 (0%)	3 (2%)
Grade 1	2 (8.7%)	1 (5.6%)	2 (12.5%)	0 (0%)	2 (28.6%)	3 (11.1%)	10 (28.6%)	1 (9.1%)	21 (14%)
Grade 2	2 (8.7%)	**6 (33.3%)**	**3 (18.7%)**	**5 (38.5%)**	**4 (57.1%)**	**21 (77.8%)**	**17 (48.6%)**	**7 (63.6%)**	65 (43.3%)
Tube feeding*	5 (21.7%)	4 (22.2%)	1 (6.2%)	4 (30.7%)	1 (14.3%)	2 (7.4%)	8 (22.8%)	2 (18.2%)	27 (18%)
Not Applicable**	13 (56.6%)	7 (38.9%)	9 (56.4%)	4 (30.7%)	0 (0%)	0 (0%)	0 (0%)	1 (9.1%)	34 (22.7%)

Group 1: Patients submitted to partial or hemi glossectomy followed by RT; Group 2: Partial or hemi glossectomy followed by CRT; Group 3: Patients submitted to other HNC surgery protocols not including tongue tissue followed by RT; Group 4: Other HNC surgery protocols not including tongue tissue followed by CRT; Group 5: Induction CT followed by RT; Group 6: Induction CT followed by CRT; Group 7: Concomitant CRT and Group 8: Exclusive RT. *Tube feeding- Patients that started feeding exclusively through tube feeding, hence dysgeusia analysis was not possible; **Not applicable - Radiotherapy treatment based on 6 weeks protocols (60Gy).

2.3 Artigo: Salivary alpha-1-antitrypsin and macrophage migration inhibitory factor may be potential prognostic biomarkers for oncologic treatment-induced severe oral mucositis

Artigo publicado no Journal of Supportive Care in Cancer.

Palmier NR, Leme AFP, De Rossi T, Telles GP, Morais-Faria K, Kowalski LP, Marta GN, Brandão TB, Arany PR, Migliorati CA, Santos-Silva AR, Prado-Ribeiro AC. Salivary alpha-1-antitrypsin and macrophage migration inhibitory factor may be potential prognostic biomarkers for oncologic treatment-induced severe oral mucositis. Support Care Cancer. 2021; 29(6):2939-2946. doi: 10.1007/s00520-020-05805-2.

*Este trabalho foi um dos trabalhos nomeados ao Robert and Kay Schattner Award na categoria de apresentação oral no congresso da Academia Americana de Medicina Oral (AAOM) realizado em Nova Orleans, EUA em 2019.

Key Words: Head and neck neoplasms, radiotherapy, oral mucositis, salivary proteins, alpha 1-antitrypsin, macrophage migration inhibitory factors.

ABSTRACT

Aims: Evaluate the abundance of the selected targets, alpha-1-antitrypsin (A1AT) and macrophage migration inhibitory factor (MIF), and correlate these findings with the risk of developing severe oral mucositis (OM).

Materials and Methods: Head and Neck squamous cell carcinoma (HNSCC) patients submitted to RT or chemo-radiotherapy (CRT) were assessed. OM grade and pain were evaluated daily during the treatments. Two protein targets, A1AT and MIF, were evaluated, using selected reaction monitoring mass spectrometry (SRM-MS), in whole saliva, collected prior to oncologic treatment. The results obtained from the targeted proteomic analysis were correlated with OM clinical outcomes.

Results: A total of 27 patients were included, of whom 21 (77.8%) had locally advanced disease (clinical stage III or IV). Most patients (70.4%) received CRT. OM grade 2 (40.8%) and 3 (33.3%) were the most prevalent during RT with a mean highest reported OM-related pain of 3.22 through the visual analogue scale (VAS). The abundance of A1AT and MIF correlated significantly with severe (grade 3 or 4, $p < 0.02$) compared to moderate-low (grade 1 or 2, $p < 0.04$) OM grade.

Conclusions: There is a correlation between the abundance of salivary A1AT and MIF and oncologic treatment induced OM. The correlation of MIF expression with severe OM appears to be compatible with its physiological pro-inflammatory role. These results open up great possibilities for the use of changes in salivary MIF and A1AT levels as prognostic markers for effective therapeutic interventions, such as photobiomodulation therapy.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) treatment is multimodal, including surgery, radiotherapy (RT) and chemotherapy (CT) [1-3]. Despite the notable benefit of RT, this treatment modality can be associated with several acute and chronic side effects that affect non-target tissues present within the radiation field [4]. In this context, oral mucositis (OM) is a highly prevalent acute toxicity that affects up to 80% to 100% of patients undergoing RT for oral and oropharyngeal cancer. According to the literature, chemoradiotherapy (CRT) protocols can increase the frequency and clinical severity of OM [4,5]. Besides the association with CRT, other factors that have been correlated as risk factors for RT-induced OM are poor oral hygiene, young age, female gender, poor renal function, poor nutritional status, total cumulative radiation dose, smoking, and decreased salivary secretion [6].

OM has a complex pathophysiology characterized by persistent oral mucosal ulcers associated with severe pain and reduced oral functions, such as swallowing, speech, taste, and chewing. These consequent morbidities can, in severe cases, lead to the interruption of cancer treatment and, consequently, cause tumor progression and negatively impact on survival rates [1-3, 7]. These comorbidities may also increase the cost of healthcare due to increased hospitalization, expensive drugs such as opioids or growth factors (e.g. Keratinocyte Growth Factor (KGF)), and the use of probes for nasogastric feeding [8-11].

Improved molecular characterization of oncologic treatment induced OM will not only aid in an improved understanding of the disease process but also enable development of robust prognostic biomarkers to improve effective interventions. A study by Jehmlich et al., [12] outlined the salivary proteomic profile of HNSCC cancer patients and noted the expression of 48 proteins related to an increased risk for the development of OM. Considering the above mentioned information and the need for better understanding of the proteins associated with OM development, the aim of the present study was to characterize two proteins, alpha-1-

antitrypsin (A1AT) and macrophage migration inhibitory factor (MIF), and correlate them with the risk of developing severe OM post-oncologic treatment.

MATERIALS and METHODS

Study Design

This was a single center cohort study designed to evaluate the salivary proteomic profile of HNSCC patients submitted to RT or CRT protocols at the Instituto do Câncer do Estado de São Paulo (ICESP, Brazil) from January 2011 to February 2018. This study was approved by the Ethics Committee of the School of Medicine, University of São Paulo, Sao Paulo, Brazil (Protocol# 2.647.153). Written informed consent was obtained from all participants and the study was conducted in accordance with the Declaration of Helsinki.

Inclusion criteria

This study included oral and oropharynx cancer patients who underwent RT or CRT protocols (with or without previous surgery) using a 6MV linear accelerator and 3-dimensional conformal or intensity-modulated radiation therapy technique (IMRT) (Synergy Platform, Elekta AB, Stockholm, Sweden). The target radiation volumes encompassed the primary site and areas of regional lymph nodes at risk and received cumulative doses that ranged from 60 to 70 Gy. We applied the recommendations for treatment planning and constraints for organs at risk, as previously reported [13-15].

All included patients completed the institutional dental conditioning protocol prior to beginning RT. Besides routine oral care, all patients were submitted to the standard-of-care daily photobiomodulation (PBM) protocol for prevention of OM as per our institutional (Dental Oncology Service, ICESP, Brazil) protocol [11]. Finally, all patients needed to have complete demographic and clinicopathological data available on electronical medical charts, including gender, age, tumor location, clinical cancer stage (according to the American Joint Committee

on Cancer Staging System, 7th edition [16]), cancer treatment modalities, and information regarding weekly dental follow-up during RT and OM outcomes.

Exclusion criteria

Patients who missed one or more RT or PBM sessions were considered to have received incomplete treatment and were excluded from the study.

Oral mucositis assessment

A trained dental surgeon conducted OM grading using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE, Version 4.0, 2010 [17]) on the last day of each week of treatment (Day 5, 10, 15, 20, 25, 30 and 35) as a part of standard of care. The highest OM grade developed throughout RT was used for comparison with the salivary proteomic profiles. Patient self-reported OM pain was recorded using the visual analogue scale (VAS) with scores ranging from 0 to 10, where 0 represents no pain and 10 is the highest level of pain. Assessments were recorded at the end of each week of RT and mean VAS values throughout RT were noted.

Saliva collection and preparation

Saliva samples were obtained voluntarily and with signed consent from HNSCC patients. Whole saliva collection was performed immediately prior to the beginning of RT. Individuals first rinsed their mouths with 5 mL of drinking water and then harvested the saliva into a sterile recipient. Saliva samples were aliquoted in 2 mL tubes and immediately frozen at − 80 °C for long-term storage.

Whole saliva protein preparation:

The preparation of saliva samples was performed as previously described [18,19]. Briefly, saliva was first centrifuged for 5 min at 1,500 g at 4 °C to remove intact cells and debris.

A volume of 100 µL of whole saliva was then used in the protein extraction procedure by homogenizing with 100 µL of urea buffer (100 mM Tris-HCl pH 7.5, 8 M urea, and 2 M thiourea) containing the complete Mini Protease Inhibitor Cocktail (Roche, Auckland, New Zealand), 5 mM EDTA, 1 mM PMSF, and 1 mM DTT. Samples were sonicated for 10 min and centrifuged at 10,000 g for 5 min. Total protein was quantified using a Bradford assay kit (Bio-Rad, São Paulo, Brazil).

Mass spectrometry and data analysis

The proteotypic peptides and their respective transitions of the proteins alpha-1-antitrypsin (A1AT) and macrophage migration inhibitory factor (MIF) were selected based on the criteria described [20,21]: MIF_HUMAN (ASVPDGFLSELTQQLAQATGK, m/z 721.04, +3 and its respective transitions [y6] m/z 575.31+; [y4] m/z 376.21+; [y2] m/z 204.13+); A1AT_HUMAN (LQHLENELTHDIITK, m/z 451.74, +4; and its respective transitions [y2] m/z 248.16+; [y8] m/z 470.77++; [y7] m/z 414.23++ and EINDYVEK, m/z 505.24,+2 and its respective transitions [y6] m/z 767.35+; [y5] m/z653.31+; [y4] m/z 538.28+).

Three proteotypic peptides were purchased as crude heavy-isotope-labeled peptide standards (Thermo Fisher Scientific). The stable isotope-labeled peptides (SIL) were synthesized with heavy isotopes on lysine (+8 Da), localized at the C-terminal of the peptide (Thermo Fisher Scientific). Three transitions were monitored for the light and heavy counterparts of each peptide. Eight or nine peptides with their respective transitions of the internal retention time standard (Pierce™ Peptide Retention Time Calibration Mixture, Thermo Fisher Scientific) were monitored as a control for retention time shifts in liquid chromatography.

In order to avoid bias in the analyses, samples were randomized using the software R (v3.4.0) and separated in blocks with each sample analyzed in triplicate for SRM analysis. All samples were assessed within each block in different orders to further reduce systematic bias.

Samples were analyzed on a Xevo TQ-XS triple quadrupole mass spectrometer (Waters,

Milford, MA, USA) equipped with an electrospray ion source (Ion Key, Waters, Milford, MA, USA) with MassLynx software (version 4.2), as described by Carnielli et al. [19]. The data analysis was manually performed in Skyline.

Statistical analysis

Demographic data, clinicopathological features, and clinical outcome results were grouped into a spreadsheet for descriptive statistical analyzes based on mean, median, standard deviation, and proportion values. For the SRM analysis, the comparison of the levels of the monitored peptides between the patients who developed grades 1 or 2 OM (M1) and those who developed grade 3 or 4 OM (M2) was performed using a Wilcoxon Mann−Whitney test (not log transformed data) with the significance level set at $\alpha = 0.05$.

RESULTS

Patient characteristics

A total of 27 patients were included. The major clinicopathological features, including age, gender, tumor site, clinical staging, treatments, and RT modality, are summarized in **Table 1**. The mean age was 57 years (range 20–81 years) and 24 (88.9%) were males. The most frequent primary tumor site was the lateral border of the tongue (12 cases, 44.5%) followed by oropharynx (10 cases, 37%), and the floor of the mouth (2 cases, 7.4%). Most patients (21, 77.8%) were diagnosed with an advanced stage of disease (III/ IV), and none of the 27 (100%) patients were submitted to surgery at the time of saliva collection (presented with visible oral cancer lesions). All included patients received complete and uninterrupted RT and 19 (70.4%) received concomitant CRT protocols. Regarding the RT technique, 20 (74.1%) of patients were treated by 3-dimensional conformal RT; 7 (25.9%) with IMRT, with an overall mean dose of 67.7 Gy (ranging from 66 Gy to 68.3 Gy).

Oral mucositis assessment

The majority of the patients (24, 88.9%) developed OM during the treatment period, even when performing PBM protocol. During the first two weeks of treatment (days 5 to 10), OM grades varied from 0 to 2. Grade 3 lesions started to develop from the third week of treatment (day 15), while grade 4 lesions developed from the 5th week (day 25) of treatment (**Figure 1**). Three (11.1%) patients did not develop OM throughout the treatment. From the patients that developed OM, a total of 14 (51.9%) patients developed minimal OM [grade 1 (3/11.1%) or 2 (11/40.8%)] and 10 (37%) patients developed severe OM [grade 3 (9/33.3%) or 4 (1/3.7%)] as the highest grade throughout the treatment. The mean highest reported VAS throughout the treatment was 3.22 (range 0 to 9) and the highest level of OM-related pain was observed on the last week of radiotherapy with a mean VAS of 1.6. Data regarding OM grades and mean VAS per week of radiotherapy are summarized in **Table 2.**

Salivary protein assessment

Targeted proteomic analysis was performed for the samples of the 27 patients. Figure 2 demonstrates, individually, the Light/Heavy intensity ratio (not log transformed) of two proteins, MIF and A1AT, between M1 and M2 saliva samples. We demonstrated that alpha-1-antitrypsin and macrophage migration inhibitory factor showed a statistically significant correlation between patients who developed severe grade 3 or 4 OM ($p < 0.02$ and 0.04, respectively) as compared to patients who developed grade 1 or 2 OM. The increase in A1AT and MIF abundance was 2.97 and 21.4-fold, respectively, in grade 3 or 4 subjects compared to patients who developed low grades 1 or 2 or no OM.

DISCUSSION

This study evaluated the proteomic profile of whole saliva in HNSCC patients treated with RT or CRT, associated or not with previous surgery for tumor resection, and correlated

this with OM severity. We found two proteins A1AT and MIF that could be possible prognostic biomarkers for the development of severe oncologic treatment induced OM. The relevance of this finding is driven by the great interest in the development of personalized treatments based on the molecular profile of diseases that could lead to increased personalized treatment efficacy [22].

The patient-related findings in the present study are consistent with those reported in the literature, with the highest prevalence of HNSCC noted among middle-aged men diagnosed with advanced squamous cell carcinomas at the lateral border of the tongue and oropharynx, with most of these lesions considered as clinically unresectable [23,24]. In terms of the frequency and severity of OM, the present study also observed conventionally reported incidences, with over 80% of patients presenting some degree of lesions for patients submitted to RT or CRT protocols and treated with prophylactic PBM therapy. Over 40% of the patients in this study developed grade 2 OM as the highest grade throughout the treatment which is similar to reports in the literature of approximately 50% of grade 2 OM incidence by the end of RT with PBM treatments [25,26]. The use of PBM therapy prophylactically is now considered standard of care and has likely provided additional benefit in this study to mitigate OM severity [11,27].

The A1AT protein, also known as serpin 1, is a major liver-derived circulating protein that functions as a natural inhibitor of various serine proteases. It serves as a key component of the acute-phase response with roles in modulating the local and systemic inflammatory responses [28]. Jehmlich et al. [12] were the first to correlate the A1AT protein with the presence of OM, based on proteome analysis of whole saliva samples [12]. We also observed a significant modulation of this marker in the present study and this appeared to correlate with increased susceptibility to develop severe OM post-oncologic treatment. However, the precise role of A1AT in the OM pathogenesis remains unclear and further studies are warranted [12].

The MIF protein is a T-cell-derived factor and its primary role is as a pro-inflammatory protein [29,30]. MIF acts by activating pathways such as mitogen-activated protein (MAP) kinase and phosphoinositide-3-kinase and enhancing the expression of pro-inflammatory genes [31]. Additionally, MIF can both act on macrophages and be secreted by them in response to stimulation by cytokines such as tumor necrosis factor alpha (TNF-α) [29,32]. Interestingly, MIF is also responsible for the production and release of TNF-α, a pro-inflammatory cytokine that, in turn, further upregulates production of MIF in an autocrine manner and thereby promoting a pro-inflammatory response [29]. MIF also regulates the innate immune response and has been shown to be important in various autoimmune diseases. It has been noted to be involved in cell proliferation, cell survival, migration, and metastasis in several types of cancers, particularly oral squamous cell carcinomas [30,31,33].

The role of MIF as a pro-inflammatory marker appears to be compatible with the results of the present study, in that we found a higher abundance of MIF in samples from subject who developed severe Grades 3 or 4 OM. This is the first clinical demonstration, to our knowledge, of this correlation in human subjects that has been previously observed in animal studies [34,35]. Other studies have reported reduced cytokine levels of TNF-α and MIF following effective OM treatment, likely reflecting a reduction in the underlying inflammatory pathophysiology [34,35].

As previously mentioned, OM presents a complex pathophysiology which involves several signaling pathways [12,34]. One of the known signaling pathways implicated in OM is the Nuclear factor kappa beta (NFκB) signal transduction pathway [36]. This pathway, when activated, enhances the expression of the pro-inflammatory cytokine TNF-α which, in turn, drives tissue damage leading to the development of OM lesions. In addition, both TNF-α and NFκB signaling have been shown to modulate MIF expression, potentially corroborating the results in this study for an important role for MIF in OM pathogenesis [34,35].

Strengths and Limitations

The major strengths of the current study were the rigorous clinical inclusion and exclusion criteria that enabled a relatively homogeneous cohort of subjects. Nonetheless, limitations of the present work include a single institution study design, availability of only pre-RT/CRT (and not intermediate and post-treatment) saliva samples and variations in tumor burden during saliva sampling. These limitations justify the need for a larger and independent cohort and long-term prospective studies that are being pursued currently.

Conclusions

This study indicates the correlation of two protein levels with the development of severe OM and noted increased MIF and A1AT levels correlated with more severe grades of OM following oncologic treatment. Besides providing potential insights into their role in OM pathogenesis, these potential salivary biomarkers could serve as valuable prognostic aids to enhance and precisely calibrate effective OM interventions such as PBM treatments.

Acknowledgments

The authors would like to gratefully acknowledge the financial support of the São Paulo Research Foundation (FAPESP 2018/04657-8; 2018/02233-6; 2016/22862-2; 2013/18402-8, 2012/06138-1, 2015/12431-1, and 2016/07846-0) as well as the National Council for Scientific and Technological Development (CNPq), Brazil. We would like to thank Waters Corporation for providing us with access to a Xevo TQ-XS triple quadrupole mass spectrometer. The authors also state that this study was financed in part by the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) - Finance Code 001.

Author's contributions

Natália Rangel Palmier, Ana Carolina Prado-Ribeiro, Alan Roger Santos-Silva and Thaís Bianca Brandão performed the conception, design of the study and draft of the manuscript. Natália Rangel Palmier, Karina Morais-Faria, Tatiane de Rossi and Guilherme Pimentel Telles performed the analysis and interpretation of data. Adriana Franco Paes Leme, César Augusto Migliorati, Gustavo Nader Marta, Luiz Paulo Kowalski and Praveen R. Arany revised the manuscript for important intellectual content. Final approval of the version to be published was performed by Ana Carolina Prado-Ribeiro, Alan Roger Santos-Silva and Thaís Bianca Brandão.

Conflict of interest

None to declare. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors also would like to state that we have full control of all primary data and agree to allow the journal to review the data if requested.

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Table 1. Clinicopathological features of 27 patients included in the study.

Variables	N(%)
Age (mean)	57 years (range 20-81)
Gender	
Male	24 (88.9%)
Female	3 (11.1%)
Clinical Stage	
Stage I	0 (0%)
Stage II	6 (22.2%)
Stage III	3 (11.1%)
Stage IV	18 (66.7%)
Primary tumor site	
Tongue (lateral border)	12 (44.5%)
Oropharynx	10 (37%)
Floor of the mouth	2 (7.4%)
Retromolar area	1 (3.7%)
Buccal mucosa	1 (3.7%)
Tonsil	1 (3.7%)
Treatment	
Surgery + RT	6 (22.2%)
Surgery + CRT	3 (11.1%)
CRT	16 (59.3%)
Exclusive RT	2 (7.4%)
RT Modality	
3DRT	20 (74.1%)
IMRT	7 (25.9%)
Mean radiation dose	67.7Gy (range 66Gy-68.3Gy)

Abbreviations used: RT - Radiotherapy; CRT - Chemoradiotherapy; 3DRT - 3-dimensional conformal radiotherapy; IMRT - Intensity modulated radiotherapy.

Table 2: Oral mucositis grades and mean VAS scores per radiotherapy weeks

Oral Mucositis Grades*	D5 N(%)	D10 N(%)	D15 N(%)	D20 N(%)	D25 N(%)	D30 N(%)	D35 N(%)**	Highest Grade
0	23 (85.2%)	11 (40.8%)	6 (22.2%)	5 (18.5%)	7 (25.9%)	6 (22.2%)	7 (30.4%)	3 (11.1%)
1	4 (14.8%)	9 (33.3%)	3 (11.1%)	5 (18.5%)	1 (3.7%)	3 (11.1%)	1 (4.3%)	3 (11.1%)
2	0 (0%)	7 (25.9%)	17 (63%)	14 (51.9%)	10 (37.1%)	9 (33.3%)	9 (39.1%)	11 (40.8%)
3	0 (0%)	0 (0%)	1 (3.7%)	3 (11.1%)	9 (33.3%)	8 (29.7%)	6 (26.2%)	9 (33.3%)
4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (3.7%)	0 (0%)	1 (3.7%)
Mean VAS	0	0.3	1.12	1	0.88	1.48	1.6	3.22

.

Abbreviations used: D - Day of radiotherapy considering the end of each week of treatment; VAS - Visual analogue scale. *Oral mucositis grading was performed as per the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE, Version 4.0, 2010); **Total number of patients submitted to 7 weeks (33 to 35) of radiotherapy sessions - N=23

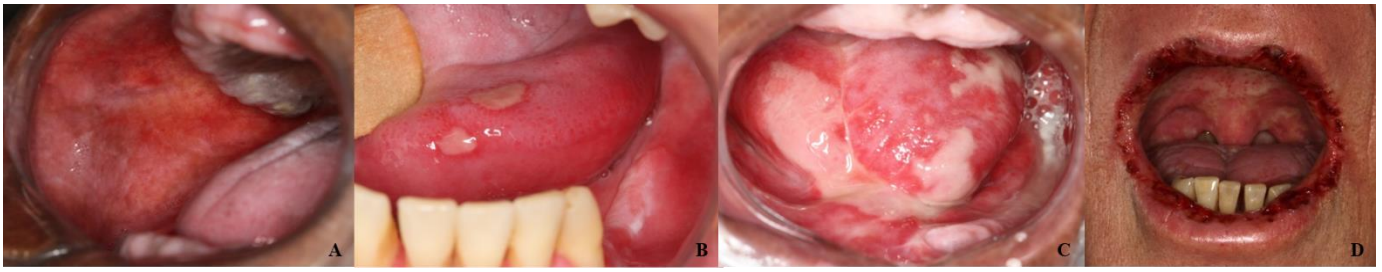

Figure 1: Representative clinical images of subjects in the present study who developed oral mucositis lesions. **A.** Subject presenting with Grade 1 OM demonstrating erythema on the right buccal mucosa; **B.** Subject presenting with Grade 2 OM demonstrating small, punctuate ulcers covered with a pseudomembrane; **C.** Subject presenting with Grade 3 OM demonstrating confluent ulcers with pseudomembrane affecting the lateral border, dorsum, and ventral surface of the tongue and **D.** Subject presenting with Grade 4 OM demonstrating deep ulcers and bleeding, note the crust formations on the upper and lower lip.

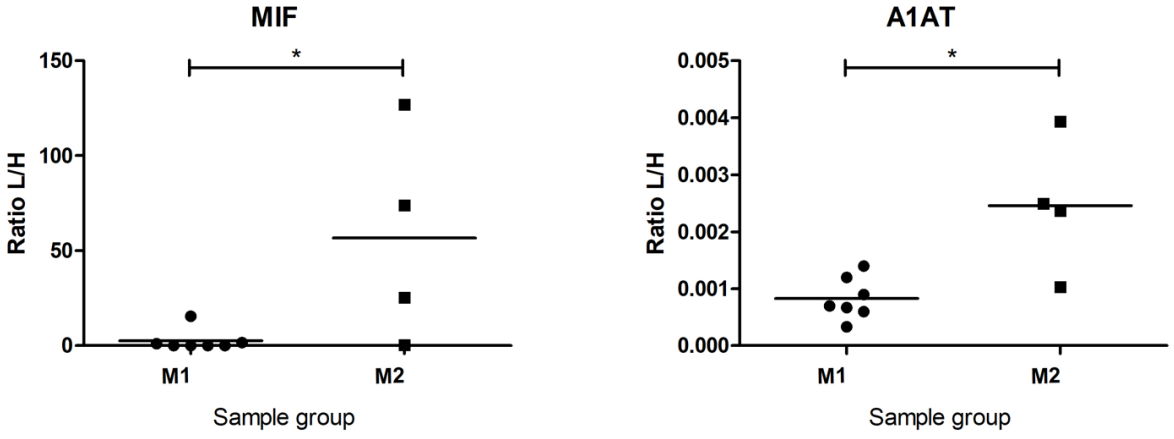

Figure 2: The graph demonstrates individually the Light/Heavy intensity ratio (not log transformed) of two proteins, MIF and A1AT, between M1 and M2 saliva samples. *p-value < 0.05, Wilcoxon Mann−Whitney test.

2.4 Artigo: Salivary proteins as possible biomarkers of acute oncotherapy-induced oral toxicities

*Este trabalho está concorrendo ao Prêmio IADR Unilever Award (Hatton) da 38a. Reunião Anual Virtual da SBPqO a ser realizada em setembro de 2021.

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Abstract word count: 250

Word count: 3,700

References: 52

Figures: 3

Tables: 3

Key Words: Head and neck cancer, head and neck radiotherapy, dysgeusia, dysphagia, xerostomia, oral candidiasis, salivary proteins, proteomics

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ABSTRACT

Aims: There's been a great search for molecular-based treatments. Thus, the aim of this study was to characterize the salivary proteomic profile of patients treated for oral squamous cell carcinoma (OSCC) and its correlation with the risk of developing severe radiation-related oral toxicities. **Material and Methods:** 35 OSCC patients submitted to radiotherapy (RT) or chemoradiotherapy (CRT) were included. Xerostomia, dysphagia, dysphagia-related pain (DRP), dysgeusia and oral candidiasis (OC) were daily evaluated. Liquid biopsy was performed by subjecting whole saliva of 35 advanced OSCC patients to targeted proteomic analysis through selected reaction monitoring-mass spectrometry (SRM-MS) including 56 targeted proteins, and their abundance was correlated with clinical outcomes (Mann-Whitney and ANOVA with post-hoc Tukey HSD tests). **Results:** 80% of patients presented stage III/IV OSCC. 63% were submitted to CRT protocols, mean RT dose of 66.7Gy. 68.6% presented grade 2 dysgeusia, 60% of patients presented with severe (grades 2-3) dysphagia, 35.3% presented severe DRP. 25.7% presented OC over 4 weeks during RT and 42.9% presented with severe xerostomia. SRM-MS results indicated that 27 proteins, mainly related to biological processes such as innate immune responses, inflammatory response, cell migration, peptidase inhibitor activity, and iron coordination and were correlated with more severe grades of dysgeusia, dysphagia, xerostomia, odynophagia, and OC in 68%, 60%, 43%, 34%, and 26% of patients, respectively. **Conclusions:** The present study is pioneer in characterizing possible biomarkers that may allow the identification of patients that are more likely to develop severe RT oral toxicities. Further studies are necessary to validate and better understand the role of these proteins in the pathophysiology of radiation-related oral toxicities.

INTRODUCTION

Most of head and neck cancer (HNC) patients (90%-100%) will be affected by at least one acute oral complication of head and neck radiotherapy (HNRT) or concomitant chemoradiotherapy (CRT) protocols for the treatment of malignant oral and oropharyngeal tumours [Sciubba and Goldenberg, 2006; Rogulj et al., 2017]. Acute oral complications of oncotherapy usually start within the first weeks of HNRT and encompass oral mucositis, dysgeusia, dysphagia and oral infections [Huber e Terezhalmy, 2003; Kielbassa et al., 2006; Sciubba and Goldenberg, 2006; Faria et al., 2014; Sroussi et al., 2017]. Additionally, patients usually develop xerostomia that, although it is categorized as a chronic toxicity, it usually develops in the first weeks of HNRT and becomes chronic due to the irreversible damage to salivary glands [Huber e Terezhalmy, 2003; Kielbassa et al., 2006; Rogulj et al., 2017].

The severity of acute oncotherapy oral toxicities can range from a slight discomfort to severe intense pain reports, difficulty in nutrition due to dysphagia-related pain (DRP) and taste loss resulting in severe oral morbidity leading to an important impact on treatment schedules that in some cases can be interrupted or discontinued, increase in need for hospitalization, probes for nasogastric tube feeding and expensive medications, consequently representing a negative impact on patient's prognosis [Elting et al., 2003; Sciubba and Goldenberg, 2006; Sroussi et al., 2017; Brandão et al., 2018].

Advances in diagnosis and treatment based on the molecular profile of diseases have been increasing in the past few years, especially in the cancer field [Cohen et al., 2016]. Nevertheless, studies regarding the salivary proteomic profile of oncotherapy-related oral toxicities are still scarce and mainly focused on the proteomic profile of oral mucositis [Jehmlich et al., 2015].

A better characterization of the salivary molecular profile of oncotherapy-related acute oral toxicities may improve the understanding of the disease process and consequently may enable the development of more accurate management techniques. Considering this, the aim of the

present study was to characterize the salivary proteomic profile of patients with oral cavity and oropharyngeal tumours and correlate it with the risk of developing dysgeusia, dysphagia and DRP, oral candidiasis (OC) and xerostomia. Finally, the ultimate goal was to assess possible biomarkers to aid in customized strategies for early diagnosis and treatments for acute oncotherapy-related oral toxicities management.

MATERIALS and METHODS

Study Design

This was a single center cohort study designed to evaluate the salivary proteomic profile of HNC patients submitted to HNRT or CRT protocols at the Sao Paulo State Cancer Institute (ICESP, Brazil) from January 2011 to February 2018. This study was approved by the Ethics Committee of the School of Medicine, University of Sao Paulo, Sao Paulo, Brazil (Protocol# 2.647.153). Written informed consent was obtained from all participants and the study was conducted in accordance with the Declaration of Helsinki.

Inclusion criteria

This study included oral and oropharynx cancer patients subjected to HNRT or CRT protocols (with or without previous surgery) using a 6MV linear accelerator (Synergy Platform, Elekta AB, Stockholm, Sweden). All included subjects were submitted to HNRT protocols with radiation volumes that encompassed the primary site and areas of lymph nodes at risk and received cumulative doses that ranged from 60 to 70 Gy. All included subjects completed the institutional dental conditioning protocol prior to the beginning HNRT. Besides routine oral care, all subjects were submitted to the standard-of-care daily photobiomodulation (PBM) protocol for prevention of OM as per our institutional (Dental Oncology Service, ICESP, Brazil) protocol [Brandão et al., 2018]. Finally, all subjects must have had complete demographic and

clinicopathological data available on electronical medical charts including gender, age, tumor location, clinical cancer stage (According to the American Joint Committee on Cancer Staging System, 7th edition (Edge and Comptom., 2010), cancer treatment modalities, information regarding weekly dental follow-up during HNRT and oncotherapy-related acute oral toxicities outcomes.

Exclusion criteria

Subjects who missed one or more HNRT or PBM sessions were considered to have received incomplete treatment and were excluded from the study. Subjects that started HNRT with exclusively diet with nasogastric feeding tube or gastrostomy making it impossible to perform an accurate assessment of dysgeusia were also excluded. Subjects that presented with tumor sites other than oral cavity and oropharynx, who were not eligible for prophylactic PBM treatments as per institutional protocol, were also excluded.

Oncotherapy-related acute toxicities assessment

A trained dental surgeon conducted oncotherapy-related acute oral toxicities grading on the last day of each week of treatment (Day 5, 10, 15, 20, 25, 30 and 35) as part of standard of care. Dysgeusia (Grades 0 to 2) and dysphagia (Grades 0 to 4) grading was performed according to National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE, Version 4.0, 2010). Patient self-reported DRP was recorded using the visual analogue scale (VAS) with a scores ranging from 0 to 10 where 0 is painless and 10 is the highest level of pain. DRP was grouped as follows: Low VAS 0-2; Moderate VAS 3-7; Severe VAS 8-10. OC assessment was performed according to the presence or absence of OC clinical subtypes throughout HNRT weeks [Millsop and Fazel, 2016] and divided by the number of weeks patients presented OC as follows: 0 weeks; 1-3 weeks and over 4 weeks. Xerostomia was

assessed according to the adapted criteria described by Eisbruch et al., [2013] (Grades 0 to 3). Assessments were recorded at the end of each week of HNRT and mean VAS values throughout HNRT were noted. The highest oncotherapy-related acute oral toxicities grades developed throughout HNRT were used for comparison with the salivary proteomic profiles.

Saliva collection and preparation

Saliva samples were obtained voluntarily from HNC patients, including patients who had undergone surgical resection and patients who had active oral malignant lesions at the time of saliva collection. Whole saliva collection was performed immediately prior to the beginning of HNRT. Individuals first rinsed their mouths with 5 mL of drinking water and then harvested the saliva into a sterile recipient. Saliva samples were aliquoted in 2 mL tubes and immediately frozen at − 80 °C for long-term storage.

Whole saliva protein extraction

Proteomic analysis of saliva samples was performed as previously described by our group [Wick et al., 2015; Carnielli et al., 2018]. Briefly, saliva was first centrifuged for 5 min at 1,500 g at 4 °C to remove intact cells and debris. A volume of 100 μL of whole saliva was then used in the protein extraction procedure by homogenizing with 100 μL of urea buffer (100 mM Tris-HCl pH 7.5, 8 M urea, and 2 M thiourea) containing the complete Mini Protease Inhibitor Cocktail (Roche, Auckland, New Zealand), 5 mM EDTA, 1 mM PMSF, and 1 mM DTT. Samples were sonicated for 10 min and centrifuged at 10,000 g for 5 min. Total protein was quantified using a Bradford assay kit (Bio-Rad, São Paulo, Brazil).

Mass spectrometry analysis

The proteotypic peptides and their respective transitions of 56 proteins were selected

based on the criteria described [Lange et al., 2008; Gallien et al., 2011]. Three proteotypic peptides were purchased as crude heavy-isotope-labeled peptide standards (Thermo Fisher Scientific). The stable isotope-labeled peptides (SIL) were synthesized with heavy isotopes on lysine (+8 Da), localized at the C-terminal of the peptide (Thermo Fisher Scientific). Three transitions were monitored for the light and heavy counterparts of each peptide. Eight or nine peptides with their respective transitions of the internal retention time standard (Pierce™ Peptide Retention Time Calibration Mixture, Thermo Fisher Scientific) were monitored as a control for retention time shifts in liquid chromatography [Palmier et al., 2020].

To avoid bias in the analyses, samples were randomized using the software R (v3.4.0) and separated in blocks with each sample analyzed in triplicate for SRM analysis. All samples were assessed within each block in different orders to further reduce systematic bias.

Samples were analyzed on a Xevo TQ-XS triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ion source (Ion Key, Waters, Milford, MA, USA) with MassLynx software (version 4.2), as previously described [Carnielli et al.,2018; Plamier et al., 2020]}. The data analysis was manually performed in Skyline.

Statistical analysis

Demographic data, clinicopathological features and clinical outcomes results were grouped into a spreadsheet for descriptive statistical analyzes based on mean, median, standard deviation and proportion values. For the proteomic analysis, the proteomic archive was exported to the Skyline software [McLean et al., 2010] and a script was developed using the R (v 3.4.0) tool. Data from the group of subjects that developed low grade and severe grade of dysgeusia, and xerostomia were compared using the Mann-Whitney test with the significance level set at $\alpha = 0.05$ and fold change analysis. Data from group oncotherapy-related acute oral toxicities grouped in more than 2 assessment groups (dysphagia, DRP and OC total of weeks) were compared using the ANOVA with post-hoc Tukey HSD test with the significance level set at

α=0.05, for these cases fold change analysis was not possible due to categorization of oral toxicities in 3 subcategories.

RESULTS

Patient characteristics

A total of 35 subjects with oral cavity and oropharynx cancer met the inclusion criteria and were included in the present study. The clinicopathological features including age, gender, tumor site, clinical staging, treatments, HNRT are summarized (**Table 1**). The mean age was 54.8 years (range 20-78 years) and 85.7% were males. The most frequent primary tumor site was the lateral border of tongue (51.5%) followed by the soft palate (25.7%) and floor of the mouth (8.6%) and most of the subjects (80%) were diagnosed with advanced stage of disease III or IV. All included subjects received complete and uninterrupted HNRT and 62.8% received concomitant CRT protocols. Subjects were treated by 3-Dimensional conformal HNRT (74.3%) and intensity modulated HNRT (IMRT, 25.7%) with an overall mean dose of 66.7 Gy (67.3Gy and 64.9Gy, respectively).

Oncotherapy-related acute toxicities assessment

Over 90% of the subjects (94.2%) developed some degree of taste alteration during the treatment period with most of the subjects (68.5%) presenting with severe grade 2 dysgeusia. 97.2% of the subjects presented with some degree of dysphagia from which 42.8% presented with swallowing difficulty with need for alterations to soft diet and 17.3% presented need for nasogastric tube feeding placement. DRP assessment revealed that 80% of the sample presented 3-10 VAS from which 34.3% of the subjects reported severe 8-10 VAS values during HNRT.

OC assessment revealed that over half the sample developed OC infection throughout

HNRT weeks from which the trained dentist assessing the oncotherapy observed OC infection for over 4 weeks of treatment in 25.7% of subjects. Finally, 91.4% of subjects reported xerostomia during HNRT with 42.9% presenting with severe grades 2 or 3 xerostomia (37.1% and 5.8%, respectively). Detailed information of Oncotherapy-related acute toxicities assessment is reported in **Table 2.**

Saliva proteins assessment

A total of 56 proteins were analyzed and monitored with target proteomic analysis, from which 27 were statistically correlated ($p<0.05$) with severity of oncotherapy-related acute oral toxicities (**Figure 1)**. Target proteomic analysis results revealed 1 protein Leucine-rich alpha-2-glycoprotein (A2GL) concomitantly correlated with severe dysgeusia and OC infection for over 4 weeks, 1 protein High mobility group protein B2 (HMBG2) exclusively correlated with severe grade 2-3 dysphagia, 4 proteins exclusively correlated with severe 8-10 VAS for DRP, as follows: Laminin subunit gamma-2 (LAMC2), Complement C3 (CO3), Nidogen-1 (NID1) and Fascin (FSCN1).

As for OC infection, 10 proteins exclusively correlated with over 4 weeks of OC which were: Lipolysis-stimulated lipoprotein receptor (LSR), Alpha-actinin-4 (ACTN4), Apolipoprotein A-I (APOA1), Antileukoproteinase (SLPI), Tumor-associated calcium signal transducer 2 (TACD2), Kallikrein-1 (KLK1), Kininogen-1 (KNG1), 8- Peptidyl-prolyl cis-trans isomerase A (PPIA), Serotransferrin (TRFE), Tubulointerstitial nephritis antigen-like (TINAL).

Three proteins exclusively correlated with severe grade 2-3 xerostomia, Desmoglein-3 (DSG3), Neutrophil gelatinase-associated lipocalin (NGAL) and Plectin (PLEC). Finally, 8 proteins were concomitant to OC infection for over 4 weeks and severe grade 2-3 xerostomia, Zinc-alpha-2-glycoprotein (ZA2G), Complement factor B (CFAB), Talin-1 (TLN1), BPI fold-

containing family B member 2 (BPIB2), Immunoglobulin heavy constant alpha 1 (IGHA1), Cysteine-rich secretory protein 3 (CRIS3), Serine protease inhibitor Kazal-type 5 (ISK5), Triosephosphate isomerase (TPIS). **Table 3** shows detailed information on statistical p values and fold changes for all proteins observed in the present study.

DISCUSSION

The lately increase for the better understanding of the molecular profile involved in human disease influenced our team to develop the present study aiming to assess the salivary proteomic profile of acute oncotherapy-related toxicities [Cohen et al., 2016]. A total of 27 proteins were statistically correlated with severity of oral toxicities and could possibly be regarded as potential salivary biomarkers of the studied acute oral toxicities.

Results related to the clinicopathological profile of subjects included in the present study are compatible with literature reports with HNC affecting mostly middle-aged men diagnosed with advanced squamous cell carcinomas (SCC) at the lateral border of the tongue and oropharynx [Kowalski et al., 2005; Scully et al., 2006]. Acute oncotherapy-related oral toxicities have been reported to occur in the form of symptoms clusters which has the impact to overlap and act synergistically exacerbating overall symptom perception [Gouvêa Vasconcellos et al., 2020]. Results of the present study are compatible in this context with the majority of the subjects reporting some degree of each oral toxicity assessed throughout HNRT weeks.

Dysgeusia or taste disturbance is one of the most common oral toxicities virtually affecting all HNC patients submitted to HNRT or CRT protocols. It can be related to direct radiogenic effect inducing the loss on taste buds, irradiated tongue volume, impairment in oral neural structure, presence of OM, shift in oral microflora and salivary flow rates [Sciubba and Goldenberg, 2006; Cohen et al., 2016; Ridner et al., 2018; Gouvêa Vasconcellos et al., 2020]. In the present study the presence of severe grade 2 dysgeusia was correlated with a higher

abundance of the A2GL protein. Besides its role in inflammation, cellular adhesion, infection, and as a possible biomarker for oral SCC [Tung et al., 2013], the A2GL protein have been recognized as a modulator of Transforming growth factor-beta (TGF- β) [Honda et al., 2017]. Studies report that TGF- β3 is expressed in tongue taste buds and with the *in vitro* increase of TGF- β3 levels inhibition of cellular proliferation in taste buds occur [Nakamura et al., 2009]. Although the correlation of A2GL and TGF-β have been reported, so far, no studies have reported the specific role of this protein on oncotherapy-related dysgeusia.

Dysphagia is characterized by the difficult in swallowing which in many cases leads to alterations in the type of diet with a shift for softer or liquid food and in the more severe cases may require the use of nasogastric tube feeding [Van der Laan et al., 2015; Cohen et al., 2016]. Pain, presence of mucous secretion and edema of soft tissue are known causes related to the development of HNRT induced dysphagia [Nevens et al., 2017; Ridner et al., 2018; Santa Cruz et al., 2018]. A total of 5 proteins were correlated to more severe levels of dysphagia and DRP (1 and 4 proteins, respectively). The HMGB2 protein correlated to severe dysphagia is known to be overexpressed in HNSCC cell lines and to affect cisplatin (the most used chemotherapy medication in HNC) cell sensitivity [Syed et al., 2015]. A study by Syed et al., [2015] reported that by silencing HMGB2 there is an increase in sensitivity to cisplatin and 5-Fluoracil enhancing their efficacy. From the reported result, it is possible to suggest that since 62.8% of the subjects of the present study were submitted to adjuvant chemotherapy protocols, the increase in HMGB2 observed in the present study could be correlated to a lower treatment response and consequently higher rates of oncotherapy-related toxicity such as dysphagia.

Interestingly, all four proteins correlated to severe swallowing pain (CO3, FSCN1, LAMC2 and NID1) are known proteins correlated to the presence of advanced staged HNSCC, presence of metastasis and poor prognosis with lower rates of disease free and overall survival rates requiring more aggressive treatment [Lee et al., 2015; Routray et al., 2017; Lee et al.,

2018; Chen et al., 2019; Hsu et al., 2019; You et al., 2019; Zhong et al., 2019]. In addition, CO3 and LAMC2 proteins are correlated to cellular resistance to cisplatin and worse radiotherapeutic outcomes, respectively, contributing to oncotherapy resistance [Chen et al., 2019; You et al., 2019]. Although no studies reported a specific correlation of the observed proteins with dysphagia-related pain, their well-known role in tumour progression and treatment response could explain the reports for severe pain commonly reported by HNC patients [Murphy et al., 2007], nevertheless further studies are required in order to verify this correlation.

Oral and oropharyngeal candidiasis is one of the most common opportunistic infection in immunosuppressed patients, such HNC patients undergoing oncological treatment protocols and is mainly caused by *Candida albicans* infection [Chattopadyay et al., 2004; Sroussi et al., 2017]. OC may cause oral burning sensation and pain, taste alteration usually described as metallic taste and when the infection extends through oropharynx and esophagus can lead to swallowing problems [Sroussi et al., 2017]. In the present study 10 proteins were exclusively correlated with over 4 weeks of OC infection during HNRT, 1 as concomitantly correlated to dysgeusia and 8 concomitantly to xerostomia. From the 10 proteins exclusively correlated to OC, 5 proteins (KLK1, KNG1, PPIA, SLPI, TRFE) presented literature reports reporting correlation with candidiasis infection.

Studies report that *C. albicans* secretes proteinases to increase KNG1 generation to facilitate dissemination via vasodilation, additionally, it also activates the Kallikrein-Kinin contact system starting the cascade of activating the complement system by the cleavage of CO3 and CFAB (a protein that correlated to both OC and xerostomia) justifying our findings that KLK1, KNG1 CO3 and CFAB were more abundant in our subjects samples [Chattopadyay et al., 2004; Held et al., 2008; Karkowska-Kuleta et al., 2016; Ramani et al., 2016; Karkowska-Kuleta et al., 2017; Irmscher et al., 2018].

As previously mentioned, KNG-1 is a vasodilator in result from inflammation and tissue

damage, in this context, Bencharit et al., [2012] observed a correlation in overexpression of KNG and PPIA in patients that developed *C. albicans* denture stomatitis (DS) compared with patients that did not develop this condition. Although the main etiological factor for the development of DS and oncotherapy-related OC differs, the similarity of the salivary proteomic results compared with the present study support the KNG-1 and PPIA proteins as possible biomarkers of candidiasis infection and should be further investigated.

The SLPI, TPIS and TRFE proteins have been described to present antifungal activity [Watanabe et al., 1997; Chattopadyay et al., 2004; Sweryn et al., 2015]. Chattopadyay et al., [2004] observed that immunosuppressed patients with history of OC presented higher levels of salivary SLPI, on the other hand, studies observed that transferrin inhibited *in vitro* growth of *C. albicans* and TPIS antigen could induce protective Imunoglobulin G2a antibody against *C. albicans* representing a potential for future development of vaccines [Fernandez- Arenas et al., 2004a; Fernandez- Arenas et al., 2004b; Lin et al., 2014]. Our results showed a higher abundance these proteins correlated to over 4 weeks of OC infection and although more studies are required, the results support the theory that such proteins could be potential indicators of OC status in immunocompromised subjects [Chattopadyay et al., 2004].

Direct radiation damage to salivary glands can lead to qualitative and quantitative (dry mouth) alterations in HNC patients and can be correlated to total dose of HNRT received by salivary glands, tumour location and treatment modalities that include chemotherapy or other medications know to decrease salivary flow [Santos-Silva et al., 2015; Cohen et al., 2016]. Xerostomia presents a rapid onset and is one of the most persistent toxicities of oncotherapy for HNC [Sciubba and Goldenberg, 2006; Gouvêa Vasconcellos et al., 2020]. Changes in salivary proteomic composition could represent an important impact not only on the development of xerostomia but in all oncotherapy-related oral toxicities considering the important role saliva presents in maintaining a health oral environment [Dawes et al., 2015]. In the present study 3

proteins were exclusively correlated to severe xerostomia (DSG3, NGAL and PLEC), and 8 concomitantly correlated with OC from which 3 (BIPB2, CFAB and CRIS3) presented studies correlated to dry mouth related to Sjogren Syndrom and autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy (APECED) [Fischibah et al., 1980; Tapinos et al., 2002; Laine et al., 2007; Burbelo et al., 2019]. Although correlated to presence of dry mouth in the mentioned autoimmune conditions, up to date no studies have so far studied the proteomic profile, or the role of the proteins observed in the present study and their correlation with oncotherapy-induced xerostomia.

STRENGTHS AND LIMITATIONS

The major strengths of the current study were rigorous clinical inclusion and exclusion criteria that enabled a relatively homogeneous cohort of subjects. Nonetheless, limitations of the present work include a single institution study design, availability of only pre-HNRT (and not intermediate and post-treatment) saliva samples and availability of retrospective clinicopathological information on electronic medical charts. These limitations justify the need for better designed, long-term prospective studies that are being pursued currently.

CONCLUSIONS

Salivary biomarkers are currently representing an important role in a better understanding of diseases pathophysiology and consequently early diagnosis, prognosis and development of therapeutic targets and prediction to drug response. The present study is pioneer in characterizing possible biomarkers that may allow the identification of patients that are more likely to develop severe HNRT acute oral toxicities. A total of 27 proteins correlated to oncotherapy-related acute oral toxicities. The correlation of A2GL with dysgeusia, HMGB2 with dysphagia and of the proteins CO3, FSCN1, LAMC2 and NID1

with DRP seems promising. The correlation of the KLK1 and KNG-1 via the Kallikrein-Kinin contact system with the presence of OC seems to be the most well-stablished correlation based on literature reports. Further studies are necessary to clinically validate the role of the observed proteins in the pathophysiology of oncotherapy-related acute oral toxicities.

Funding Sources

The authors would like to gratefully acknowledge the financial support of the São Paulo Research Foundation (FAPESP 2018/04657-8; 2018/02233-6; 2016/22862-2; 2013/18402-8 and 2012/06138-1) as well as the National Council for Scientific and Technological Development (CNPq), Brazil. The authors also state that this study was financed in part by the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) - Finance Code 001.

Conflict of interest

None to declare.

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Table 1. Clinicopathological features of 35 patients included in the study

Variables	Total (N%)
Age (mean)	54.8 years (range 20-78)
Gender	
Male	30 (85.7%)
Female	5 (14.3%)
Clinical Stage	
Stage I	0 (0%)
Stage II	7 (20%)
Stage III	7 (20%)
Stage IV	21 (60%)
Primary tumor site	
Tongue (lateral border)	18 (51.5%)
Soft palate	9 (25.7%)
Floor of the mouth	3 (8.6%)
Retromolar area	2 (5.8%)
Buccal mucosa	1 (2.8%)
Tonsil	1 (2.8%)
Base of tongue with oral extension	1 (2.8%)
Treatment	
Surgery + HNRT*	11 (31.4%)
Surgery + CRT**	8 (22.8%)
CRT	14 (40%)
Exclusive HNRT	2 (5.8%)
HNRT Modality	
3DRT***	26 (74.3%)
IMRT****	9 (25.7%)
Mean radiation dose	66.7Gy

Abbreviations used: * HNRT - Head and Neck Radiotherapy; CRT - Chemoradiotherapy; 3DRT - 3-dimensional conformal radiotherapy; Intensity modulated radiotherapy - IMRT.

Table 2. Oncotherapy-induced acute oral toxicities highest grades throughout HNRT*

Oral toxicities	Total (N%)
Dysgeusia	
Grade 0	2 (5.8%)
Grade 1	9 (25.7%)
Grade 2	24 (68.5%)
Dysphagia	
Grade 0	1 (2.8%)
Grade 1	13 (37.1%)
Grade 2	15 (42.8%)
Grade 3	6 (17.3%)
Grade 4	0 (0.0%)
Dysphagia-related Pain	
Low (VAS** 0-2)	7 (20%)
Moderate (VAS 3-7)	16 (45.7%)
Severe (VAS 8-10)	12 (34.3%)
Oran Candidiasis (number of weeks)	
0	16 (45.7%)
1 to 3	10 (28.6%)
Over 4	9 (25.7%)
Xerostomia	
Grade 0	3 (8.6%)
Grade 1	17 (48.5%)
Grade 2	13 (37.1%)
Grade 3	2 (5.8%)

Abbreviations used: *HNRT: Head and Neck Radiotherapy; **VAS: Visual Analogue Scale

Table 3: List of the 56 proteins identified by target proteomic analysis

Protein accession/gene name	Fold change Dysgeusia	p.value.MW* Dysgeusia	p.value.anova Dysphagia	p.value.anova DRP**	p.value.anova OC***	Fold change Xerostomia	p.value.MW Xerostomia
sp|P13796|PLSL_HUMAN	0.0872	0.2256	0.2538	0.4525	0.2817	0.0576	0.2721
sp|P02750|A2GL_HUMAN	**0.4230**	**0.0264**	0.2813	0.9922	**0.0225**	0.6849	0.5287
sp|Q86X29|LSR_HUMAN	0.1037	0.9479	0.6355	0.4107	**0.0289**	0.3785	0.1941
sp|P05121|PAI1_HUMAN	0.6442	0.9474	0.5277	0.0814	0.8337	1.4100	0.4751
sp|P09228|CYTT_HUMAN	0.1330	0.5714	0.6353	0.8247	0.1543	0.0931	0.1810
sp|Q13751|LAMB3_HUMAN	0.1839	0.2140	0.7586	0.1944	0.0960	0.6795	0.9529
sp|P25311|ZA2G_HUMAN	0.4134	0.4894	0.2031	0.9667	**0.0181**	**0.1040**	**0.0120**
sp|P09871|C1S_HUMAN	0.3759	0.4707	0.4481	0.9786	0.1687	0.3243	0.3143
sp|P32926|DSG3_HUMAN	0.7134	0.8513	0.6736	0.3598	0.0626	**0.1386**	**0.0256**
sp|P01023|A2MG_HUMAN	0.3428	0.1700	0.2598	0.9689	0.0753	0.1936	0.2884
sp|P52790|HXK3_HUMAN	0.1696	0.1377	0.3805	0.6245	0.2169	0.1730	0.2238
sp|Q9NUQ9|FA49B_HUMAN	0.1712	0.1040	0.3895	0.6130	0.3053	0.2727	0.5287
sp|P09211|GSTP1_HUMAN	0.2721	0.3429	0.4346	0.8574	0.0841	0.1729	0.1447
sp|P01037|CYTN_HUMAN	0.2542	0.4894	0.5731	0.8947	0.0960	0.1056	0.0879
sp|P00751|CFAB_HUMAN	0.8941	0.5714	0.4561	0.9104	**0.0034**	**0.2548**	**0.0360**
sp|O43707|ACTN4_HUMAN	0.1024	0.7531	0.3597	0.8287	**0.0010**	0.3783	0.0663
sp|P02790|HEMO_HUMAN	0.3547	0.4117	0.1858	0.7263	0.1526	0.1339	0.0879
sp|Q02413|DSG1_HUMAN	0.6360	0.5714	0.5075	0.2833	0.4155	0.7369	0.5287
sp|P12830|CADH1_HUMAN	0.6705	0.0777	0.3641	0.7164	0.1053	0.9959	0.8639
sp|P02647|APOA1_HUMAN	0.7488	0.4894	0.1085	0.7429	**0.0290**	0.3426	0.3277
sp|Q9Y490|TLN1_HUMAN	0.9255	0.5714	0.1345	0.8193	**0.0336**	**0.6327**	**0.0048**
sp|P02766|TTHY_HUMAN	0.4823	0.4894	0.3747	0.9708	0.0833	0.3243	0.2721
sp|P55072|TERA_HUMAN	0.5468	0.3940	0.6451	0.8571	0.2671	0.5417	0.6358
sp|P08670|VIME_HUMAN	0.6299	0.2799	0.4543	0.8568	0.0607	0.6332	0.2721
sp|P37802|TAGL2_HUMAN	0.1370	0.1021	0.5447	0.6604	0.2972	0.3120	0.8594
sp|Q8N4F0|BPIB2_HUMAN	0.6796	0.8513	0.4577	0.7156	**0.0025**	**0.1710**	**0.0496**
sp|P01876|IGHA1_HUMAN	0.5454	0.8513	0.3627	0.9760	**0.0006**	**0.0481**	**0.0360**
sp|P04083|ANXA1_HUMAN	0.4620	0.4117	0.8252	0.8876	0.7028	0.2106	0.6070
sp|P26583|HMGB2_HUMAN	0.0115	0.1024	**0.0283**	0.3094	0.6751	0.9079	0.7234

Abbreviations used: *MW: Mann-Whitney test; **DRP: Dysphagia-related pain; ***OC: Oral Candidiasis

Table 3: List of the 56 proteins identified by target proteomic analysis (continued)

Protein accession/gene name	Fold change Dysgeusia	p.value.MW Dysgeusia	p.value.anova Dysphagia	p.value.anova DRP*	p.value.anova OC**	Fold change Xerostomia	p.value.MW Xerostomia
sp|P01009|A1AT_HUMAN	0.8847	0.4893	0.5623	0.3590	0.1317	0.5213	0.3884
sp|P03973|SLPI_HUMAN	0.9796	0.4321	0.6146	0.3034	**0.0065**	0.1667	0.7230
sp|Q13753|LAMC2_HUMAN	0.6972	1.000	0.1495	**0.0325**	0.7509	0.1274	0.7662
sp|P09758|TACD2_HUMAN	0.5624	0.4723	0.3508	0.8090	**0.0397**	0.4104	0.0872
sp|P01024|CO3_HUMAN	0.5785	0.2370	0.7747	**0.0452**	0.2843	0.1398	0.7669
sp|P12830|CADH1_HUMAN	0.6690	0.6608	0.2445	0.3653	**0.0398**	0.1048	0.1810
sp|P01042|KNG1_HUMAN	0.6306	0.3270	0.2422	0.9526	**0.0099**	0.5615	0.0768
sp|P80188|NGAL_HUMAN	0.5107	0.1772	0.5867	0.5080	0.1757	0.4298	**0.0076**
sp|P14174|MIF_HUMAN	0.3020	0.8512	0.9266	0.6662	0.2855	0.1947	0.8639
sp|Q9HC84|MUC5B_HUMAN	0.2001	0.8512	0.3065	0.8344	0.8659	0.6997	0.6889
sp|P14543|NID1_HUMAN	0.5743	0.2391	0.8402	**0.0218**	0.4741	0.7414	0.4086
sp|P02763|A1AG1_HUMAN	0.3852	0.4117	0.1649	0.6280	0.1997	0.2029	0.0663
sp|Q15149|PLEC_HUMAN	0.8181	0.1864	0.1684	0.4425	0.1141	**0.5800**	**0.0564**
sp|P62937|PPIA_HUMAN	0.4913	0.1772	0.3129	0.8353	**0.0045**	0.2240	0.0663
sp|P24158|PRTN3_HUMAN	0.5712	0.1772	0.8903	0.4155	0.0610	0.2471	0.2721
sp|P04217|A1BG_HUMAN	0.1659	0.4893	0.3243	0.6777	0.3123	0.0488	0.1447
sp|P02765|FETUA_HUMAN	0.2438	0.2132	0.3828	0.6161	0.3922	0.2816	0.0668
sp|P54108|CRIS3_HUMAN	0.4780	0.4893	0.0771	0.5673	0.0184	0.1649	0.0360
sp|P02679|FIBG_HUMAN	0.3087	0.6608	0.1567	0.5341	0.2126	0.1383	0.1810
sp|P01591|IGJ_HUMAN	0.3741	0.2798	0.4067	0.8836	0.2291	0.3391	0.2238
sp|Q9NQ38|ISK5_HUMAN	0.1252	0.8512	0.7225	0.2787	**0.0342**	**0.1913**	**0.0256**
sp|P02787|TRFE_HUMAN	0.5443	0.4893	0.1205	0.6737	**0.0311**	0.1057	0.2238
sp|Q9GZM7|TINAL_HUMAN	0.5866	0.2919	0.0988	0.7434	**0.0023**	0.4342	0.4395
sp|Q16658|FSCN1_HUMAN	0.1117	0.6471	0.2294	**0.0451**	0.4581	0.8377	0.6367
sp|P78536|ADA17_HUMAN	0.3937	0.6474	0.7338	0.7138	0.2900	0.4619	0.4791
sp|P63104|1433Z_HUMAN	0.8657	0.7436	0.0957	1.3109	0.8382	0.1254	0.7232
sp|P60174|TPIS_HUMAN	0.4387	0.3428	0.5137	0.8173	**0.0111**	**0.1437**	**0.0360**

Abbreviations used: *MW: Mann-Whitney test; **DRP: Dysphagia-related pain; ***OC: Oral Candidiasis

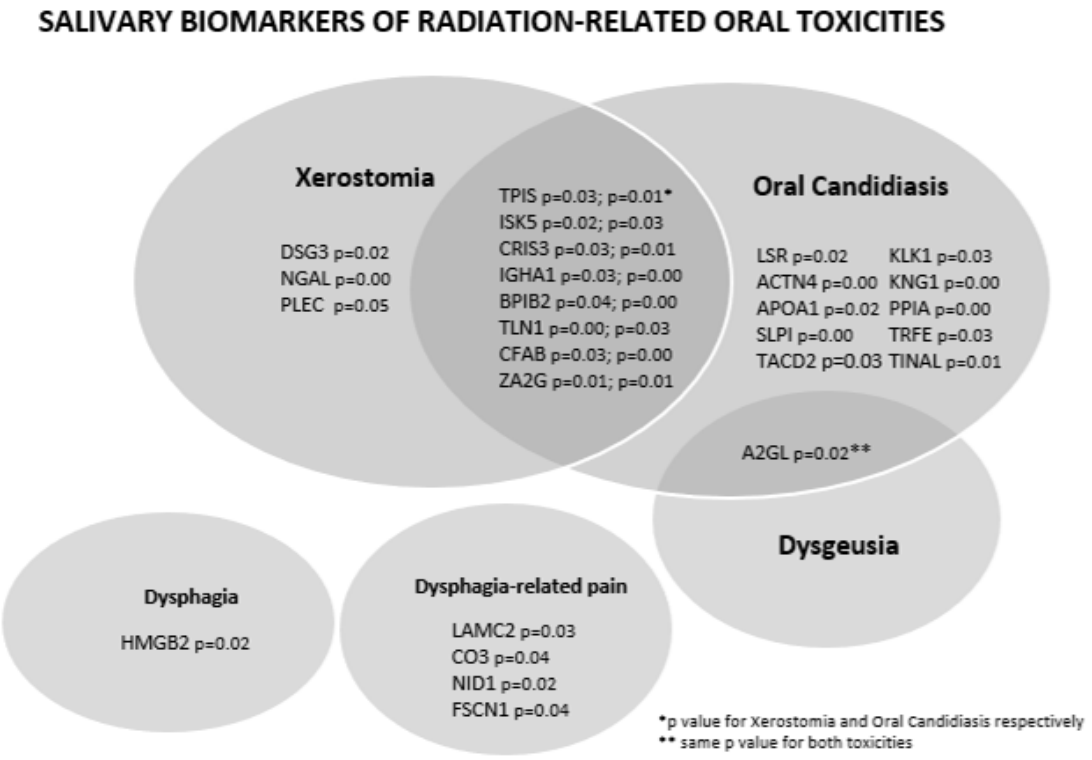

Figure 1: Venn diagram representing the statistically significant proteins observed in the present study and their correlation with the specific oncotherapy-induced acute oral toxicities.

2.5 Artigo: Radiation-related caries: current diagnostic, prognostic, and management paradigms

Artigo publicado no Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology.

Palmier NR, Migliorati CA, Prado-Ribeiro AC, de Oliveira MCQ, Vechiato Filho AJ, de Goes MF, Brandão TB, Lopes MA, Santos-Silva AR. Radiation-related caries: current diagnostic, prognostic, and management paradigms. Oral Surg Oral Med Oral Pathol Oral Radiol. 2020;130(1):52-62. doi: 10.1016/j.oooo.2020.04.003.

Key Words: Radiation caries, head and neck radiotherapy, oral cancer.

Abstract

Radiation-related caries (RC) is an aggressive disease affecting approximately 30% of post-head and neck radiotherapy (HNRT) patients. RC mainly affects tooth cervical areas and incisal/cuspal tips and develops 6–12 months post-HNRT. Early RC signs include black/brownish tooth color alteration and enamel cracks, which progress to enamel delamination, exposing underlying dentin to a highly cariogenic oral environment and rapid tooth destruction/dental crown amputation. As RC advances and deems the tooth non-restorable, it may lead to osteoradionecrosis spontaneously or upon extraction if the tooth is in a highly irradiated field of the oral cavity. This would require aggressive treatment, having a negative impact on a cancer survivor's quality of life, and contributing to the incremental cost of cancer care. Chlorhexidine mouth rinses and topical fluoride applications are effective agents used in RC prevention; however, there are no well-established protocols for treatment. Once RC progresses, dental restorations should be performed based on adhesive materials associated with systematic fluoride application, as illustrated in the clinical case presented in this narrative review. Post-HNRT patients should be closely followed up for optimal RC prevention, early diagnosis, and prompt treatment. Future clinical studies are necessary to establish a contemporary clinically validated protocol for RC management.

Overview

Treatment protocols for head and neck cancer (HNC) are known to be multimodal, usually including surgery, head and neck radiotherapy (HNRT), chemotherapy (CT) or chemoradiotherapy (CRT) that are often combined.[1] Although the effectiveness of HNRT is key for disease control and survival rate improvement, it can be associated with several acute and chronic toxicities to non-target tissues, including radiation caries (RC).[2]

Frist described by Del Regato,[3] RC is an aggressive type of tooth decay that can lead to generalized tooth destruction, loss of masticatory efficiency, persistent chronic oral infections and osteoradionecrosis (ORN), which can have a great impact on patients' quality of life.[4] In this context, a study reported that the presence of RC was associated with increased scores of the Decayed, Missing and Filled Teeth (DMFT) index (reflecting a poorer oral health status) and lower scores of the Quality of Life questionnaire in patients subjected to RT, especially regarding complaints of xerostomia, which has been reported as one of the most significant aetiologic factors for RC development.[5]

Although well recognized as an important chronic toxicity of HNRT, RC aetiology is still very controversial, which consequently leads to difficulty in the diagnosis and management of this entity and therefore poorer overall oral function for post-HNRT patients.[6] In addition, recent data suggest that RC management costs are high, varying from $192 to $4,500 and therefore representing an important impact on global cancer treatment costs.[7] In addition to the management cost of RC, the increased risk for the development of ORN also represents an important impact on both patients' quality of life, due to the need for the use of antibiotics, debridement and in some cases surgical resections and hospitalization, and also impact cancer treatment costs as reported by Elting and Chang[7] which showed that ORN management can range from $4800 for debridement of necrotic bone to up to $78 000 when reconstructions are required.[7]

In this context, it is important to emphasize that according to the National Institute of Dental and Craniofacial Research[8], dental caries is the most prevalent chronic disease in both children and adults affecting up to 95.62% of patients between 50-64 years of age, and in addition to this perspective, HNC patients usually have a poor oral health status even prior to cancer diagnosis and HNRT. This correlates to poor oral hygiene, smoking and drinking habits, increasing the risk for the development of periodontal disease, generalized caries and multiple tooth loss **(Figure 1)**,[9,10] a complex dental scenario that may play a role in the increased risk for RC onset.

Aetiologic factors

The aetiologic factors that affect the development of RC lesions are still controversial and are usually divided into direct and indirect effects of HNRT on teeth.[11] Studies report that cumulative radiation doses may be as high as 99% of the overall dose prescribed to the primary tumour site and that even more sophisticated radiation techniques can deliver doses which are still high (50 Gray) and considered at risk for dental impact.[12,13]

In vitro studies report that irradiated teeth present biomechanical alterations, such as a reduction in resistance to tensile and compressive stress forces, and decreased microhardness, which can lead to destabilization of the dentin–enamel junction (DEJ) and an increase of matrix metalloproteinase-20 (MMP-20) at the DEJ, which in turn may lead to degradation of the protein components of the DEJ, consequently leading to enamel cracks and delamination.[14,15] *In vitro* studies also report disorganized patterns of intertubular and peritubular dentin that could be correlated with alterations in dentin microhardness favouring the propagation of enamel cracks and interfering with the adhesion of resin-based restorative materials.[16-18] The literature also reports that HNRT may lead to the destruction of connective tissue and morphological changes in odontoblast processes, which could impact the pulp response to cariogenic damage.[19]

In addition, studies report that HNRT can cause inflammation and ischaemia in a dose-dependent manner, temporally decreasing the pulp response to sensory tests.[20,21]

Contrary to that observed in the previously mentioned studies, our team using *ex vivo*-irradiated teeth observed that alterations of enamel prisms and the interprismatic zone were mostly interpreted as sub-superficial demineralization related to radiation-induced hyposalivation. In addition, our team found no differences in dentin and DEJ morphological patterns or microvascularization, innervation and extracellular matrix of the dental pulp of *in vivo*-irradiated teeth when compared to homologue control teeth.[2,22-25]

There is a well-established theory of the impact of the indirect effects of HNRT/CRT in the form of a 'cluster of oral symptoms' in the onset and progression of RC.[26] HNRT/CRT protocols can be associated with two sets of clusters, the head and neck (HN)-specific cluster composed of dysphagia, xerostomia/hyposalivation, pain, dysgeusia, fatigue, oral mucositis and radiodermatitis, and the gastrointestinal (GI) cluster composed of nausea, vomiting and dehydration.[26,27]

Hyposalivation and xerostomia caused by damage to the salivary glands are the indirect effects that can be mostly associated with RC.[11] With a reduction of salivary flow and alteration of the salivary components, oral pH is lowered due to the loss of saliva buffer capacity, which favours biofilm accumulation and a shift to a more cariogenic oral microbiota mainly composed of *Lactobacillus* sp. and *Streptococcus mutans*.[28] Additionally, these patients experience difficulties in performing oral hygiene due to trismus, pain and oral mucositis and usually start on a highly cariogenic diet with softer carbohydrate-rich food in order to combat the weight loss due to dysphagia, pain and oral/oropharyngeal mucositis and shift to sweet foods which is usually the last flavour to disappear during the treatment (as reported by patients).[4,10]

Although there is a lack of studies focused on better understanding of the aetiologic factors for RC, Palmier et al.[6] found a high percentage of larynx cancer patients affected by RC,

supporting the theory that the clustering of oral symptoms may be more relevant to RC onset and progression than the direct effects of irradiation on teeth.[11]

Clinical presentation and diagnosis

RC is a very aggressive and multifocal type of tooth decay that appears around 6 to 12 months post-HNRT and previous estimates reported a range of RC incidence of 24% to up 57% in a Northern Ireland population.[2,6,29] A recent systematic review observed an overall incidence of 29% that can be increased up to 37% after two years post-HNRT conclusion, which can represent a great contribution to overall statistics of dental caries incidence.[29] RC lesions present patterns of clinical presentation and progression that differ from those of conventional caries in non-irradiated patients, such as in their different topography and clinical features **(Figure 2)**.[6,28] In addition to this perspective, Palmier et al.[6] observed that RC lesions are usually misdiagnosed having a potential impact on incidence reports of RC. Considering this, future prospective multicentre studies are necessary to better understand incidence rates of this important post-HNRT chronic toxicity.

RC lesions usually start as alterations in the translucency and colour of enamel which tends to develop brown/blackish pigmentation on tooth smooth surfaces.[11,19] The literature reports that these areas of pigmentation represent microscopic areas of sub-superficial demineralization, representing areas of incipient caries on non-cavitated enamel.[2]

RC in the initial stage also presents with enamel cracks and fissures. Palmier et al.[30] observed a higher prevalence of enamel craze lines (ECL) in the cervical area of irradiated non-carious and RC teeth when compared to homologue control teeth. The increased incidence of ECL in irradiated patients may be explained by the theory that under dry conditions, such as hyposalivation, enamel biomechanical properties become weaker and enamel becomes more brittle; dentin and DEJ properties are also altered, which, in addition to a low oral pH, can

increase tooth demineralization and cracks **(Figure 3)**.[28,31]

The main tooth areas affected by RC lesions are cervical areas (near the amelocemental junction) surrounding the teeth, which can be explained by incipient alterations to microstructure in the cervical enamel of teeth irradiated *in vivo*,[25] and wear on incisal edges and cusp tips. RC especially affects the lingual surfaces of anterior mandibular teeth, which are not usually common sites for conventional caries **(Figure 4)**.[6,19]

As RC progresses, enamel delamination tends to occur, exposing the underlying dentin to a highly cariogenic oral environment and favouring fast and aggressive progression of tooth destruction **(Figure 4)**.[11] As destruction of the tooth structure surrounding the cervical area and through the incisal/cuspal edges progresses (axial/transversal progression), there is a decrease in support of the dental crown that exfoliates, leaving the root remains exposed to the oral environment **(Figures 5 and 6)**.[6,25,28]

The atypical clinical patterns of RC also apply to its radiographic presentation. The RC demineralization process tends to occur fast without revealing meaningful clinical changes. Morais-Faria et al.[12] observed by microtomography that clinical incipient RC lesions may be represented by extensive and deep demineralization with pulp tissue involvement, representing a much more aggressive lesion than observed by clinical examination only **(Figure 7)**. Radiographic representation of RC onset and progression is represented in **Figure 8**.

Currently, there are no clinically validated systems for the diagnosis of RC.[6] Walker et al.[32] developed the post-radiation dental index (PRDI) and, although specific for RC, this index did not take into consideration important clinical aspects of RC such as blackish enamel discoloration, carious lesions on incisal edges or cusp tips, enamel cracks, delamination and crown amputation. Considering this, we propose herein a clinical guide for the assessment and management of RC lesions **(Table 1)**.

Management

Pre-RT dental management

In order to prevent RC, oral conditioning regimens for patients who will be treated with HNRT should ideally begin immediately after the diagnosis of cancer. Patients must be submitted to complete dental examination and treatment. During this phase, the dental professional can remove existing decay and perform new restorations, adjustment of pre-existing restorations, endodontic and periodontal treatment, and extractions when teeth present extensive structural or extensive periodontal bone loss deeming the tooth unrestorable. Additionally, patients must be orientated on adequate oral hygiene with atraumatic tooth brushing using soft-bristled toothbrushes at least 2 to 4 times a day, interdental cleaning with dental floss, and fluoride supplementation with the use of fluoride trays or topical fluoride application.[33,34]

Besides dental follow-up, another very important step in preventing and managing RC is sparing the salivary glands during HNRT planning.[33] As previously mentioned, hyposalivation plays an essential role in RC development, and studies have reported that sparing of salivary glands, or their preservation by stimulation with cholinergic muscarinic agonists, can decrease the impact of HNRT on saliva production and, therefore, enhance the protection of teeth against carious processes.[33] In this scenario, the Quantitative Analyses of Normal Tissue Effects in the Clinic (QUANTEC) group recommends limiting the mean dose to at least one parotid gland to <25 Gy and the mean dose to both parotid glands to <20 Gy with a threshold for xerostomia development of 26Gy.[35,36] In addition, although the imitation of saliva features and composition is complex, prescription of commercial saliva substitutes can help improve oral lubrication, lowering patients' discomfort in performing oral hygiene. *In vitro* studies have reported that polymers used as thickening agents of saliva substitutes may inhibit dental demineralization, which may play an important role in RC prevention.[37]

Post-RT dental management

During and after radiation therapy, patients must be closely followed, ideally every 3 months. In patients with higher caries risk and non-ideal oral hygiene, more frequent appointments may be necessary. The goal is to maintain good oral hygiene status and to perform prevention, early diagnosis and management of RC.[33,34,38]

The role of chlorhexidine and fluoride in preventing RC is well recognized, and studies report that daily applications of 0.12% chlorhexidine mouth rinses and 1% neutral sodium fluoride gel with custom-made trays can help reduce RC.[39] Studies show that chlorhexidine reduces plaque index and salivary levels of *S. mutans* during RT. The antibacterial rinse can be absorbed by tooth cracks, an important clinical feature of RC, presenting a prolonged effect of tooth protection.[34,40] Daily fluoride application, in the form of mouth rinses or gel in a tray used for a few minutes daily, can increase the re-hardening effect of softened enamel surfaces by enhancing remineralization. *In vitro* reports suggest that fluoride preserves the hardness and mineralization of enamel and cementum during HNRT.[40] Although the importance of these substances in preventing RC is recognized, studies report that patient compliance with fluoride and chlorhexidine use decreases over time, reinforcing the need for close dental follow-up.[34]

RC prevention strategies

Remineralizing toothpastes have been reported as having a positive effect on RC prevention. Studies report that by adding casein phosphopeptide–amorphous calcium phosphate to remineralizing toothpaste increases the bioavailability of calcium and phosphate ions that can supplement saliva and help balance the de/remineralization process, especially in cervical areas. This process, therefore, prevents the formation of caries in roots, the most affected and challenging tooth surfaces to treat in patients who develop RC.[41]

In addition to mouth rinses and remineralizing toothpastes, fluoride varnishes also have a protective effect on enamel at risk for RC development. The use of fluoride varnishes for

surface protection as a coating material can improve the depth of enamel protected against the demineralization process caused by acid exposure.[42] In this scenario, resin-modified glass ionomer cement (RMGIC) with calcium varnish protects against demineralization. It also releases calcium, which may enhance enamel re-hardening and durability when compared to conventional varnishes. This is due to the chemical bonding to the tooth representing an interesting material for the management of areas of demineralization typical of incipient RC lesions.[42]

Another fluoride modality is through the silver diamine fluoride (SDF) application. SDF is a clear liquid used for professional topical fluoride application that combines the antibacterial effects of silver and the remineralizing effects of fluoride.[43-45] *In vitro* studies report its effectiveness in reducing specific cariogenic bacteria and its remineralizing potential on enamel and dentin and *ex vivo* studies report that SDF may increase the microhardness and mineral density of caries lesions, reduces loss of calcium and phosphate ions and lessen collagen damage. [43-45]

The fluoride component of SDF strengthens the tooth structure against acid bacterial products and may also interfere with biofilm composition. Unfortunately, the precipitation of silver products in the dental tissues stain the lesions in black which is an important drawback in aesthetic visible areas. [43-45]

Although SDF is known to be a safe and effective, caries control agent, especially in caries arrest in primary teeth, and arrest and prevention of new root caries lesions, it is a relatively new material, and a consensus on its use has not yet been reached. [43-45] Up to date there aren't studies reporting the effectiveness of SDF in the prevention and management of RC, and, although one of SDF applications is its use in root/cervical caries, future prospective clinical trials are required in this matter.

Stablished RC treatment and restorative materials

The post-HNRT oral environment represents a challenge for the restoration of RC lesions. These patients have high rates of decay development and premature deterioration of tooth structures, leading to the failure of dental restorations.[46-48] In general, the management of RC is extremely challenging to dentists. Root caries is difficult to restore due to limited access, trismus or surgical defects. Excavation of caries may be incomplete, preparation of restorative cavities may present poor mechanical retention due to difficult margin preparation and, finally, contouring and polishing are also impaired due to accessibility.[40,48]

When RC is established and tooth structure loss is clinically observed, the use of permanent restorative materials is key to the success of the restoration. Unfortunately, the choice of the best restorative material for the treatment of RC is still very controversial and usually based on the dentist's clinical experience rather than scientifically established protocols.[6]

Adhesive systems such as resin composites, glass ionomer cement (GIC) and RMGIC have biocompatibility, optical and micromechanical properties similar to those of the dental structure. In addition, their fluoride-releasing property makes these materials the choice for the treatment of RC lesions.[16,47] Studies report that hyposalivation can impair the survival of GIC and RMGIC restorations. [16,47] Nevertheless, Galetti et al.[49] found no differences in the properties of adhesive systems in irradiated teeth, supporting the use of insoluble dental materials such as resin composites for RC management.

Studies report high rates of restoration failure in the 2 years post-HNRT, with rates of 50% for RC, 75% for RMGIC and up to 96% for GIC restorations, which are significantly higher when compared to non-irradiated patients that usually present a mean survival time of 15 years.[16,48,50]

Studies evaluating post-HNRT dental restoration failure suggest that the main reason for failure is displacement of the material. Secondary caries was not found.[46-48] Additionally, studies found that fluoride use has a negative impact on the survival of GIC and RMGIC restorations, probably due to the structural alterations suffered by these materials under topical applications of fluoride gel.[16] These results support the idea that resin composites may be the best-indicated material for restorations in the treatment of RC due to their adhesive and insoluble properties. Nevertheless, studies that evaluated survival rates of post-HNRT restorations date back 10 years or longer.[16,47,48] Dental material properties have improved over time; considering this, more studies are necessary to establish a contemporary clinically validated protocol for the treatment of RC lesions. With this in mind, we show an example of a clinical case of a patient diagnosed with RC lesions 3 months post-HNRT conclusion, and treatment of the lesions performed with a protocol based on the use of resin composites **(Figure 9)**.

Complications and prognosis

As RC lesions progress, tooth loss is observed, leading to eating and speaking difficulties and aesthetic complaints among post-HNRT patients. The rehabilitation of these patients is usually challenging due to trismus and surgical defects that may preclude an adequate prosthetic rehabilitation, presenting an important impact on the quality of life of these patients.[51]

One of the most concerning complications in post-HNRT patients is the development of ORN that can occur spontaneously, in response to RC progression, or due to RC-related tooth extractions **(Figure 10)**.[38] ORN is reported to affect between 2.6% and 44% of patients submitted to HNRT protocols and as ORN progresses, more aggressive treatment approaches are needed, such as jaw block resection, in order to control the dissemination of bone necrosis. This complication presents an important impact on patients' quality of life. Therefore, strategies

to prevent this important toxicity should include dental management of RC lesions.[52] The ideal scenario would include the stabilization or elimination of dental disease prior to the start of HNRT, minimizing the future necessity for tooth extractions which increase the risk for ORN.[38]

Palmier et al.[6] observed that RC lesions are usually underdiagnosed or unrecognized by clinicians and patients and therefore mistreated. Considering this, patients should be closely followed. This would allow for early diagnosis and management of RC lesions. In cases where dental extractions due to RC are needed, the procedure should be performed avoiding trauma to the adjacent tissues in order to minimize the risk for ORN development.[2,53] Unfortunately, few cancer treatment centres provide adequate preparation of patients to receive radiation therapy and frequent dental follow-up visits. This could be the result of a lack of dental oncologists as members of the oncology teams and poor patient compliance with maintaining oral health.[10]

Funding

The authors gratefully acknowledge the financial support of the São Paulo Research Foundation (FAPESP) (process numbers 2018/04657-8; 2018/02233-6; 2016/22862-2; 2013/18402-8; and 2012/06138-1) as well as the National Council for Scientific and Technological Development (CNPq), Brazil. The authors also state that this study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasil – Finance Code 001.

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Table 1: Clinical guide for RC* lesion diagnosis and management

Clinical stage	Correspondent clinical features	Management and treatment
At risk	Patients submitted to HNRT** protocols	Close dental follow-up (3 months), oral hygiene orientation and dental prophylaxis, 0.12% chlorhexidine mouth rinse and 1% neutral sodium fluoride application, remineralizing toothpastes, prescription of saliva substitutes.
Initial stage	Incipient caries presenting black/brownish colour alterations and increased number of enamel cracks and fissures	These patients should have closer follow-up visits (less than 3-month interval), oral hygiene orientation and dental prophylaxis, 0.12% chlorhexidine mouth rinse and 1% neutral sodium fluoride application, remineralizing toothpastes, prescription of saliva substitutes, fluoride vanishes or RMGI*** with calcium as coating material.
Established stage	Small areas of established decay with enamel structure loss and dentin involvement; areas of delamination	In addition to the measures mentioned above, tooth restoration with insoluble adhesive materials. Resin composites seem to be the best alternative.
Advanced stage	Extensive areas of dentin involvement/delamination; tooth crown amputation	In addition to the measures mentioned above, in cases of extensive tooth damage/tooth structure loss, decoronation or root submergence should be performed, atraumatic dental extraction should be carefully evaluated, and patients should be closely followed up, clinically and radiographically, to prevent and diagnose early the possible development of osteoradionecrosis.

*RC: radiation caries; **HNRT: head and neck radiotherapy; ***RMGI: Resin-modified glass ionomer.

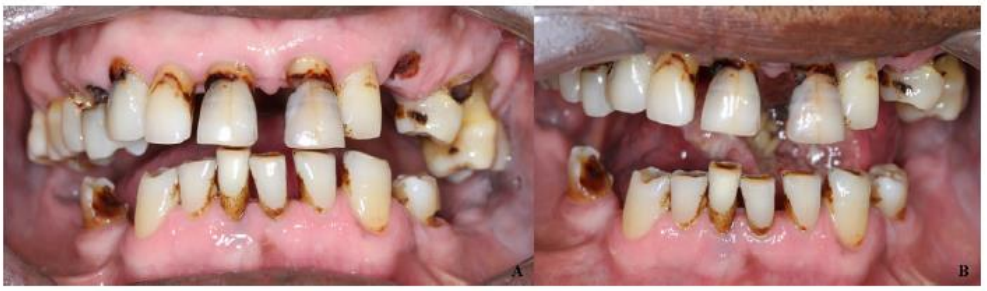

Fig. 1. Patient before the beginning of oncological treatment (head and neck radiotherapy) presenting carious lesions that are clinically similar to radiation-related caries (RC). (**A**) Areas of black/brownish discoloration, enamel cracks, cervical and incisal caries, and dental crown amputation of tooth #11. (**B**) Mandibular teeth presenting incisal and cervical caries, enamel cracks, black/brownish discoloration patterns. Note the active tongue tumor lesion behind the mandibular incisors.

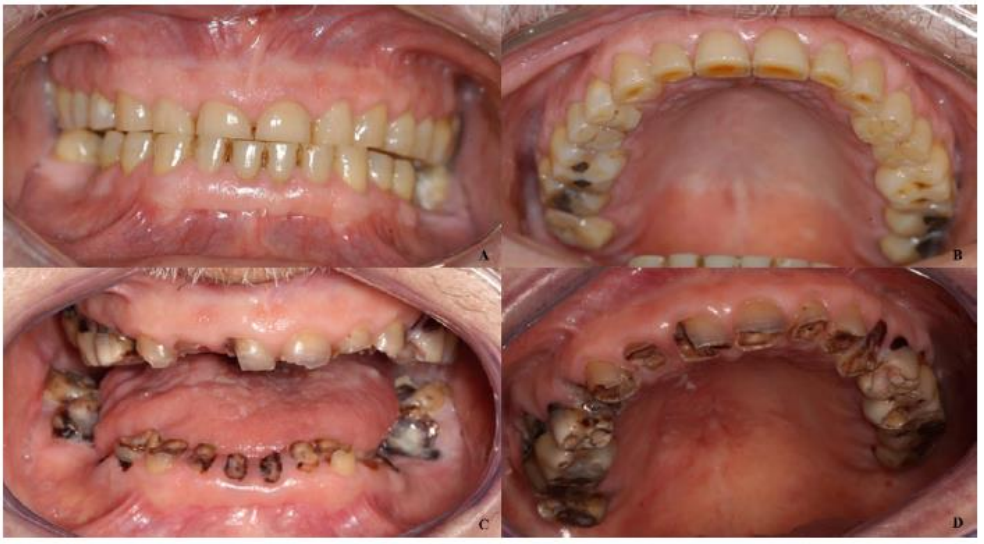

Fig. 2. Clinical case of radiation-related caries (RC) tooth destruction in a 1-year period. (**A**) Pre-head and neck radiotherapy oral status of a cancer patient who did not adhere to pre-treatment dental management protocol. (**B**) Inferior view of maxillary teeth of patient in Figure 2A, presenting wear facets and brownish discoloration on the incisal/cuspal surface of teeth. (**C**) Clinical image of patient in Figure 2A, 1 year following radiotherapy conclusion, presenting extensive black/brownish discoloration and several dental crown amputations. (**D**) Inferior view of maxillary teeth of patient in Figure 2C, presenting intense black/brownish discoloration, several incisal/cuspal and cervical caries.

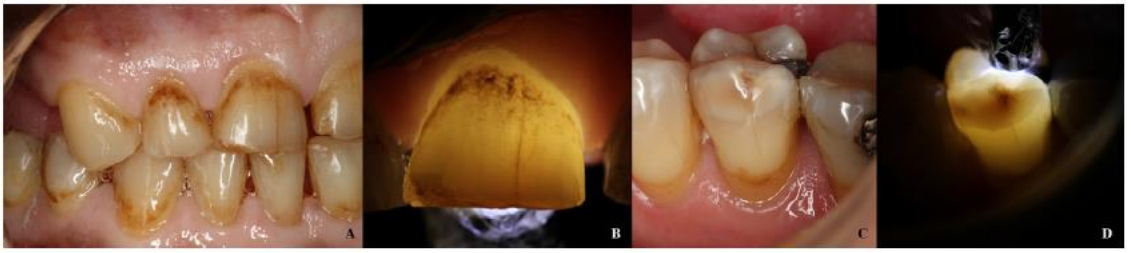

Fig. 3. Patients after head and neck radiotherapy, presenting clinical images of the initial stage of radiation caries. (**A**) Brownish discoloration surrounding the cervical area of teeth and the presence of several enamel cracks. (**B**) Transillumination of anterior maxillary teeth,[32] presenting an extensive enamel crack extending from the cervical to the incisal area and presence of smaller enamel cracks localized on the incisal area. Note the altered pattern of light reflection on the cervical area associated with the clinical area of discoloration pattern, which could indicate the process of subsuperficial demineralization. (**C**) Enamel crack extending from cervical to incisal area. Note the brownish discoloration associated with the crack on the vestibular smooth surface of the tooth. (**D**) Transillumination[32] image of the tooth presented in Figure 3C. Note the extension of the enamel crack from the cuspal tip to the cervical area. In addition, note the alteration of the transillumination pattern correspondent to the brownish discoloration area.

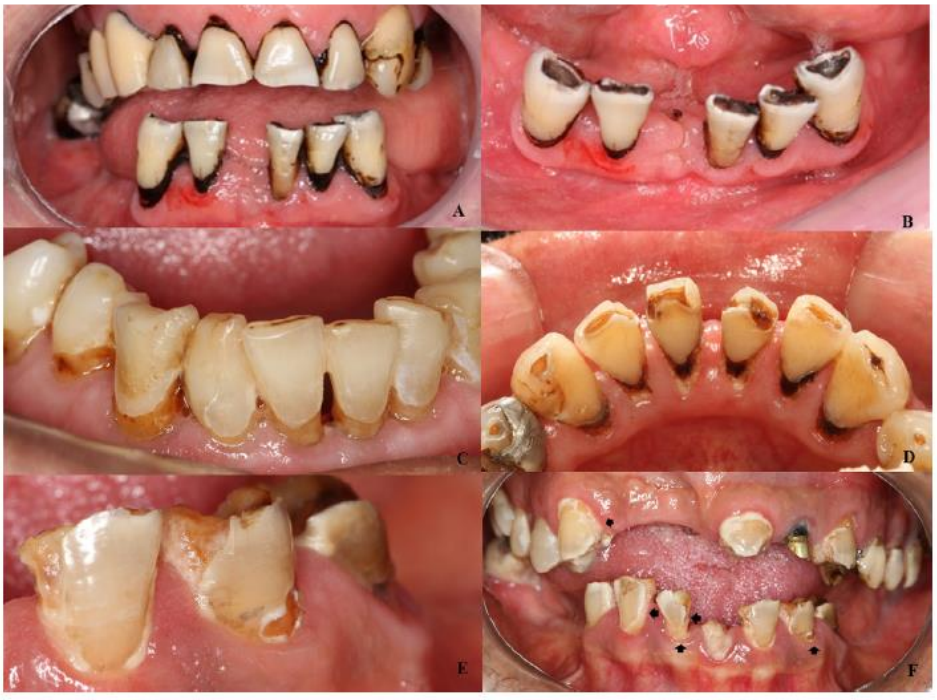

Fig. 4. Patients after head and neck radiotherapy, presenting clinical images of the established stage of radiation caries. (**A**) Patient presenting several areas of black/brownish discoloration; cervical caries on all anterior teeth. (**B**) Superior view of mandibular teeth of patient in Figure 3A, presenting generalized cervical and incisal tooth involvement of radiation-related caries (RC). (**C**) Patient presenting generalized cervical tooth involvement of RC; note the areas of enamel delamination near the cementoenamel junction. (**D**) Lingual view of patient presenting generalized cervical and incisal involvement of RC with intense black/brownish discoloration; note that the carious process extends through the proximal areas surrounding the cervical area of the teeth. (**E**) Clinical image of teeth presenting areas of enamel delamination on the proximal and vestibular surfaces. (**F**) Patient presenting several areas of enamel delamination *(arrows)* and tooth structure loss in the cervical and incisal tooth areas.

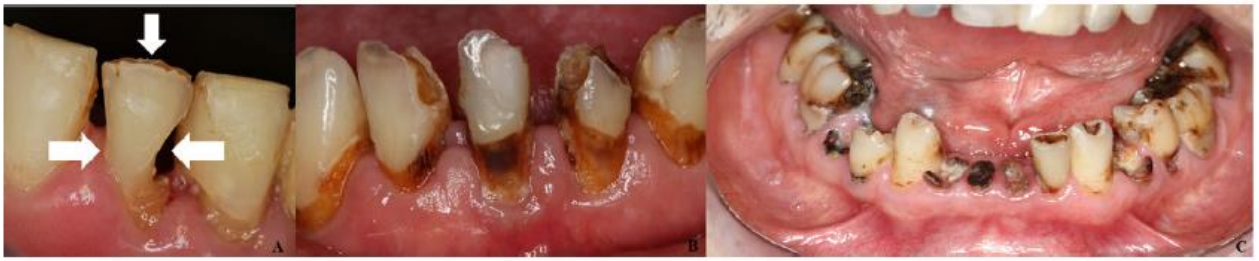

Fig. 5. Patients after head and neck radiotherapy, presenting clinical images of the advanced stage of radiation caries. **(A)** Clinical image representing the characteristic axial/transversal radiation-related caries (RC) evolution process with caries surrounding the cervical and incisal areas of the tooth. **(B)** Teeth presenting black/brownish discoloration, several enamel cracks, and intense tooth structure loss and exposure of the dentin to the oral environment. **(C)** Patient presenting RC involvement in all mandibular teeth with the presence of cervical and incisal/cuspal caries, black/brownish discoloration, and dental crown amputation involving 5 teeth.

Fig. 6. Representative images of spontaneous dental crown amputation. Patient presented the amputated crowns at his follow-up visit at the Dental Oncology Service, São Paulo State Cancer Institute (ICESP, São Paulo, Brazil).

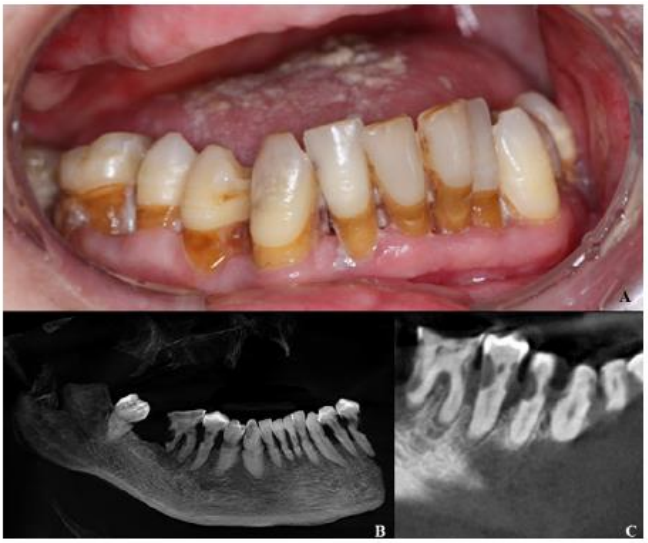

Fig. 7. Clinical and radiographic findings of radiation-related caries (RC) lesions. **(A)** Patient presenting clinically small cervical areas of RC involvement, the presence of enamel cracks, and brownish discoloration in the cervical area. **(B)** Panoramic reconstruction of the patient's computed tomography (CT) scan shown in Figure 7A. Note that in this image, it is possible to observe hypodense images suggestive of a more aggressive process of tooth destruction by RC compared with the clinical image. **(C)** Frontal section of CT scan showing in detail the depth of the RC process in teeth #28, #29, and #30, incompatible with the clinical aspect of the lesions as shown in Figure 7B.

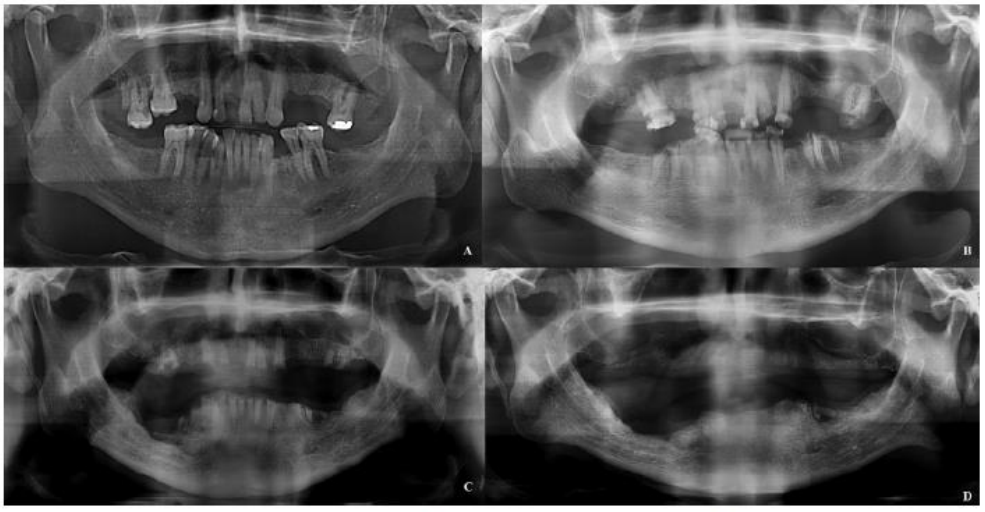

Fig. 8. Radiographic image of radiation-related caries (RC) onset and progression. **(A)** Pre-head and neck radiotherapy (HNRT) patient prior to mouth conditioning protocols. **(B)** Radiographic image 1 year after HNRT. Note the loss of dental crowns on teeth #22, #27, #31, #32, #35, #36, #41, #45, and #46. **(C)** Radiographic image 3 years after HNRT. Patient presents dental crown amputation of all teeth. Note the areas of bone reabsorption on the right and left mandible consistent with the radiographic features of osteoradionecrosis. **(D)** Radiographic image 5 years after HNRT. Patient is edentulous.

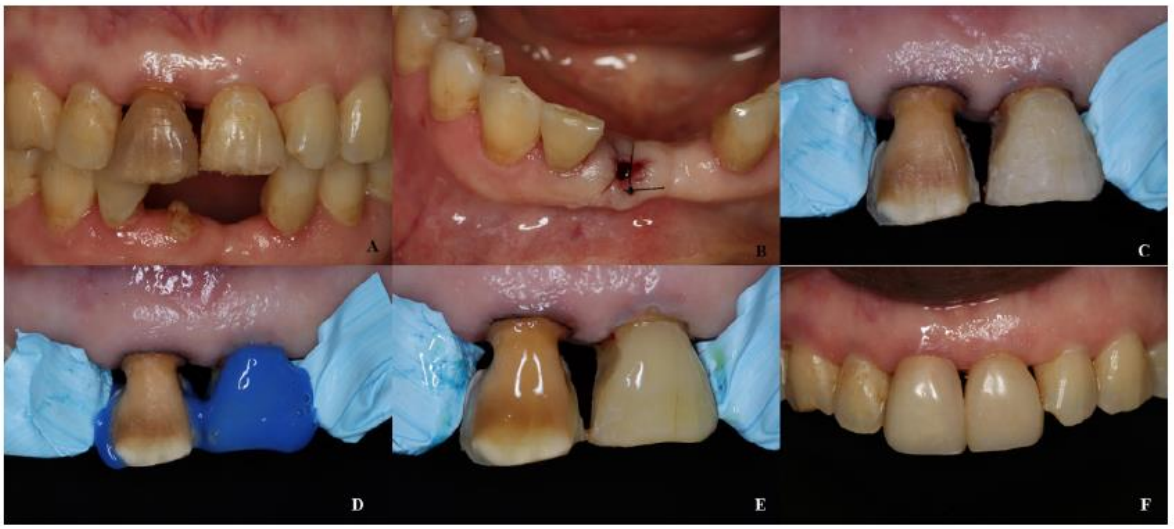

Fig. 9. Clinical case of a patient presenting radiation-related caries (RC) lesions 3 months after head and neck radiotherapy conclusion. (**A**) Areas of initial-stage RC with brownish discoloration and enamel cracks, areas of established-stage RC with cervical tooth involvement, and advanced-stage RC with dental crown amputation of tooth #25. (**B**) First step of RC management with the extraction of tooth #25. (**C**) Exposure of RC tissue using No. 00 retraction cord. (**D**) After the RC tissue was removed, the enamel bezel was made, followed by cavity cleaning, and selective enamel conditioning with 37% phosphoric acid. (**E**) Dental conditioning with adhesive system. Once again, note the extension of the enamel crack of tooth #9. (**F**) Final aspect of tooth restoration with resin composite and polish with sanding disks (3M Sof-Lex, 3M ESPE, Seefeld, Germany). Note the cervical adaptation of the restoration and the fully covered smooth vestibular surfaces without the presence of enamel cracks.

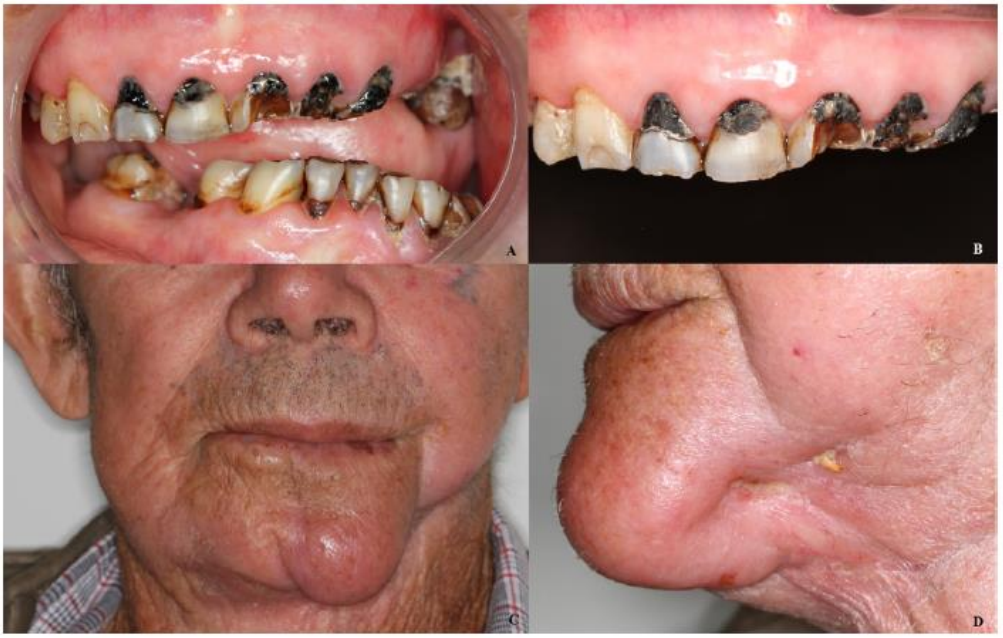

Fig. 10. Clinical images of spontaneous osteoradionecrosis (ORN) after untreated radiation-related caries (RC) in a patient that did not adhere to pre- and post-radiotherapy dental management protocols. (**A**) Patient presenting several areas of black/brownish discoloration, enamel cracks, enamel delamination, incisal/cuspal and cervical caries, and dental crown amputation. (**B**) Detail of anterior maxillary teeth showing intense black/brownish discoloration and tooth structure destruction. (**C**) Extraoral frontal view of the patient showing facial asymmetry caused by the tumor and oncologic treatment. (**D**) Extraoral view of ORN process presenting in the context of extraoral fistula and exposed mandibular bone.

2.6 Artigo: Impact of radiation-related caries in morbidity and mortality outcomes of head and neck cancer patients

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Abstract word count: 249
Word count: 4,347
References: 46
Figures: 2
Tables: 3

Key Words: Radiation-related caries; Head and neck cancer; head and neck radiotherapy; osteoradionecrosis; morbidity.

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Abstract

Objective: The aim of the present study was to assess the impact of RRC on the morbidity and mortality outcomes of head and neck squamous cell carcinoma (HNSCC) patients submitted to head and neck radiotherapy (HNRT).

Methods: Patients were divided into 3 groups: RRC group (N=20), control (n=20) and edentulous (N=20). Information regarding number of dental appointments, of dental procedures, osteoradionecrosis (ORN) development, prescriptions performed by the dental team, and hospital admissions were collected. Mortality outcomes were assessed through disease free survival (DFS) and overall survival (OS) rates.

Results: RRC patients required more dental appointments ($p<0.001$); dental restorations ($p<0.001$); higher number of restored teeth ($p=0.025$); and higher number of tooth extractions ($p=0.001$). Kaplan-Meier subgroup analyses showed a significant impaired risk of ORN in RRC when compared to edentulous ($p=0.015$), and when the groups of patients with teeth are combined the risk of ORN development was also higher ($p=0.036$). RRC group required more antibiotic and analgesic prescriptions ($p<0.001$). Although not statistically significant, RRC group presented lower DFS rates (46.5 months) when compared to control and edentulous (53.5 and 52.8 months).

Conclusions: RRC impacts the morbidity outcomes of post-HNRT patients. RRC patients are prone to develop more ORN consequently increasing the number of drug prescriptions and surgical procedures for its management. Although RRC did not impact OS or DFS mortality rates, it increases morbidity outcomes due to increased need for specialized dental appointments, invasive surgical dental procedures and hospital admissions.

Introduction

Radiation-related caries (RRC) is a well-known chronic complication of head and neck radiotherapy (HNRT) affecting approximately 29% of patients within the first 6 to 12 months post-HNRT (Moore et al., 2020; Palmier et al., 2020).

Despite the negative impact on patients' quality of life (QoL) (Paglioni et al., 2020), a recent publication showed that the level of awareness of this important condition is low among dentists, physicians and head and neck cancer patients which can lead to both misdiagnosis and undertreatment in addition to lack of compliance of patients with preventive measures, therefore increasing the risk for the development of RRC (Martins et al., 2021).

Difficulties in the diagnosis and treatment of RRC represents an important issue for the oral health of post-HNRT patients due to the risk of persistent dental and oral infections, dental destruction and loss of masticatory efficiency with consequent impact on patients' nutritional status and increased risk for the development of osteoradionecrosis (ORN) (Hong et al., 2010; Lalla et al., 2017; Paglioni et al., 2020; Palmier et al., 2020).

RRC treatment protocols are still lacking and RRC lesions treated mostly based on dentists' clinical experience (Palmier et al., 2017). In this context, a recent systematic review showed that composite resin associated with fluoride supplementation may be the best alternative for the management of RRC (Palmier et al., 2021). Nevertheless, although this combination presented better results in comparison with other adhesive materials, survival of dental adhesive restorations in patients post-HNRT are remarkably lower therefore requiring the replacement of restorations more frequently, leading to increased management costs that can vary from $192 to $4,500 for RRC and from $4,800 to up to $78,000 when treatment of ORN is required greatly impacting overall oncologic treatment costs (Wood, et al., 1999; McComb et al., 2002; De Moor et al., 2011; Elting et al., 2019; Palmier et al., 2020; Palmier et al., 2021).

Considering the myriad of local and potential systemic complications associated with

the progression of RRC, the aim of the present study was to assess the impact of RRC on the morbidity and mortality outcomes of head and neck squamous cell carcinoma (HNSCC) patients submitted to HNRT.

Material And Methods

Study Design

This was a single center cohort study designed to evaluate the impact of RRC on the morbidity and mortality outcomes of patients submitted to HNRT or concomitant chemoradiotherapy (CRT) protocols at the Instituto do Câncer do Estado de São Paulo (ICESP, Brazil) from April 2010 to April 2017. This study was approved by the Ethics Committee of the School of Medicine, University of São Paulo, Sao Paulo, Brazil (Protocol# 53869216.2). Written informed consent was obtained from all participants and the study was conducted in accordance with the Declaration of Helsinki.

Inclusion criteria

This study included HNSCC patients who underwent HNRT or CRT protocols (with or without previous surgery) using a 6MV linear accelerator and 3-dimensional conformal or intensity-modulated radiation therapy technique (IMRT) (Synergy Platform, Elekta AB, Stockholm, Sweden). The target radiation volumes encompassed the primary site and areas of regional lymph nodes at risk and received cumulative doses that ranged from 60 to 70 Gy. We applied the recommendations for treatment planning and constraints for organs at risk, as previously reported (Grégoire et al., 2014; Mendez et al., 2016; Grégoire et al., 2018).

Before the beginning of HNRT, patients were submitted to a comprehensive dental treatment with the goal of stabilizing dental disease (if necessary, they were submitted to extractions, periodontal therapy and restorative care). During HNRT, all patients were instructed to use alcohol-free 0.12% chlorhexidine gluconate and 0.05% sodium fluoride mouth

rinse (15 mL, twice a day). Besides routine oral care, all oral and oropharyngeal cancer patients were submitted to the standard-of-care daily photobiomodulation (PBM) protocol for prevention of oral mucositis (OM) as per institutional (Dental Oncology Service, ICESP, Brazil) protocol (Brandão et al., 2018). Patients diagnosed with different tumor topographies other than the oral cavity or oropharynx were followed-up once a week during HNRT. After the HNRT conclusion, all patients from groups RRC and control were placed on a protocol for follow up every 3 months and edentulous patients placed on a protocol for follow up every 6 months, done by the hospital dental team.

All patients needed to have complete demographic and clinicopathological data available on electronical medical charts, including sex, age, tumor location and clinical cancer stage (according to the American Joint Committee on Cancer Staging System, 7th edition; Edge and Comptom., 2010), cancer treatment modalities and information regarding dental appointments post-HNRT.

Sample size was calculated using G*Power 3 software (Faul et al, 2007), with the following input data: “Effect size f” = 0.45, “α error probability” = 0.05, “Power (1-β error probability)” = 0.8, and “Number of groups” = 3; resulting in a total sample size of 60 patients, and an actual power of 0.86. Included patients were divided into three groups, as follows: RRC group (N=20) encompassed patients that developed RRC based on the described characteristics of cervical or incisal caries (Palmier et al., 2020) within the first 12 months post-HNRT; control group (N=20) encompassed patients that developed conventional characteristics of caries, such as a few points on the occlusal area (Paglioni et al., 2020), that did not developed RRC or the ones who developed RRC after the first year post-HNRT and the edentulous groups (N=20) which encompassed patients who were submitted to HNRT presenting no teeth in the oral cavity. Information on the development of RRC and conventional caries was collected according to the information available at the electronical medical charts.

Exclusion criteria

Patients who received incomplete HNRT treatment, that did not present complete information on electronical medical charts or that did not return for dental follow-up post-HNRT were excluded from the study.

Dental and morbidity assessment

Electronical medical charts from the included patients were screened and information regarding number of dental appointments, number of dental procedures such as tooth restorations, periodontal and endodontic treatment and tooth extractions, number of antibiotics prescriptions, analgesics and opioids prescribed by the dentists, number of hospital admissions due to dental/oral complications and outcomes regarding the onset and progression of ORN were collected. ORN presence was identified by characterizing the presence of exposed necrotic bone or significant bone alterations present in radiographic images as described by the dentists in the electronical medical charts. Digital mandible panoramic X-Rays from each dental appointment were also screened.

Mortality assessment

Information regarding disease free survival (DFS) including tumor persistence, recurrence, second primary tumors and distant metastasis, and overall survival (OS) rates were collected from electronical medical charts.

Statistical analysis

Variables were analyzed using specific statistical tests, with a level of significance set at 5% ($p < 0.05$). All analyses were performed using SPSS version 22.0 (IBM, Chicago, IL, USA).

Results

Patient clinical features

The clinicopathological features, including age, gender, tumor site, clinical staging, treatments, and HNRT modality, are summarized in **Table 1**. The mean overall age was 56.8 years (range 28–83 years) and 46 (76.7%) patients were males. The most frequent primary tumor sites were base of tongue and oropharynx (13 cases, 21.6% each), followed by the lateral border of the tongue and larynx (6 cases, 10% each). Most patients (55, 91.6%) were diagnosed with an advanced stage of disease (III/ IV). All included patients received complete and uninterrupted HNRT, the main treatment modality observed were induction chemotherapy followed by CRT (19, 31.7%), followed by RCT (16, 26.7%) and surgery followed by HNRT (12, 20%) protocols. Regarding the HNRT technique, 54 (90%) of patients were treated by 3-dimensional conformal RT; 6 (10%) with IMRT, with an overall mean dose of 67.7Gy (ranging from 66.6Gy to 67.7Gy). The overall mean follow-up time was 64 months (varying from 61 to 66 months between groups).

Patients' dental profile pre-HNRT

RRC group presented a total of 343 teeth (mean 17 teeth per patient) of which 76 (22.2%) were restored, 26 (7.5%) extracted, 2 (0.6%) submitted to endodontic treatment, and a total of 20 periodontal treatments were performed in 16 patients. Three (15%) patients did not conclude mouth conditioning protocols. The mean number of dental appointments during mouth conditioning was 3 (ranging from 0 to 5).

Control group presented a total of 415 teeth (mean 21 teeth per patient) of which 111 (26.7%) were restored, 29 (7.0%) were extracted, none submitted to endodontic treatment, and a total of 21 periodontal treatments were performed in 15 patients. Five (25%) patients did not conclude mouth conditioning protocols and 2 (10%) performed conditioning protocol outside

the hospital facilities. The mean number of dental appointments during mouth conditioning was 3 (ranging from 0 to 5). Control group presented more teeth pre-RT than the RRC group ($p<0.001$).

Edentulous group presented a total of 45 teeth previous to mouth conditioning protocol (mean 2 teeth per patient) of which 40 (88.9%) were extracted and the remaining 5 (11.1%) were removed along with tumor resection surgery. A total of 4 periodontal treatments were performed in 3 patients prior to dental extractions. All patients of the edentulous group (20, 100%) concluded mouth conditioning protocol. The mean number of dental appointments during mouth conditioning was 2 (ranging from 1 to 4).

Dental and morbidity assessment post-HNRT

Detailed information including dental appointments and procedures post-HNRT, dental/oral complications and prescriptions performed by the dental team are available in **Table 2.**

The mean time for the development of RRC in the RRC group was 8.2 (ranging from 3 to 12 months) and 26.2 (ranging from 13 to 41 months) in the control group. Two (10%) patients of the control group did not develop RRC lesions. Infections of odontogenic origin were observed in both groups, mainly dental abscess and post-extraction oroantral fistula in the RRC group; while the control group presented periodontal fistula, apical periodontitis, and endodontic-periodontal lesions.

RRC group required more dental appointments (521) when compared to control (473) and edentulous groups (270) ($p<0.001$). From the 318 teeth observed in the RRC group after mouth conditioning protocol, a total of 215 (67.6%) required dental restorations while only 171 (44.7%) of the 382 teeth from the control group were restored ($p=0.025$). A total of 457 dental restorations were performed in the RRC group with 32 (14.9%) teeth requiring over 4

restorations on the same teeth during the follow-up period while only 292 dental restorations were required for the control group (<0.001) and 10 (5.9%) teeth restored more than 4 times (p=0.001). One (0.5%) tooth from the RRC group had to be restored over 7 times over the follow-up period.

A total of 166 (52.2%) teeth from 18 patients of the RRC group and 110 (28.7%) from 14 patients of the control group were extracted post-HNRT (p=0.001). The main reasons for tooth extractions were extensive caries, periodontal disease, mandible resection due to ORN and root fracture.

The development of ORN was observed in 11 (55%) patients from RRC group after a mean follow-up time of 32 (ranging from 13 to 76 months); 8 (40%) patients of control group with a mean time of 26.9 (ranging from 0 to 48 months) and 4 (20%) patients from edentulous group with a mean time of 30 (ranging from 9 to 49 months) and an overall incidence rate of 38.3%. Interestingly, incidence rates of ORN increased up to the third-year post-HNRT, on the first-year overall incidence was 5% followed by 10% on the second year and a peak of incidence of 13.3% on the third year. After the third year of follow-up, incidence rates decreased to 6.66% on the fourth year and 3.33% at 76 months post-HNRT. Kaplan-Meier analyses showed a higher risk of ORN development in RRC and control groups when compared to edentulous patients. In 5 years, 52.6% of RRC, 41.6% of control, and 22.9% of edentulous patients had developed ORN **(Figure 1a)**. Although log-rank test was not statistically significant (p=0.069), there was a significant log-rank test for trend (p=0.02). Thus, we performed subgroup analyses, which showed a significant impaired risk of ORN in RRC when compared to edentulous (HR: 3.654; 95%CI: 1.29-10.35; p=0.015) **(Figure 1b)**, but not when compared to control (HR: 1.488; 95%CI: 0.6-3.687; p=0.3) **(Figure 1c)**. Additionally, when the groups of patients with teeth are combined (RRC and control), the risk of ORN development was also higher (HR: 2.468; 95%CI: 1.058-5.756; p=0.036) **(Figure 1d)**.

The main reasons for the development of ORN in the RRC group were post dental extractions, associated with dental infection caused by extensive RRC and associated with dental implants, it is worth to mention that the dental implants had been performed prior do to HNRT. The main reasons for the development of ORN in the control group was post dental extractions, associated with periodontal disease, oncologic surgery for recurrent tumor, spontaneous and one patient developed ORN associated with the tumor resection surgery site during HNRT. Finally, the main reasons observed for the development of ORN in the edentulous group were associated to oncologic surgery for recurrent or persistent tumors and one patient presented spontaneous ORN.

Local sequestrectomy was performed in 6 (54.5%) out of the 11 patients from the RRC with a total of 15 procedures (mean 2.5 per patient); 4 (50%) out of 8 patients of the control group with a total of 6 procedures (mean 1.5 per patient); 3 (75%) out of the 4 patients from the edentulous group with a total of 7 procedures (mean 2.3 per patient). Bone debridement was required in 6 (54.5%) patients of the RRC group, 5 (62.5%) patients of the control group and 3 (75%) patients of the edentulous group. Mandible resection due to ORN was observed in 2 (18.2%) of the patients of RRC group that developed ORN and 1 (25%) patient from the edentulous group. No patients from the control group required bone resection due to ORN.

A total of 6 hospital admissions were observed in the RRC group from which 4 were to perform bone debridement in operating room and 2 for mandible resection due to ORN. For the control group a total of 2 hospital admissions were observed to perform bone debridement in operating room. For the edentulous group, a total of 3 hospital admissions were observed to perform 2 bone debridement in operating room and 1mandible resection due to ORN.

Antibiotics prescriptions were performed 69 times for the RRC group while only 53 times for control and 17 for the edentulous group ($p<0.001$). The most prescribed antibiotic was amoxicillin followed by clindamycin, amoxicillin combined with clavulanic acid and

metronidazole. The main reasons for antibiotic prescriptions were tooth extractions, ORN and dental abscess. All patients submitted to tooth extractions were submitted to pre- and post-operative antibiotics prescriptions.

Analgesic prescriptions, mostly dipyrone, were performed 57 times for the RRC group while 48 times for the control and 15 times for the edentulous group ($p<0.001$). The main reasons for analgesic prescription were for pain control related to tooth extractions, ORN, and biopsy performed in the oral cavity. Opioid prescription including codeine, tramadol or morphine, was observed 4 times in the RRC and the edentulous group and 3 times for the control group. For the RRC group, all opioid prescriptions were associated with ORN while for the control group all opioid prescriptions were associated to tooth extraction procedures. Finally, for the edentulous group most of the opioid prescriptions (75%) were due to ORN-related pain and one (25%) was due to pain secondary to dental prosthetic surgery.

Mortality assessment

Detailed information regarding oncological outcomes of each group are presented in **Table 3.** RRC group presented lower DFS rates (46.5 months) when compared to control and edentulous (53.5 and 52.8 months, respectively), although Kaplan-Meier analysis showed no statistically significant differences **(Figure 2a)**. OS rates were also lower for RRC group which presented a mean OS of 61.4 months when compared to control and edentulous groups (66 and 65 months respectively), with no statistically significant differences found in OS rates (**Figure 2b)**.

Discussion

This study evaluated the impact of the development of RRC on morbidity and mortality outcomes of HNSCC submitted to HNRT. From the present results it was possible to observe

that RRC represent an important impact on morbidity outcomes of post-HNRT patients and, although mortality rates presented no significant differences, the complications associated with the presence of RRC could contribute to the negative impact on these patients' quality of life as previously demonstrate by our group (Paglioni et al., 2020).

The clinicopathological features of the sample is compatible with HNSCC patients' profile with tumors affecting mostly middle-aged men with the most common primary tumor sites as oropharynx and lateral border of the tongue, diagnosed in advanced stages of disease consequently requiring multimodal treatment which includes HNRT protocols (Kowalski et al., 2005).

Dental profile observed pre-HNRT showed that, when compared to literature reports for mean number of teeth present in the oral cavity (mean of 22.9) (Brennan et al., 2017) and the control group of the present study (mean of 21), RRC patients presented a lower mean number of teeth (mean 17) pré-HNRT. This result also corroborates with a recent published work that shows that patients with lower mean number of teeth presented lower level of awareness of RRC which could directly impact on patients' compliance to preventive measures and therefore increase the risk for the development of RRC (Martins et al., 2021). Additionally, when added up, results show that 8 (13.3%) out of 60 patients did not conclude mouth conditioning protocols prior to HNRT, similar to the results of Brennan et al., 2017, which reported that approximately 14.1% did not conclude mouth conditioning protocols beginning HNRT with untreated carious lesions or even teeth with infectious foci that required dental extractions pre-HNRT. This result shows that although free oral care was provided by the Dental Oncology Service (ICESP), compliance of patients to dental appointments remains an important issue even prior to HNRT, and measures for increasing patients' awareness of the importance of dental assessment and adequate follow-up should be improved (Brennan et al., 2017; Martins et al., 2021). In this context, it is worth mentioning that up to date there are no stablished standards for dental care

prior to HNRT with procedures being based mainly on the removal of oral foci of infection to prevent post-HNRT complications (Schuurhuis et al., 2015; Brennan et al., 2017).

Elevated risk for the development of RRC is well stablished in the literature (Hong et al., 2010). Results for the mean time for the development of RRC are compatible with the literature with a mean time of 8.2 months ranging from 3 to 12 months (Palmier et al., 2017; Moore et al., 2020; Palmier et al., 2020). In terms of dental restoration needs, RRC group presented with 67.6% of teeth requiring tooth restorations compared to only 44.7% of control group. Additionally, a considerable number of teeth of the RRC group needed to be restored over 4 times during the follow-up period and one tooth needed to be restored over 7 times, these results corroborate with a recent systematic reporting low survival rate for adhesive dental restorations in post-HNRT patients (Palmier et al., 2021). Difficulties in maintaining adequate oral hygiene due to the cluster of oral symptoms, such as hyposalivation, oral pain, trismus, among others, usually observed in post-HNRT patients may be regarded as one of the reasons for the low longevity of dental restorations observed in the present study (Gouvea et al., 2020; Palmier et al., 2021). Additionally, results also corroborate with literature reports of impaired adhesive properties after HNRT. Nevertheless, most studies that assess adhesive properties post-RT were performed under simulated RT protocols, additionally, studies that assessed *in vivo* irradiated teeth are limited and controversial in the literature (Galleti et al., 2014; Madrid et al., 2017).

Periodontal therapy needs were high for both RRC and control groups which also corroborates with previous literature reports of worsening of periodontal disease post-HNRT (Epstein et al., 1998; Marques et al., 2004; Ammajan et al., 2013). The high need for periodontal treatment can be correlated with increased risk for plaque accumulation due to the altered oral microbiome in addition to a dry oral environment due to qualitative and quantitative salivary alterations presented by these patients. In addition to the elevated risk for periodontal disease,

plaque accumulation on the cervical area of teeth also plays an important role in the development of RRC (Irie et al., 2018; Palmier et al., 2020).

A remarkably higher need for tooth extractions post-HNRT was observed in the RRC group when compared to control, this result is in accordance with the aggressive profile of RRC that leads to extensive tooth structure destruction, dental crown amputation and dental infections consequently justifying the need for tooth extractions (Kiealbassa et al., 2006; Palmier et al 2020).

The major issue associated with the elevated number of tooth extractions is the increased risk for the development of ORN. As observed in the present study, an overall incidence of ORN of 38.3% was observed, with the RRC group presenting the highest per group incidence (55%), values that, although compatible with literature reports for the range of ORN incidence, are considered high for the post-HNRT population (Monnier et al., 2011; Nabil et al., 2011; Crombie et al., 2012; Chronopoulos et al., 2018). It is important to mention that present ORN overall incidence values may be increased when compared to overall literature reports due to the extended time for dental follow-up performed in the present study, most literature reports present a follow-up period post- dental extraction of 1 to 12 months while patients in the present study were followed-up for a longer period with mean time for ORN development of approximately 30 months post-HNRT (Nabil et al., 2011).When comparing incidence of ORN within the first and second year post-HNRT, results show incidence rates of 5% and 10%, respectively, which is compatible with most literature reports for overall incidence rates of ORN that range from 5% to 10% (Monnier bet al., 2011). The extended follow-up period of the present study (mean follow-up varying from 61 to 66 months between groups) could directly increase ORN rates as observed by Monnier et al., 2011 and Crombie et al., 2012 which observed ORN incidence rates of 40% and 36%, respectively, when a 5-year follow-up was performed.

The main reason for ORN development in both RRC and control groups were related to tooth extractions due to poor overall oral health status which is compatible to etiologic factors for ORN development in the literature (Nabil et al., 2011; Moon et al., 2017). Interestingly, although almost all cases of ORN for the RRC group were correlated to the progression of RRC and consequent tooth extraction, for the control group a myriad of factors were associated with ORN development with the second most correlated cause being periodontal disease, which is also one of the main factors for ORN development reported in the literature (Nabil et al., 2011). As for the edentulous group the main reason for ORN development was associated with mandibular surgery for tumor resection, either primary tumor pre-HNRT or recurrent tumor post-HNRT. Interestingly, although the association with tumor resection is a known factor for ORN (Arup-Kristensen et al., 2019), edentulous patients presented a remarkably lower ORN rate when compared do RRC and control groups. The increased incidence of ORN mostly correlated to RRC-related tooth extractions is an important factor that support the impact of RRC on the morbidity outcomes of HNSCC patients, especially its correlation with the development of ORN and its complications (Niewald et al., 2013; Paglioni et al., 2020; Palmier et al., 2020).

The treatment for ORN required several surgical procedures including local sequestrectomy, bone debridement and mandible resection, as according to reported literature for measures for ORN management (Rice et al., 2015), from which most were performed in the RRC group. The increased need for surgical management of ORN lead to increased hospital admissions to perform ORN debridement in the operating room and mandible resection. The increased necessity for invasive surgical treatment contributes to the morbidity outcomes of RRC and consequently its negative impact on patients QoL (Lee et al., 2014; Silva et al., 2014; Paglioni et al., 2020; de Oliveira et al., 2020; Palmier et al., 2020). Although RRC and ORN management cost were not assessed in the present study, the increased need for the management

of RRC, increased tooth extractions and increased invasive surgical management of ORN with the need with hospital admissions for mandible resection reinforces previous reports that RRC can lead to increased overall oncological-related treatment costs that can vary from $192 for the management of RRC up to $78,000 when bone reconstructions are required after mandibulectomy due to the progression of ORN (Elting et al., 2019; Palmier et al., 2020).

Increased number of antibiotics prescriptions observed in the RRC is compatible to both increased need for tooth extractions considering that all patients submitted pre- and post-operative antibiotics prescriptions, this is an important result considering literature reports that 86% surgeons advocate pre-operative, and 89% surgeons advocate post-operative antibiotic prescriptions in post-HNRT patients that require tooth extractions (Kanatas et al., 2002). The use of penicillin agents and clindamycin as pre- and post-operative antibiotic regimens are compatible with literature reports of medications of choice for preventing infection in post-HNRT patients that require surgical procedures including tooth extractions and prevention of ORN (Nabil et al., 2011).

Results of the present study regarding DFS, and OS rates are compatible with the profile of HNSCC patients with patients presenting low survival rates in 5 years (Guidi et al., 2018; dos Santos et al., 2021; Sung et al., 2021). Although the results did not show a direct impact of the presence of RRC on DFS and OS rates, the complications associated with its development can potentially impair several physical, emotional and social aspects of patients leading to a poor overall QoL status as previously reported in the literature (Paglioni et al., 2020).

Limitations

Limitations of the present study include retrospective and single institution study design, availability of subjective information regarding the presence of RRC and grading of ORN and only two patients that did not develop RRC lesion during the whole follow-up period. Additionally, cost analysis was not possible to be performed. These limitations justify the need

for multicentric cohort and long-term prospective studies which includes adequate grading of RRC and cost analysis of RRC management and the cost of management of complications associated with RRC such as ORN.

Conclusions

RRC represents an important morbidity factor in post-HNRT patients. Increased number of dental appointments and dental restorations in the RRC group supports the low longevity of post-HNRT restorations. The remarkably higher need for dental extractions in the RRC group reinforces its clinical aggressiveness. RRC patients are more prone to develop ORN, consequently increasing the number of drug prescriptions and surgical procedures for its management. Overall, although RRC did not represent an important factor associated with mortality rates, it increases morbidity outcomes due to increased need for specialized dental appointments, drug prescriptions, surgical dental procedures, and hospital admissions.

Funding Sources

The authors would like to gratefully acknowledge the financial support of the São Paulo Research Foundation (FAPESP 2018/04657-8; 2018/02233-6; 2016/22862-2; 2013/18402-8 and 2012/06138-1) as well as the National Council for Scientific and Technological Development (CNPq), Brazil. The authors also state that this study was financed in part by the Coordination for the Improvement of Higher Education Personnel — Brazil (CAPES) — Finance Code 001.

Conflict of interest

None to declare.

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Table 1: Clinicopathological profile of patients included in the present study divided by group RRC, control and edentulous.

Variables	RRC (N=20)	Control (N=20)	Edentulous (N=20)	Total (N=60)
Gender				
Female	6 (30%)	5 (25%)	3 (15%)	14 (23.3%)
Male	14 (70%)	15 (75%)	17 (85%)	46 (76.7%)
Mean age	56.15 (range from 28 to 71 years)	53.35 (range from 36 to 77 years)	61.1 (range from 49 to 83 years)	56.8 (range from 28 to 83 years)
Tumor topography				
Base of Tongue	7 (35%)	3 (15%)	3 (15%)	13 (21.6%)
Oropharynx	5 (25%)	5 (25%)	3 (15%)	13 (21.6%)
Lateral border of the tongue	2 (10%)	2 (20%)	2 (20%)	6 (10%)
Gingiva	2 (10%)	0 (0%)	2 (20%)	4 (6.7%)
Retromolar trigone	0 (0%)	1 (5%)	2 (20%)	3 (5%)
Palate	1 (5%)	2 (10%)	1 (5%)	4 (6.7)
Tonsil	0 (0%)	2 (10%)	1 (5%)	3 (5%)
Mouth floor	0 (0%)	0 (0%)	2 (20%)	2 (3.3%)
Larynx	2 (10%)	3 (15%)	1 (5%)	6 (10%)
Nasopharynx	1 (5%)	1 (5%)	1 (5%)	3 (5%)
Hypopharynx	0 (0%)	1 (5%)	1 (5%)	2 (3.3%)
Occult primary tumor with cervical metastasis	0 (0%)	0 (0%)	1 (5%)	1 (1.7%)
Smoking				
Yes	2 (10%)	5 (25%)	8 (40%)	15 (25%)
No	3 (15%)	6 (30%)	0 (0%)	9 (15%)
Former	15 (75%)	9 (45%)	12 (60%)	36 (60%)
Alcohol				
Yes	2 (10%)	4 (20%)	2 (10%)	8 (13.3%)
No	6 (30%)	4 (20%)	3 (15%)	13 (21.6%)
Former	11 (55%)	12 (60%)	15 (75%)	38 (63.4%)
NI	1 (5%)	0 (0%)	0 (0%)	1 (1.7%)
TNM staging				
I	0 (0%)	1 (5%)	0 (0%)	1 (1.7%)
II	0 (0%)	2 (10%)	2 (10%)	4 (6.7%)
III	7 (35%)	2 (10%)	4 (20%)	13 (21.6%)
IV	13 (65%)	15 (75%)	14 (70%)	42 (70%)
Oncologic treatment				
Surgery + RCT	4 (20%)	3 (15%)	0 (0%)	7 (11.6%)
Surgery + RT	2 (10%)	2 (10%)	8 (40%)	12 (20%)
Induction CT+ Surgery + RCT	0 (0%)	1 (5%)	0 (0%)	1 (1.7%)
Induction CT +RT	2 (10%)	1 (5%)	0 (0%)	3 (5%)
Induction CT + RCT	7 (35%)	7 (35%)	5 (25%)	19 (31.7%)
RCT	5 (25%)	5 (25%)	6 (30%)	16 (26.7%)
Exclusive RT	0 (0%)	1 (5%)	1 (5%)	2 (3.3%)
RT modality				
3DRT	20 (100%)	17 (85%)	17 (85%)	54 (90%)
IMRT	0 (0%)	3 (15%)	3 (15%)	6 (10%)
Mean dose	66.65Gy	67.75Gy	66.9Gy	67.7Gy

3DRT= Three-dimensional conformal radiotherapy; CT= Chemotherapy; Gy= Gray; IMRT= Intensity-modulated radiotherapy; NI = Not informed; RCT= Radiochemotherapy; RRC= Radiation-related caries.

Table 2: Dental profile and dental needs post-HNRT among RRC, control and edentulous groups.

Variables	RRC (N=20)	Control (N=20)	Edentulous (N=20)	p§
Teeth post mouth conditioning protocols	318	382	NA	0.016
Dental appointments post-HNRT	521	473	270	<0.001
Total number of restorations	457	292	NA	<0.001
Number of restored teeth	215 (67.6%)	171 (44.7%)	NA	0.025
Teeth restored 1 to 3 times	183 (85.1%)	161 (94.1%)	NA	ns
Teeth restored 4 times or more	32 (14.9%)	10 (5.9%)	NA	0.001
Teeth restored >7 times	1 (0.5%)	0 (0%)	NA	-
Periodontal therapy	131	160	NA	ns
Endodontic treatment	11 (3.4%)	11 (2.8%)	NA	ns
Tooth extractions	166 (52.2%)	110 (28.7%)	NA	0.001
Prescriptions				
Antibiotics	69	53	17	<0.001
Analgesic	57	48	15	<0.001
Opioids	4	3	4	ns
Antibiotic prescriptions				
Tooth extractions	45 (65.2%)	40 (75.5%)	NA	ns
ORN	18 (26.1%)	10 (18.8%)	13 (76.5%)	ns
Dental abscess	4 (5.8%)	1 (1.9%)	NA	ns
Other procedures	2 (2.9%)	2 (3.8%)	4 (23.5%)	ns
Analgesic prescriptions				
Tooth extractions	43 (74.4%)	35 (72.9%)	NA	ns
ORN	13 (22.8%)	9 (18.8%)	12 (80%)	ns
Biopsy in the oral cavity	1 (1.8%)	4 (8.3%)	3 (20%)	ns
Opioid prescription				
ORN	4 (100%)	0 (0%)	3 (75%)	-
Tooth extraction	0 (0%)	3 (100%)	NA	-
Dental prosthetic surgery	0 (0%)	0 (0%)	1 (25%)	-

HNRT= Head and neck radiotherapy; ns= Not statistically significant; NA= not applicable; ORN= Osteoradionecrosis; RRC= Radiation-related caries. § Non-parametric chi-square test

Table 3: Oncological outcomes, disease free survival and overall survival rates of patients included in the present study.

Variables	RRC (N=20)	Control (N=20)	Edentulous (N=20)	Total (N=60)
Tumor persistence	1 (5%)	1 (5%)	2 (10%)	4 (6.7%)
Recurrence	5 (25%)	0 (0%)	3 (15%)	8 (13.3%)
Second primary tumor	2 (10%)	6 (30%)	2 (10%)	10 (16.7%)
Distant metastasis	1 (5%)	0 (0%)	1 (5%)	2 (3.3%)
None	11 (55%)	13 (65%)	12 (60%)	36 (60%)
DFS (mean)	46.5 (1 a 93 meses)	53.4 (4 a 102 meses)	52.8 (0 to 104 months)	50.8 (0 to 104 months)
OS (mean)	61.4 (27 a 96 meses)	66.8 (29 a 110 meses)	65 (28 a 104 meses)	64.4 (27 to 104 months)
Deaths	5 (25%)	7 (35%)	5 (25%)	17 (28.3%)

DFS= Disease free survival; OS= Overall survival; RRC= Radiation-related caries.

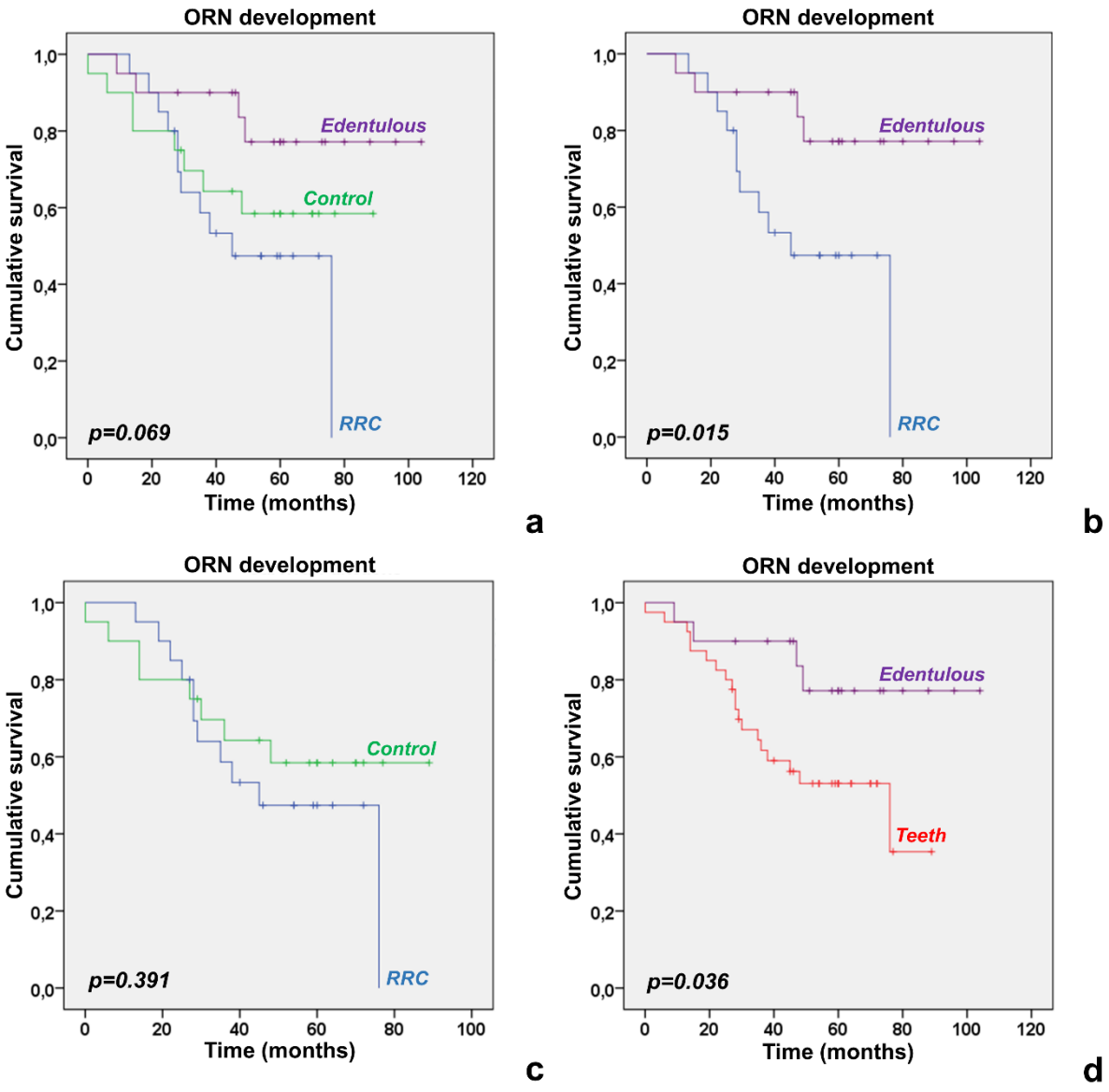

Figure 1: Kaplan-Meier analyses of the risk of osteoradionecrosis (ORN) development in radiation-relate caries (RRC), control and edentulous groups. **A:** Kaplan-Meier analyses showed a higher risk of ORN development in RRC and control groups when compared to edentulous patients. Although log-rank test was not statistically significant (p=0.069), there was a significant log-rank test for trend (p=0.02). **B:** Kaplan-Meier analysis showed a significant impaired risk of ORN in RRC when compared to edentulous (HR: 3.654; 95%CI: 1.29-10.35; p=0.015). **C:** Kaplan-Meier analysis showed no statistical differences when comparing ORN among RRC and control (HR: 1.488; 95%CI: 0.6-3.687; p=0.3). **D:** Kaplan-Meier analysis showed that when the groups of patients with teeth are combined (RRC and control), the risk of ORN development was also higher (HR: 2.468; 95%CI: 1.058-5.756; p=0.036).

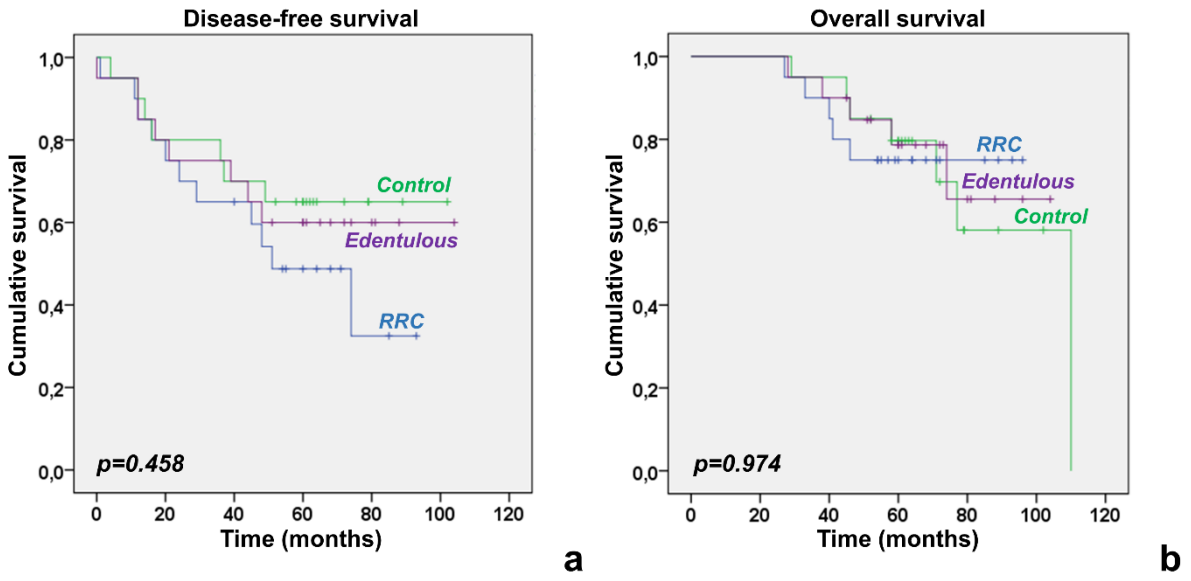

Figure 2: Disease free survival (DFS) and overall survival (OS) rates among radiation-relate caries (RRC), control and edentulous groups. **A:** RRC group presented lower DFS rates (46.5 months) when compared to control and edentulous (53.5 and 52.8 months, respectively), although Kaplan-Meier analysis showed no statistically significant differences. **B:** OS rates were also lower for RRC group which presented a mean OS of 61.4 months when compared to control and edentulous groups (66 and 65 months respectively), again no statistically significant differences were found in OS rates.

3. DISCUSSÃO

Os resultados da presente tese sugerem a correlação das toxicidades orais induzidas pela RT através da formação do agrupamento de sintomas orais assim como seu possível impacto no desenvolvimento e progressão da CR. Adicionalmente as descobertas relacionadas ao perfil proteômico das toxicidades orais agudas de pacientes com CEC de cavidade oral e orofaringe submetidos à RT também suportam essa teoria através dos achados nos quais parte das proteínas salivares foram correlacionadas com a gravidade de mais de uma toxicidade oral induzida pela RT.

Disgeusia, ou alteração no paladar, é uma das principais toxicidades agudas pós-RT para o CCP. Resultados da presente tese corroboram com a literatura em relação ao padrão de desenvolvimento nas semanas iniciais e progressão para disgeusia severa a partir da terceira semana de RT (Zecha et al., 2016; Palmieri et al., 2019). Interessantemente, também foi possível observar de forma efetiva o impacto da xerostomia e MO na progressão da disgeusia corroborando com artigos prévios que sugeriam tal correlação assim como reforçando a presença do agrupamento de sintomas orais (Ogama et al., 2010; Bressan et al., 2016; Barnhart et al., 2018; Gouvêa Vasconcelos et al., 2020).

Análise do perfil molecular demonstrou a correlação da proteína Alfa-2- glicoproteína rica em leucina (A2GL) com a presença de disgeusia severa, a literatura sugere que tal proteína seria um modulador do Fator de transformação de crescimento beta (TGF-β) o qual por sua vez, já foi correlacionado com inibição da proliferação de papilas gustativas *in vitro* (Nakamura et al., 2009; Honda et al., 2017). Este é o primeiro estudo a correlacionar diretamente a presença da proteína A2GL com a presença de disgeusia severa, entretanto os padrões e vias de sinalização envolvidos nessa correlação são necessários.

Adicionalmente, resultados também demonstraram a correlação de proteínas pró-inflamatórias e pró-tumorais assim como proteínas consideradas como marcadores de

resistência a cisplatina, como modalidade de QT utilizada. com a presença de MO e disfagia severa/odinofagia (Syed et al., 2015; Ribeiro et al., 2017; Araújo et al., 2018; You et al., 2019). Considerando que grande parte dos pacientes com CCP são diagnosticados em estadio avançado necessitando, portanto, de protocolos de tratamento multimodal os quais incluem protocolos de QT, tais proteínas poderiam ser utilizadas como biomarcadores não só de resposta ao tratamento oncológico, mas também possíveis preditores de pacientes propensos a desenvolver MO e disfagia severa (Murphy et al., 2007; Lee et al., 2018; Palmier et al., 2020).

De forma interessante, resultados da presente tese revelaram 3 proteínas que foram previamente correlacionadas à boca seca relacionada à Síndrom de Sjogren e polendocrinopatia autoimune- distrofia candidíaseectodérmica (APECED) (Tapinos et al., 2002; Laine et al., 2007; Burbelo et al., 2019), entretanto, ainda que correlacionadas à presença de boca seca nas doenças autoimunes mencionadas, até o momento nenhum estudo estudou o perfil proteômico ou o papel das proteínas observadas no presente estudo e sua correlação com a xerostomia induzida por RT.

Nesse contexto, vale ressaltar que os danos da radiação direta nas glândulas salivares podem levar a alterações qualitativas e quantitativas (hipossalivação) em pacientes com CCP e podem ser correlacionados à dose total de radiação aplicada nas glândulas salivares, localização do tumor e modalidades de tratamento que incluem quimioterapia ou outros medicamentos conhecidos por diminuir o fluxo salivar (Santos-Silva et al., 2015; Cohen et al., 2016). A xerostomia é uma das toxicidades mais persistentes da RT para o tratamento de CCP, e mudanças na composição no perfil proteômico salivar podem representar um impacto importante no desenvolvimento da xerostomia, assim como em todas as toxicidades orais relacionadas à RT, como a disgeusia, mucosite oral e cárie por radiação, especialmente considerando o importante papel que a saliva apresenta na manutenção de um ambiente bucal saudável através da lubrificação, controle do pH por meio da neutralização de ácidos de

bactérias e alimentos e proteção e limpeza dos tecidos orais (Sciubba e Goldenberg, 2006; Vidotto et al., 2011; Ogama et al., 2012; Dawes et al., 2015; Esteves et al., 2020; Gouvêa Vasconcellos et al., 2020).

A partir dos resultados do perfil molecular das toxicidades orais agudas induzidas pela RT foi possível observar que parte das proteínas foram correlacionadas com mais de uma toxicidade, o que além de corroborar com padrão de sobreposição clínica das toxicidades orais induzidas pela RT, também demonstram a possibilidade destas proteínas serem classificadas como biomarcadores destas toxicidades e consequentemente tornando-as alvo para o futuro desenvolvimento de medidas de prevenção e tratamento das toxicidades.

A correlação entre disgeusia, xerostomia e MO além de corroborar com a teoria do agrupamento de sintomas orais, pode também ser associada ao seu impacto, através dos efeitos indiretos da RT, no desenvolvimento e progressão da RC (Gouvêa Vasconcelos et al., 2020). A CR é um dos efeitos colaterais crônicos mais comuns que ocorre usualmente nos primeiros 6 a 12 meses pós-RT e afeta cerca 29% destes pacientes (Moore et al., 2020). Apesar de bem reconhecida, estudos recentes revelam que o nível de conhecimento da CR é baixo entre médicos, dentistas e pacientes com CEC de boca e orofaringe submetidos à RT (Martins et al., 2021). A falta de conhecimento sobre a CR pode representar um impacto direto na forma como os pacientes cumprem as medidas para prevenção, tais como manutenção de higiene oral adequada e comparecimento nas consultas odontológicas de rotina, assim como o diagnóstico precoce por parte dos dentistas (Palmier et al., 2020; Martins et al., 2021). Ainda que a CR seja uma condição reconhecida na literatura, ainda não existem índices de classificação clínicos estabelecidos e validados que orientem o clínico no correto diagnóstico e tratamento da CR (Palmier et al., 2017). A presente tese propõe um índice de classificação clínico associado a medidas de tratamento por grau de classificação e evolução clínica da CR, contudo, a validação por meio de estudos clínicos prospectivos que confirmem a aplicabilidade dele se faz necessária.

Adicionalmente às dificuldades diagnósticas, resultados da presente tese demonstram de forma original o impacto da CR na morbidade de pacientes com CCP submetidos à RT, resultados revelam maior necessidade de consultas odontológicas especializadas, número elevado de restaurações com dentes apresentando necessidade de troca de restauração por até 7 vezes durante o período de acompanhamento, maior necessidade de exodontias pós-RT o que representou um impacto direto em uma incidência elevada de casos de ORN quando comparado com grupos controle e de pacientes edêntulos pré-RT e na necessidade de procedimentos cirúrgicos invasivos. Esses resultados corroboram com a literatura que demonstra uma longevidade baixa em 2 anos de restaurações realizadas pós-RT (Palmier et al., 2021) assim como o padrão agressivo desta condição levando à necessidade de extrações dentárias pós-RT (Kielbassa et al., 2006; Palmier et al., 2020) e o impacto negativo que a CR pode representar para esse grupo de pacientes (de Pauli Paglioni et al., 2020).

As novas perspectivas clínicas observadas através do agrupamento de sintomas das toxicidades orais induzidas pela RT e o impacto da CR na morbidade de pacientes CCP pós-RT corroboram com a atual necessidade de melhor entendimento não só do perfil de correlações clínicas, mas também do perfil molecular das toxicidades de forma a promover tratamentos individualizados de forma a reduzir as taxas de morbidade a longo prazo (Cohen et al., 2016).

4. CONCLUSÃO

Os resultados da presente tese de doutoramento demonstram novas perspectivas em relação ao padrão de desenvolvimento assim como o impacto que as toxicidades agudas induzidas pela RT podem representar no desenvolvimento e agravamento uma das outras na forma do recém descrito agrupamento de sintomas orais. Dessa forma, considerando que pacientes com CCP raramente apresentam um único sintoma oral, a compreensão e tratamento adequado do agrupamento de sintomas orais são de suma importância para a preservação da qualidade de vida dos sobreviventes do câncer. Adicionalmente, resultados também apresentam de forma original preditores proteômicos salivares de toxicidades orais agudas debilitantes induzidas por RT em pacientes com CEC oral e de orofaringe em estadio avançado. De forma interessante, oito biomarcadores foram associados a gravidade clínica de xerostomia e candidose oral e um biomarcador associado a disgeusia e candidose oral, trazendo evidências originais, em termos biológicos, para a existência de um agrupamento de sintomas e toxicidades orais resultantes da RT, apresentando, portanto, potencial de aprimoramento dos protocolos clínicos de suporte odontológico personalizados em Oncologia. Por fim, descobertas quanto aos padrões de agrupamento de sintomas orais suportam a teoria de que o principal fator etiológico da CR seja relacionado aos efeitos indiretos da RT, e através dos resultados observados foi possível propor uma nova metodologia para guiar os dentistas no diagnóstico precoce e tratamento adequado da CR de forma a minimizar seu potencial impacto nos desfechos de morbidade em pacientes CCP submetidos à RT.

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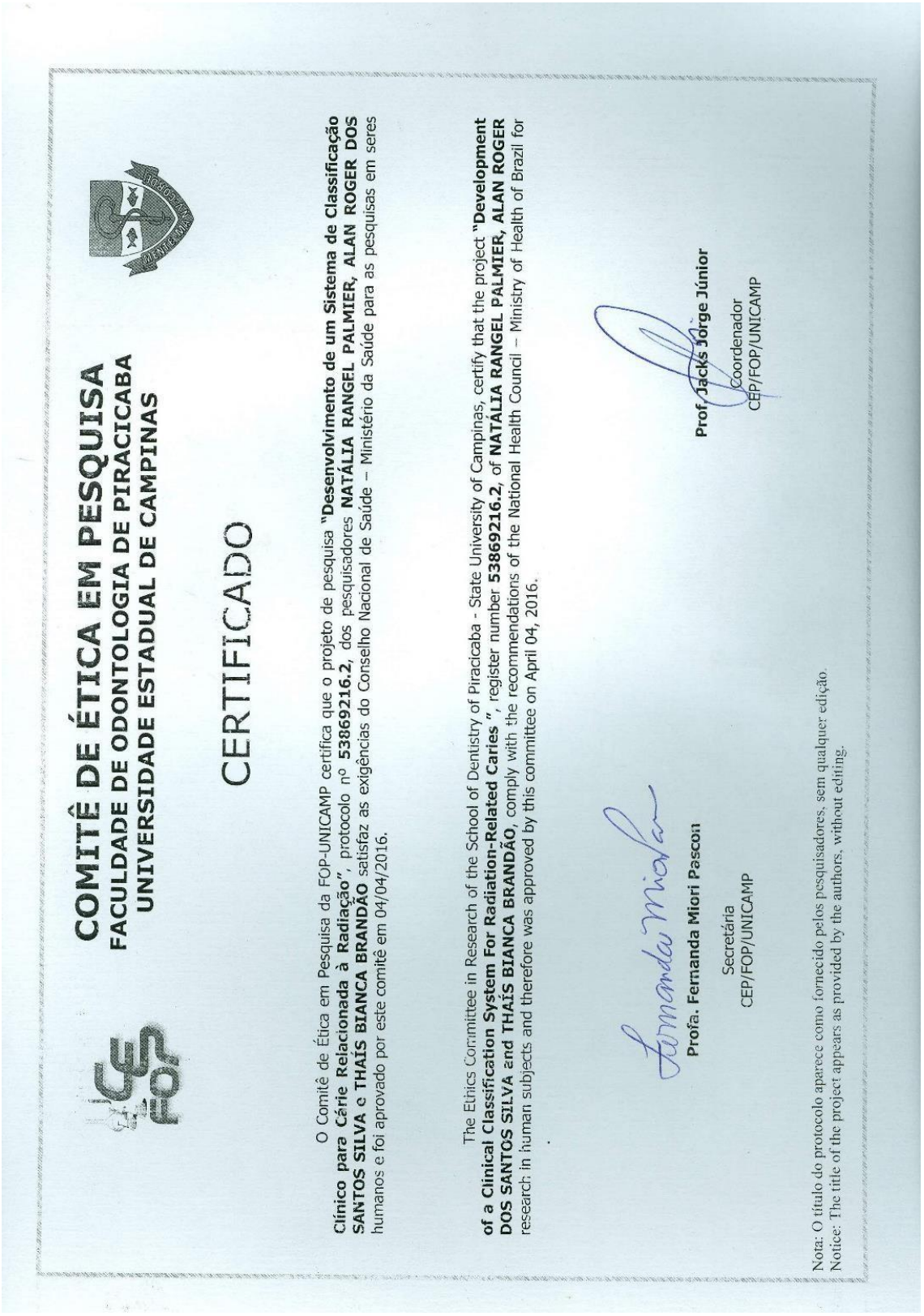
ANEXOS

Anexo 1 – Aprovações do Comitê de Ética em Pesquisa



COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Desenvolvimento de um Sistema de Classificação Clínico para Cárie Relacionada à Radiação"**, protocolo nº **53869216.2**, dos pesquisadores **NATÁLIA RANGEL PALMIER, ALAN ROGER DOS SANTOS SILVA e THAÍS BIANCA BRANDÃO** satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 04/04/2016.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Development of a Clinical Classification System For Radiation-Related Caries "**, register number **53869216.2**, of **NATÁLIA RANGEL PALMIER, ALAN ROGER DOS SANTOS SILVA and THAÍS BIANCA BRANDÃO**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on April 04, 2016.

Profa. Fernanda Miori Pascon
Secretária
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

USP - FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO - FMUSP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise retrospectiva de proteínas salivares candidatas a marcadores de toxicidade bucal associada à radioterapia

Pesquisador: Ana Carolina Prado Ribeiro e Silva

Área Temática:

Versão: 1

CAAE: 88742918.1.0000.0065

Instituição Proponente: FUNDACAO FACULDADE DE MEDICINA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.647.153

Apresentação do Projeto:

Trata-se de projeto original visando identificar por espectrometria de massas, as proteinas da saliva de paciente submetidos a radioterapia local ou regional e assim predizer de forma adequada eventuais complicações decorrentes doprocesso. O estudo é transversal com 50 pacientes e visa apenas a coleta de saliva e a recuperação dos dados evolutivos nos pacientes sob tratamento.

Objetivo da Pesquisa:

Análise retrospectiva de proteínas salivares candidatas a marcadores de toxicidade bucal associada à radioterapia

Avaliação dos Riscos e Benefícios:

O projeto não tem aspectos éticos pertinentes a intervenções, sendo apenas um estudo observacional e de coleta de saliva apenas.

Comentários e Considerações sobre a Pesquisa:

O projeto tem uma abordagem metodologica de espectrometria de massas para a determinação de proteínas, que acredito vá dar pouco resultado prático devido a sua baixa disponibilidade. Mais interessante seria determinar algumas proteínas inflamatórias locais com proteína C reativa ou outros marcadores imunolgicos em saliva. Estes comentários são apenas cientificos e não afetam os aspectos éticos do trabalho.

Endereço: DOUTOR ARNALDO 251 21º andar sala 36
Bairro: PACAEMBU **CEP:** 01.246-903
UF: SP **Município:** SAO PAULO
Telefone: (11)3893-4401 **E-mail:** cep.fm@usp.br

USP - FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO - FMUSP

Considerações sobre os Termos de apresentação obrigatória:

O TCLE é adequado e explicito tanto quanto ao projeto como a atuação dos pesquisador

Recomendações:

Aprovação

Conclusões ou Pendências e Lista de Inadequações:

Nenhuma

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1112152.pdf	26/04/2018 18:04:41		Aceito
Outros	AdrianaPaesLeme.pdf	26/04/2018 18:04:17	Natália Rangel Palmier	Aceito
Outros	AlanSantosSilva.pdf	26/04/2018 18:04:03	Natália Rangel Palmier	Aceito
Declaração de Pesquisadores	Formularioassinado.pdf	26/04/2018 18:03:47	Natália Rangel Palmier	Aceito
Declaração de Instituição e Infraestrutura	NucleoPesquisa.pdf	26/04/2018 18:03:21	Natália Rangel Palmier	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	3TCLE.pdf	26/04/2018 18:02:48	Natália Rangel Palmier	Aceito
Projeto Detalhado / Brochura Investigador	2Projeto.pdf	26/04/2018 17:53:23	Natália Rangel Palmier	Aceito
Folha de Rosto	1Folhaderosto.pdf	26/04/2018 17:50:34	Natália Rangel Palmier	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

São Paulo, 29 de março de 2018

Registro: NP 1300/18

Ref. Solicitação de execução de estudo: **"Análise retrospectiva de proteínas salivares candidatas a marcadores de toxicidade bucal associada à radioterapia"**

Pesquisador Responsável: Ana Carolina Prado Ribeiro
Pesquisador Executante: Natália Rangel Palmier

CONSIDERAÇÕES

Tumores malignos da cavidade oral são o sexto tipo de neoplasia mais prevalente no mundo, sendo 90% diagnosticados como carcinoma espinocelular (CEC). Anualmente são diagnosticados mais de 550.000 novos casos e aproximadamente 300.000 óbitos em função da doença no mundo (Haddad et al., 2008; Marron et al., 2009, Chi et al., 2015). No Brasil, estimam-se 15.490 novos casos de CEC para o ano de 2017 (INCA, 2016).
Objetivo deste estudo atualmente há uma grande busca por tratamentos personalizados de acordo com o perfil molecular das doenças com o intuito de aumentar a eficiência do tratamento e diminuir as taxas de toxicidade. O presente estudo pretende caracterizar o perfil proteômico salivar de pacientes tratados do câncer de cabeça e pescoço (CCP) a fim de correlacionar os resultados do proteoma salivar com o risco de desenvolvimento de toxicidades orais da radioterapia (RT) e com a gravidade clínica destas complicações.
Por se tratar de um estudo retrospectivo, a coleta de saliva foi realizada de forma independente a idealização deste projeto de pesquisa; sendo inicialmente executada para o desenvolvimento do estudo intitulado "Análise molecular de saliva humana aplicada a identificação de "bioperfis" associados ao câncer de boca" aprovado no Núcleo de Pesquisa do ICESP sob o número 509/14 e no Comitê de Ética em pesquisa sob o parecer número 675.999.

RESPONSABILIDADES DO PESQUISADOR
Antes do início das atividades relacionadas ao estudo:

- Apresentar a aprovação do Comitê de Ética em Pesquisa da FMUSP para execução no ICESP conforme a Resolução 466/12 do Conselho Nacional de Saúde, Ministério da Saúde;
- Enviar anualmente o *status* ou relatório do estudo. Projetos sem informações por mais de 1 ano serão cancelados automaticamente e impedirá a submissão de novos projetos pelos investigadores;
- Enviar resultados do projeto (publicações, defesa de tese, apresentação em congressos e outros);

Informamos que sua solicitação foi DEFERIDA

Atenciosamente,

Prof. Dr. Paulo M. Hoff
Diretor Geral

Versão 2.0 28 de Dezembro de 2017.

Prof. Dr. Paulo M. Hoff
Diretor Geral

UNICAMP - FACULDADE DE ODONTOLOGIA DE PIRACICABA DA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Proteínas salivares candidatas a marcadores de toxicidade bucal associada à radioterapia

Pesquisador: Natália Rangel Palmier

Área Temática:

Versão: 4

CAAE: 90210418.1.0000.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.786.746

Apresentação do Projeto:

Transcrição editada do conteúdo do registro do protocolo e dos arquivos anexados à Plataforma Brasil

METODOLOGIA: Trata-se de estudo clínico observacional, longitudinal, com 12 meses de duração por paciente, que envolverá 50 indivíduos, pacientes do Serviço de Odontologia Oncológica e Oncologia Clínica do ICESP diagnosticados com CEC de cavidade oral (C02; C03; C04; C05; C06), em estadio clínico III ou IV de acordo com o sistema TNM (International Union Against Cancer), que foram submetidos à adequação odontológica antes do início do tratamento oncológico, que iniciarão RT (com planejamento em base tridimensional conformacional) como forma de tratamento oncológico isoladamente ou em combinação com cirurgia ou QT e que estejam se alimentado exclusivamente por via oral.

Os mesmos passarão por avaliação clínica/gradação de disgeusia, disfagia, mucosite, e xerostomia, para eventual coleta de amostras de saliva total (diariamente durante a radioterapia e nas consultas de acompanhamento odontológico em D+30 e D+90, D+180, D+270 e D+360). Serão avaliadas ainda as CRR e a ORN por meio clínico e imagenológico (estas a partir de material de arquivo). As amostras serão processadas para análise do perfil proteômico salivar.

Delineamento da pesquisa:

Critérios de inclusão: Para a realização desse estudo serão selecionados aleatoriamente 50 pacientes diagnosticados com CEC de cavidade oral (C02; C03; C04; C05; C06), em estadio clínico

UNICAMP - FACULDADE DE ODONTOLOGIA DE PIRACICABA DA

Continuação do Parecer: 2.786.746

Outros	4ComentariosResposta.pdf	03/07/2018 15:05:10	Natália Rangel Palmier	Aceito
Projeto Detalhado / Brochura Investigador	2ProjetoResposta.pdf	03/07/2018 15:04:48	Natália Rangel Palmier	Aceito
Folha de Rosto	1Folhaderosto.pdf	03/07/2018 15:04:24	Natália Rangel Palmier	Aceito
Outros	CEPcompleto.pdf	23/05/2018 16:03:31	Leny Cecilia Faro Pereira	Aceito
Outros	61Anexo2.pdf	23/05/2018 15:15:10	Natália Rangel Palmier	Aceito
Outros	61Anexo1.pdf	23/05/2018 15:14:16	Natália Rangel Palmier	Aceito
Outros	55AutArq.pdf	23/05/2018 15:13:59	Natália Rangel Palmier	Aceito
Outros	54AltInfra.pdf	23/05/2018 15:13:45	Natália Rangel Palmier	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	4TA.pdf	23/05/2018 15:12:45	Natália Rangel Palmier	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PIRACICABA, 26 de Julho de 2018

Assinado por:
jacks jorge junior
(Coordenador)

INSTITUTO DO CÂNCER DO ESTADO DE SÃO PAULO
OCTAVIO FRIAS DE OLIVEIRA

FUNDAÇÃO FACULDADE DE MEDICINA

São Paulo, 17 de julho de 2018.

Registro: NP 1318/18

Ref. Solicitação de execução de estudo: **PROTEÍNAS SALIVARES CANDIDATAS A MARCADORES DE TOXICIDADE BUCAL ASSOCIADA À RADIOTERAPIA.**

Pesquisador Responsável: Dra. Ana Carolina Prado Ribeiro
Pesquisador Executante: Dra. Natália Rangel Palmier

CONSIDERAÇÕES

Trata-se de um estudo prospectivo coma finalidade de obtenção de título acadêmico.
O projeto visa realizar uma pesquisa clínico/molecular para categorizar o perfil proteômico salivar de pacientes tratados com câncer de cabeça e pescoço (CCP) a fim de correlacionar os resultados do proteoma salivar com o risco de desenvolvimento de toxicidades orais da radioterapia (RT) e com a gravidade clínica destas complicações.
Serão selecionados pacientes com CEC e que serão submetidos à RT nos Serviços de Odontologia Oncológica e Oncologia Clínica do ICesp.

RESPONSABILIDADES DO PESQUISADOR
Antes do início das atividades relacionadas ao estudo:

- Apresentar a aprovação do Comitê de Ética em Pesquisa da FMUSP para execução no ICESP conforme a Resolução 466/12 do Conselho Nacional de Saúde, Ministério da Saúde;
- Enviar anualmente o *status* ou relatório do estudo. Projetos sem informações por mais de 1 ano serão cancelados automaticamente e impedirá a submissão de novos projetos pelos investigadores;
- Enviar resultados do projeto (publicações, defesa de tese, apresentação em congressos e outros);

Informamos que sua solicitação foi DEFERIDA

Atenciosamente,

Prof. Dr. Paulo M. Hoff
Diretor Geral

Prof. Dr. Paulo M. Hoff
Diretor Geral
ICESP

Anexo 2 – Aprovações para uso de dados publicados.

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Expected presentation date	Aug 2021

Additional Data

Anexo 3 – Certificado de verificação de similaridade

Tese Doutorado

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Publicação

Excluir citações	Desligado	Excluir correspondências	Desligado
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