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JESSICA MONTENEGRO FONSECA

EFEITO DA RADIOTERAPIA SOBRE O COMPLEXO DENTINO-PULPAR

RADIOTHERAPY EFFECT ON THE DENTIN-PULP COMPLEX

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Tese apresentada à Faculdade de Odontologia de Piracicaba
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Orientador: Prof. Dr. Mário Fernando de Góes
Coorientador: Prof. Dr. Alan Roger dos Santos Silva

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ORIENTADA PELO PROF. DR. MÁRIO FERNANDO DE GÓES E
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RESUMO

A cárie de radiação (CR) é uma toxidade crônica que afeta cerca de 30% dos pacientes submetidos à radioterapia na região de cabeça e pescoço (RDTCP), podendo gerar destruição generalizada dos dentes e consequente dificuldade na mastigação, infecção crônica e risco aumentado para o desenvolvimento da osteorradionecrose. Permanece incerto se o efeito da radiação age diretamente sobre o complexo dentino-pulpar (CDP). Com o objetivo de melhor compreender as mudanças que ocorrem no CDP em resposta ao desenvolvimento da cárie após RDTCP, esta tese de doutorado se propôs a realizar uma revisão sistemática que incluiu estudos *in vitro* e *in vivo* que avaliaram efeitos da RDTCP na junção amelodentinária (JAD) e testou a imunoexpressão das proteínas colágeno tipo I (COL I), sialoproteína óssea (SPO) e proteína morfogenética óssea 4 (PMO4), em dentes irradiados *in vivo* e dentes não irradiados de pacientes com câncer de cabeça e pescoço. 154 estudos foram encontrados na revisão sitemática, dos quais 8 atenderam aos critérios de inclusão. A maior parte dos estudos incluídos (5) foram clasificados como alta qualidade geral de evidencia. Somente dois estudos não demonstraram alterações na JDE após HNRT, enquanto os outros seis artigos descreveram várias alterações orgânicas e inorgânicas na JDE de amostras de dentes irradiados. Para o estudo experimental 22 dentes desmineralizados (grupo irradiado *in vivo* n=11, versus grupo controle n=11), extraídos de pacientes com câncer de cabeça e pescoço tratados por radioterapia foram analisados por meio de microscopia óptica convencional e imunoistoquímica a fim de investigar a preservação da micromorfologia dentinária e os padrões de imunolocalização das proteínas: COL I, SPO e PMO4. Não foram detectadas diferenças significativas entre os grupos (grupo irradiado *in vivo* n=11, versus grupo controle n=11) na micromorfologia do CDP que possa ser atribuída ao impacto direto da radioterapia. Além disso, os padrões de expressão imunoistoquímica e a imunolocalização das proteínas estudadas não diferiram entre amostras irradiadas e controle para COL I, BSP e BMP4.

Apesar da maior parte dos artigos incluídos na revisão sistemática terem identificado alterações orgânicas e inorgânicas na JAD de amostras de dentes irradiados, o estudo experimental incluído nesta tese sugere que a RDTCP seja incapaz de alterar diretamente a composição do CDP, predispondo os pacientes com câncer de cabeça e pescoço ao desenvolvimento da CR.

Palavras – chave: câncer oral, cárie de radiação, imunoistoquímica, revisão sistemática.

ABSTRACT

Radiation caries (RC) is a chronic toxicity that affects about 30% of patients undergoing head and neck radiotherapy (HNRT) and can lead to widespread tooth destruction and subsequent difficulty in chewing, chronic infection, and increased risk for the development of osteoradionecrosis. It is unclear whether the effect of radiation directly affects the dentin-pulp complex (DPC). In order to better understand the changes that occur in DPC in response to the development of caries after HNRT, this doctoral thesis proposed a systematic review that included in vitro and in vivo studies that evaluated the effects of HNRT on the dentin-enamel junction (DEJ) and tested the immunoexpression of collagen type I (COL I), Bone sialoprotein (BSP) and bone morphogenetic protein 4 (BMP4) in the in vivo irradiated teeth and non-irradiated teeth of patients with head and neck cancer. 154 studies were found in the systematic review, of which 8 met the inclusion criteria. Most (5) of the included studies were classified as high overall quality of evidence. Only two studies showed no changes in the DEJ after HNRT, while the other six articles described a variety of organic and inorganic changes in the DEJ of irradiated tooth samples. 22 demineralized teeth (in vivo irradiated group n=11, versus control group n=11) extracted from head and neck cancer patients treated by radiotherapy were analyzed by conventional optical microscopy and immunohistochemical to investigate the preservation of dentin micromorphology and immunolocalization patterns of the proteins: COL I, BSP and BMP4. No significant differences were detected between the groups (in vivo irradiated group n=11, versus control group n=11) in DPC micromorphology, which can be due to the direct impact of radiotherapy. In addition, the immunohistochemical expression and immunolocalization patterns of the proteins studied did not differ between irradiated and control samples for COL I, BSP, and BMP4. Although most of the articles included in the systematic review identified organic and inorganic changes in the DEJ of irradiated tooth samples, the experimental study included in this thesis suggests that HNRT is

unable to directly alter the structure of DPC, predisposing head and neck cancer patients to the development of CR.

Key-words: Oral cancer, radiation caries, immunohistochemistry, systematic review.

SUMÁRIO

1 INTRODUÇÃO

O câncer prevalece como um grande problema de saúde pública mundialmente. Calcula-se 18,1 milhões de novos casos (17,0 milhões excluindo câncer de pele não melanoma) e 9,6 milhões de mortes por câncer (9,5 milhões excluindo câncer de pele não melanoma) segundo a mais recente estimativa mundial (Bray *et al*., 2018). No Brasil, para cada ano do triênio 2020-2022 estão previstos 625 mil casos novos de câncer (450 mil, excluindo os casos de câncer de pele não melanoma). Os tumores de lábios, cavidade oral, faringe, laringe, cavidade nasal, glândulas salivares e tireoide, estão agrupados como cânceres de cabeça e pescoço (CCP) e representam hoje a uma das maiores incidências e mortalidades por câncer em homens brasileiros (INCA, 2020). O carcinoma escamocelular (CEC) é a variante histológica mais comum de tumor maligno de cabeça e pescoço e é classificado como a oitava principal causa de câncer mundialmente, apresentando alta prevalência e morbidade, com 300.000 novos casos e 145.000 mortes por ano (Bray *et al.,* 2018).

O perfil clínico dos pacientes acometidos pelo CCP, é representado principalmente por indivíduos do gênero masculino, acima de 60 anos, tabagistas e etilistas de longa data e consumo excessivo diário (Pelucchi *et al*., 2008; Neville *et al*., 2016). Outro grupo possível, foi identificado recentemente e é integrado por pacientes mais jovens (menos de 40 anos de idade) e sem fatores de risco comumente associados ao câncer de cabeça e pescoço.

Além do consumo de tabaco e bebidas alcóolicas, a infecção pelo papiloma vírus humano (HPV), principalmente o genótipo 16 e 18, tem sido citado como fator de risco para o CEC, específicamente localizado em orofaringe (tonsilas palatinas e linguais) (Scully e Felix, 2006; Sloan *et al*., 2017).

Adicionalmente, é importante esclarecer que a maior parte destes tumores é diagnosticada tardiamente, em estadiamentos clínicos avançados, levando a uma maior dificuldade no tratamento e tornando a taxa de sobrevida menor que 50% em cinco anos. Os protocolos de tratamento do CCP atualmente incluem cirurgia, quimioterapia (QT) e radioterapia (RDT) isoladas ou, mais frequentemente, combinadas (Huang e O´Sullivan, 2013; Marta *et al.,* 2015).

A RDT é caracterizada pelo uso de raios ionizantes que afetam as células cancerígenas, produzindo espécies reativas de oxigênio que interagem, por sua vez, com o

DNA, o RNA e as enzimas celulares de forma a desorganizar seus nucleotídeos e danificar seu material genético de modo que não consegue ser reparado pelos mecanismos regulatórios de células malignas, gerando apoptose, morte celular e diminuição da capacidade proliferativa do tumor. No entanto, apesar dos benefícios desta terapia no tratamento das neoplasias de cabeça e pescoço, a mesma acaba afetando as células não tumorais além das neoplasias, levando ao surgimento dos efeitos colaterais ou toxicidades (Huber e Terezhalmy, 2003; Vissink *et al*., 2003; Kielbassa *et al.,* 2006).

Tais complicações ocorrem em quase 90% dos pacientes acometidos pelo CCP e sua gravidade depende da modalidade de RDT, dose diária de radiação, abrangência do campo de radiação, além das condições individuais de cada paciente (Rolim; Costa; Ramalho, 2011).

A dose de RDT é expressa pela quantidade de energia absorvida pelo tecido irradiado. A unidade que padroniza a dose absorvida pelo tecido é conhecida como Gray (Gy = 1J/Kg), (Huber; Terezhalmy, 2003; Rolim; Costa; Ramalho, 2011). Com o objetivo de minimizar toxicidades ao paciente, a RDT em cabeça e pescoço costuma ser administrada ao longo de 5 a 7 semanas com frações diárias de 2 Gy, 5 vezes por semana com intervalos de dois dias (aos finais de semana) a fim de que os tecidos sadios adjacentes ao tumor possam se recuperar (Vissink *et al*., 2003; Kielbassa *et a*l., 2006).

A QT também tem sido benéfica no tratamento dos CCPs e, quando associada à RDT (quimiorradioterapia - QRDT), tende a elevar as chances de sucesso terapêutico, podendo ser aplicada antes (indução ou neoadjuvante), concomitantemente ou após o tratamento radioterápico (adjuvante). Desta forma, a QRDT atua reduzindo o tamanho do tumor primário, potencializando a atividade citotóxica do tratamento e reduzindo o risco de metástases (Bucheler *et al*., 2012). Apesar disso, esta modalidade pode intensificar as toxicidades bucais ao paciente, ocasionando dor intensa, necessidade de interrupção dos protocolos de tratamento oncológico, redução nas taxas de sucesso do tratamento e aumento dos custos do tratamento oncológico (Seiwert *et al*., 2007).

O tratamento radioterápico associado ou não à QT para os CCPs promove reações aos tecidos sadios adjacentes ao campo irradiado, incluindo os dentes. As toxicidades mais comuns da RDT incluem mucosite, radiodermite, disgeusia, disfagia, hipossalivação, infecções oportunistas, osteorradionecrose, cárie de radiação (CR), trismo, entre outros (Vissink *et al*., 2003; Lobo; Martins, 2009). Estas toxicidades podem se manifestar logo no início do tratamento (agudas) ou após meses da conclusão da RDT (crônicas), podendo estas últimas afetar de forma permanente aos pacientes (Vissink *et al.,* 2003).

Neste contexto, a CR é uma toxicidade crônica que surge tardiamente

(aproximadamente 12 meses da conclusão da RDT) e afeta cerca de 30% dos pacientes tratados por RDT em cabeça e pescoço (Hong *et al*., 2018). Interessantemente, os padrões clínicos de início, desenvolvimento e progressão da CR diferem da cárie convencional (CC) (Hong *et al*., 2018). Inicialmente, a CR afeta as áreas cervical e incisal dos dentes anteriores (Kielbassa *et al*., 2006), gerando perda do brilho e translucidez do esmalte da coroa dental seguida por alteração da coloração da coroa que passa a assumir tom esbranquiçado e, se não tratado, progride para marrom escuro em associação a trincas e fraturas nas superfícies livres de esmalte (Palmier *et al*., 2017).

Posteriormente, o esmalte sofre o fenômeno de "delaminação" caracterizado pelo desprendimento de grandes áreas de esmalte que acaba expondo a dentina (tecido mais frágil) ao ambiente bucal dos pacientes irradiados, marcado por alto potencial cariogênico e que permitirá rápida deterioração das coroas dentárias que acabam amputadas da raiz, expondo o canal radicular ao meio bucal, tornando-se fácil acesso para entrada de microrganismos nos tecidos periapicais (Palmier *et al*., 2018; Schweyen *et al*., 2012). Caso não seja instituído um protocolo de prevenção ou tratamento à CR, pode ocorrer destruição generalizada dos dentes de pacientes oncológicos, representando um grande risco para o desenvolvimento de focos de infecção crônica e, consequentemente, de osteorradionecrose (Vissink *et al*., 2003; Silva *et al*., 2009). Geralmente, a deterioração dos tecidos dentais relacionada à CR não está associada a queixa de dor espontânea, ou estimulada, por parte dos pacientes (Schweyen *et al*., 2012).

Cogita-se sobre o efeito da radiação sob a estrutura dentária, favorecendo a delaminação do esmalte e o início e progressão da CR. Alguns estudos sugerem danos radiogênicos diretos à dentina e ao esmalte que podem levar à CR (Franzel *et al*., 2009; McGuire *et al*., 2014a; Reed *et al*., 2015). Por outro lado, alguns autores associam o aumento do risco de CR aos efeitos indiretos da RDT na estrutura dos dentes (Faria *et al*., 2014; Deng *et al*., 2015; Gomes-Silva *et al*., 2017), como os causados por hipossalivação, alterações da microbiota oral, propriedades de auto-limpeza prejudicadas, entre outros fatores que atuam em sinergia para formar um conjunto de sintomas orais que predispõem os dentes ao desenvolvimento deste tipo de cárie (Kielbassa *et al*., 2006; Palmier *et al*., 2017).

Estudos sugerem que existe uma região de maior fragilidade entre o esmalte e a dentina, que abrange a junção amelodentinária (JAD), podendo haver uma perda de resistência mecânica caso seja submetida a tensões e impactos cíclicos repetidos. Isso geraria a delaminação do esmalte, deixando a dentina exposta vulnerável à deterioração subsequente (Springer *et al.*, 2005; Mcguire *et al*., 2014a). Adicionalmente, estudos *in vitro* mostram que os danos radiogênicos diretos à dentição também podem afetar a estabilidade da JAD, levando

à delaminação do esmalte e à progressão da CR (Lee et al, 2014; Seyedmahmoud et al., 2018). Além disso o complexo dentino-pulpar representa uma grande e importante parte da estrutura dentária (Smith *et al*., 2012), onde componentes orgânicos colagenosos e não colagenosos, como sialoproteínas, fosfoproteínas e proteoglicanos, desempenham um papel importante na sua integridade mecânica e estrutural (Bertassoni *et al*., 2014; Bertassoni *et al*., 2015). Nesse contexto, algumas proteínas são essenciais à compreensão dos eventos moleculares que ocorrem no complexo dentino-pulpar em resposta ao desenvolvimento da doença cárie.

A título de exemplo, o colágeno tipo I (COL I) é a proteína mais abundante presente em tecidos mineralizados, além do esmalte, funcionando com um guia na progressão da mineralização em seu longo eixo. Também é o constituinte mais importante da matriz extracelular do tecido conjuntivo da polpa dentária (Karjalainen *et al.* 1986, Hilmann & Geurtsen 1997), e possui papel decisivo no seu processo de mineralização (Garcia *et al*., 2003). A SPO é uma proteína sintetizada por células precursoras dos odontoblastos, pelos odontoblastos propriamente ditos e por algumas células pulpares, e é responsável pela formação de 5 a 8% da matriz extracelular da dentina (Garcia *et al.,* 2003; Chibinski *et al*., 2014).

Além dessas, a PMO4, é um fator de crescimento relacionado ao desenvolvimento dentário e atua juntamente ao COL I, na formação da matriz dentinária. A maioria dos estudos mostram que sua localização mais específica se encontra na polpa (Hosoya *et al.,* 2008; Gluhak-Heinrich *et al*., 2010).

Em virtude dos aspectos mencionados este estudo teve como objetivo testar a hipótese de que a RDT de cabeça e pescoço afeta a imunoexpressão do COL I, SPO e PMO4, levando a alterações micromorfológicas detectáveis no complexo dentino-pulpar e contribuindo para o desenvolvimento da CR. Adicionalmente, será apresentada uma revisão sistemática com o propósito de avaliar se o dano radiogênico direto pode ser considerado um fator de risco independente para danificar as propiedades físico-químicas da estrutura dentária, especificamente na região da JAD.

2 ARTIGOS

2.1 Artigo: The impact of head and neck radiotherapy on the dentine-enamel junction: a systematic review

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Title: The impact of head and neck radiotherapy on the dentine-enamel junction: a systematic review

Running title: Radiotherapy impact on the dentine-enamel junction

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Abstract

Background: Radiotherapy is widely used in contemporary head and neck cancer treatment protocols. The ability of head and neck radiotherapy (HNRT) to cause direct radiogenic destruction to the teeth is one of the most controversial topics in the field of oral oncology. Therefore, this systematic review aimed to investigate ionising radiation as an independent factor for physical and chemical changes on the dentine-enamel junction (DEJ), a pivotal dental topography for the onset and progression of radiation-related caries (RRC) and enamel delamination. *Methods:* Systematic searches were conducted on three databases: Scopus, MEDLINE (Via PubMed) and Embase (Elsevier). Laboratory studies evaluating the effects of simulated or *in vivo* HNRT on the DEJ were included. The GRADE tool adapted for *in vitro* studies was used to assess the methodological quality. *Results:* Of the 154 initially selected studies, eight met the inclusion criteria, from which five studies were graded as high quality of evidence, two studies were graded as moderate quality and one as low quality. Two studies did not demonstrate DEJ alterations following HNRT while the other six articles described several organic and inorganic changes in the DEJ of irradiated teeth samples. These radiogenic events were mostly detected through micro and nanoindentation, Raman micro-spectroscopy, confocal microscopy, Western blotting and optical coherence tomography. *Conclusion:* HNRT may have a negative impact on the physical and chemical aspects of the DEJ, predisposing cancer patients to RRC and enamel delamination.

Keywords: Cancer; Radiotherapy, Radiation-Related Caries, Dentin-enamel junction, Systematic Review.

Introduction

Head and neck cancer (HNC) is an important public health problem throughout the world and covers approximately 10% of all malignant tumours in developed countries, occupying the sixth place among the most common malignancies. Treatment protocols often involve the combination of surgery, chemotherapy and head and neck radiotherapy (HNRT). Although considered highly effective in the locoregional control of cancer, HNRT results in a myriad of acute and chronic toxicities to non-targeted tissues including oral mucositis, hyposalivation, oral opportunistic infections, trismus, radiation-related caries (RRC) and osteoradionecrosis, among others (1,2).

RRC is one of the most significant oral toxicities of head and neck radiotherapy (HNRT), which affects up to 25% of all cancer patients subjected to radiation therapy (3). The potential of ionising radiation to directly cause harmful effects on tooth structure, favouring enamel delamination and RRC onset and progression, is highly controversial. In this context, many studies have suggested direct radiogenic damage to dentine and enamel that could lead to RRC (4-6). Conversely, other studies have linked the increased risk of RRC to the indirect effects of radiation therapy on the structure of the teeth (7-9), such as those caused by hyposalivation, oral microbiota alterations, impaired self-cleaning properties, poor oral health status of HNC patients, increased dietary intake of carbohydrates and insufficient fluoride exposure, which act in synergy to form a cluster of oral symptoms that predisposes the teeth to caries onset and rapid progression (10).

RRC lesions do not follow the conventional caries patterns of clinical development; instead, there is an initial brownish discolouration of non-cavitated enamel surfaces and the cervical region of the teeth, incisal caries and enamel wear on molar cusps. When not diagnosed and promptly treated, RRC progresses as generalised cervical caries, enamel craze lines and cracks, enamel delamination and crown amputation, leading to diffuse dental destruction in only a few months (10). However, RRC and conventional caries are undistinguished by microscopic patterns of progression and dentine reactions to their progression (11).

Delamination is a type of failure mode for composite materials including the dentine-enamel junction (DEJ). Repeated cyclic stresses and impact can cause enamel delamination due to a biomechanical failure of dentine and inner enamel sites since they symmetrically span the DEJ, with a

significant loss of mechanical toughness, leaving the exposed dentine vulnerable to subsequent decay (4,12). In addition, previously published *in vitro* studies (8,13) have suggested that direct radiogenic damage to the dentition might also impact the stability of the DEJ, leading to enamel delamination and RRC progression.

The enamel organic matrix is mainly located in inner sites, the DEJ acts in synergy to preserve the adhesion between the enamel layer and the underlying dentine, dissipating mechanical stress between both hard-dental tissues and inhibiting further crack propagation into dentine (4,14). Clinical research suggests that the mechanical properties of the enamel are negatively affected by HNRT; however, no consensus has been established in the literature concerning the ability of ionising radiation to be directly injurious to the DEJ, increasing the risk for enamel delamination and RRC progression (15-17).

Despite controversial results regarding radiation-related damage to the DEJ microstructure in HNC patients (4,10,18), many studies aimed to evaluate that enzymatic expression favours RRC progression (9,15,19,20). Therefore, this systematic review aimed to evaluate if HNRT should be considered an independent risk factor to damage the physicochemical properties of the DEJ in cancer patients.

Methods

Protocol and registration

This systematic review was developed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Checklist (21). The main methodological data were previously registered at the International Prospective Register of Systematic Reviews (http://www.crd.york.ac.uk/PROSPERO/) and received the protocol number CRD42018087404.

Eligibility Criteria

The present study aimed to investigate the following question: “Does ionising radiation induce damage to the micromorphology properties of the DEJ contributing to caries progression?”.

To elucidate the mentioned clinical query, our search was based on *in vitro* or *in vivo* studies

that analysed human teeth before and after HNRT. As established by the PRISMA guidelines, the PICO framework was designed, as follows:

- Population: Head and neck adult cancer patients
- Interventions: Radiation doses from 30 to 70 Grays (Gy)
- Comparison: Irradiated teeth versus non-irradiated human teeth
- Primary outcome: To evaluate the micromorphology property changes of DEJ
- Secondary outcome: Metalloproteinases related to caries progression
- Exclusion criteria: Animal studies; studies designed to evaluate the effects of radiotherapy on independent tooth tissues (enamel, dentin, pulp and cementum) structure; total radiation doses lower than 30 Gy; comprehensive reviews, editor letters, personal opinions, book chapters, conference abstracts and patents..

Search Strategy

Electronic searches were carried out on Scopus, MEDLINE/PubMed and Embase (Elsevier) using the following strategies (adapted for each database): ('enamel-dentine junction' OR 'dentine-enamel junction') AND ('radiation therapy' OR 'radiation' OR 'ionising radiation exposure' OR 'ionising radiation' OR 'radiation dose' OR 'radiotherapy'). Articles were searched until the 19th of March, 2018. In addition, the reference lists of the selected articles were hand screened to identify potentially relevant studies that could have been missed during initial electronic database searches.

Study selection

The study selection was completed in two phases. In phase one, two authors (JMF and ARSS) independently reviewed the titles and abstracts of all the references. They selected articles that met the inclusion criteria based on their titles and abstracts. In cases of disagreement, a third author (CCMT) intervened. Studies that clearly failed the inclusion criteria were discarded, and those whose abstracts did not contain all the information needed were entered into phase two. In phase two, full articles were read to determine the research in which *in vitro* or *in vivo* studies evaluated the effects of HNRT on the DEJ. Two authors (JMF and ARSS) independently participated in phase two. The final selection was

always based on the full text. The reference lists for all the included articles were critically assessed by JMF for the new articles.

Data extraction

One author (JMF) collected the following information from the included articles: author/year, country, study design, sample size, tooth, control, assays, radiation dose, radiotherapy modality, storage solution, time storage, results and main conclusions (Table 1). A second author (ARSS) cross-checked the collected information and confirmed its veracity. Any disagreement was resolved by a discussion and mutual agreement. Figure 1 shows the search strategy of the selected studies obtained in the reviewing process.

Risk of bias in individual studies

The risk of bias in the individual studies was assessed in accordance with the GRADE tool (22) for *in vitro* studies. The GRADE tool was adapted to *in vitro* studies, according to Pavan et al. (23), given that no specific quality assessment method was developed for this type of study. The domains below were considered.

For *in vitro* studies, two authors (JMF and ARSS) categorised the articles as 'high', 'moderate', 'low' or 'very low' overall quality of evidence, according to their analysis of each study. When they did not reach a consensus, a third author (CCMT) intervened to make a final decision.

Summary measures

Effects of *in vitro* and *in vivo* HNRT on the microhardness, nanomechanical properties, indentation pattern and the micromorphological patterns, as well as the expression or activation of matrix metalloproteinases in the DEJ, were the main evaluated outcomes.

Due to methodological differences within the studies, a meta-analysis was inappropriate, but a detailed qualitative synthesis of the results was conducted.

Results

Overall, 154 articles were identified from databases; after duplicate removal, 129 articles remained. A comprehensive evaluation of the titles and abstracts resulted in the exclusion of 106 articles. A full-text review was conducted on 23 articles retrieved, and an additional article was identified by reading the reference lists of these selected studies. This process led to the exclusion of 16 studies. In the end, eight articles were maintained for the final analyses (9,15,17,18,19,20,24,25). A flow diagram detailing the selection process of the study is shown in Figure 1.

Study characteristics

The studies were conducted in two different countries: Brazil (n=3) and the United States (n=5). All the studies were published in English from 2013 to 2018; they were divided according to the radiotherapy modality: *in vitro* (n=3), *in vivo* (n = 3) or both (n = 2). All the studies evaluated the effect of radiotherapy on the microhardness and nanomechanical properties, indentation patterns, microstructure and morphological alterations in the DEJ area, as well as the expression and activation of the matrix metalloproteinases. For these evaluations, techniques such as immunohistochemistry, micro-indentation, Raman spectroscopy, confocal microscopy, *in situ* zymography, finite elements, optical coherence tomography, proteomic and enzymatic analyses were used. A summary of the descriptive characteristics as well the main results and conclusions of the included studies are presented in Table 1.

Risk of bias in individual studies

When assessed with GRADE, as seen in Table 2, five studies were graded as high quality of evidence (9,17-20); two studies were graded as moderate quality (15,25) and one as low quality (24).

Two studies presented limitations as the sample size was not reported or the study had a limited size sample (15,24). Two presented serious indirectness because of the indirectness of methods and consequently of the outcomes (24,25).

In two other studies, there was no important information regarding the characterisation of the sample including the mean radiation dose delivered; it also failed to provide data concerning the time of storage and storage solution, resulting in imprecise outcomes (15,24). Generally, the studies

presented a high quality of evidence and, as a consequence, a low risk of bias.

Synthesis of results

One study demonstrated through a microhardness test that the inner enamel (50 μm from DEJ - inner enamel and 200 μm from DEJ - middle enamel) presented decreased microhardness values following *in vitro* and *in vivo* irradiation as compared to the control group; however, this reduction was significant only ($p < 0.05$) in the middle enamel (200 μm from DEJ). The percentage of 'clean micro-indentation' patterns was also significantly higher in all the enamel regions close to DEJ of the irradiated group when compared to the control samples (18).

Reed et al. (17) demonstrated that the elastic modulus of the enamel and dentin surrounding the DEJ was significantly increased ($p \leq 0.05$) following radiation. Based on the Raman spectroscopic analysis, there was a significant decrease in the protein to mineral ratio (2931/430 cm^{-1}) following radiation at all the sites tested except in the dentine tissue at 500 μm away from the DEJ, while the carbonate to phosphate ratio (1070/960 cm^{-1}) increased at 30 μm away from DEJ, in the enamel, and decreased at 500 μm away from the DEJ, in the dentine. Finally, the phosphate peak width as measured at 960 cm^{-1} significantly decreased at both 30 μm and 500 μm away from the DEJ, in the dentine tissue, following radiation (17).

Thiagarajan et al. (24), through the Finite Element (FE) method, observed in the DEJ an increase in the principal tensile stress of less than 10% between the control and *in vivo* irradiated teeth with no variation in the shear stress. In addition, a difference of 3.2 MPa in the principal tensile stress between the control model principal stress and the *in vivo* model principal stress was observed.

Optical coherence tomography (OCT) was used by Lee et al. (25) to visualise the morphological characteristics of the caries lesions formed at the DEJ. The involvement of the DEJ and marked alterations could be clearly observed as junction continuity loss, gap formation and mineral loss.

Gomes-Silva et al. (9) used *in situ* zymography to demonstrate that the gelatinolytic activity in the DEJ and adjacent sound dentine was similar between the control and *in vivo* irradiated teeth samples ($p>0.05$). They also performed immunohistochemistry and observed similar patterns of MMP-

2 and MMP-9 expression along the DEJ of both the test and control teeth samples. Likewise, a second study of the same group of researchers observed that the immunolocalization and the expression patterns of MMP-20 were similar in the DEJ microstructure components of both the control and *in vivo* irradiated teeth samples (20).

The screening of irradiated teeth crown extracts using proteomic and enzymatic analyses demonstrated that MMP-20 is a radiation-resistant component of mature tooth crowns, which is enriched in the DEJ. MMP-20 catalysed the degradation of the enamel organic matrix following radiotherapy in cancer patients, which could lead to enamel delamination associated with HNRT (15).

Confocal microscopy and Western blotting revealed that immune-stained type IV collagen at the DEJ (5 to 10 μm in width) irradiated *in vivo* presented a severe reduction in its immunoreactivity (19), suggesting that HNRT directly degrades this essential component of the organic dentine matrix.

The synthesis of the quantitative results of the included studies is presented in Table 3.

Discussion

This systematic review included studies that evaluated the impact of HNRT on DEJ. Most of the studies did not present accurate sample characterisation (sample size, the anatomic origin of extracted teeth and modality of radiotherapy). Instead, they described through simulated or *in vivo* radiotherapy, the mechanical, physical and chemical aspects of the DEJ, which may predispose cancer patients to RRC.

Although there is a lack of literature regarding the direct radiogenic damage to dentition, the results of this systematic review suggest that HNRT may act as an independent risk factor to impact the micromorphological and biochemical features of the DEJ. One included study reported a decrease in the enamel microhardness in a region of the DEJ following *in vivo* and *in vitro* irradiation (18). In addition, another study found a significant increase in elastic modulus after simulated oral cancer radiotherapy at the evaluated sites in enamel and dentin, near the DEJ region (17); this was in agreement with two *in vitro* studies (26,27) that were not included, but in discordance with two other authors that did not observe any dental microhardness changes after *in vitro* irradiation (12, 28). This divergence in the literature might be explained by the fact that the aforementioned studies did not

evaluate the specific region of the DEJ.

Furthermore, a third included study (24) suggested that post-HNRT dentition failure appears to be in the inner enamel near the DEJ, not specifically at the DEJ. This can be explained by the fact that there were not any apparent differences in either the tensile and shear stress between the control and *in vitro* radiation group probably due to the observation that *in vitro* radiation increased the elastic modulus of all the regions of enamel including the DEJ.

Another issue under discussion is the use of the OCT for the carious evaluation. Lee et al. (25) associated the optical coherence tomography with optical light microscopy analysis and found that the irradiated tooth lesions close to the DEJ proved to be deeper than those on non-irradiated teeth. This tooth crown location (DEJ) has been chosen for the development of the lesions according to previous studies that showed a high clinical incidence of carious lesions in this dental area in irradiated head and neck patients (29,30).

The analysis assumed that the OCT method was not conclusive because it was difficult to observe the differences among the carious lesions, probably because the images of the lesions appeared somewhat tenuous and, for this reason, were not clear enough to make a comparison (25,29). Due to these difficulties in interpretation, common light microscopy analysis associated with the measurements of the carious lesions was added to this study in order to obtain quantitative data that allowed a more objective comparison (25).

In terms of the structural aspects of the DEJ, one study (19) included in this systematic review has focused, demonstrating (by confocal immunofluorescent staining and by Western blot analyses) type IV collagen as a DEJ component. Besides this, a reduction in DEJ collagen IV immunostaining, in *in vivo* irradiated teeth, that manifest DEJ instability, suggests that it may contribute functionally to uniting enamel to dentine. Thus, the type IV collagen may be destabilised following oral cancer radiotherapy, resulting in pathologic enamel loss followed by the decay of the exposed dentin (19,31,32).

McGuire et al. (19) hypothesised that *in vivo* HNRT using high doses of radiation causes induction and activation of enzymes that degrade collagens over a period of months/years and might increase the expression and activation of matrix metalloproteinases (MMPs) in various tissues (31,33),

such as MMP-20, a type IV collagenase localised in the DEJ which would have its processing altered in post-radiotherapy teeth. These authors concluded that type IV collagen is a novel biomarker of the DEJ in mature human teeth and its loss following *in vivo* radiotherapy may represent a mechanism of post-irradiation DEJ instability observed in oral cancer patients, which leads to enamel delamination and dentition breakdown.

In contrast to these conclusions, another included study (20), which carried out the immunohistochemical expression of MMP-20 in post-HNRT teeth, showed that overexpression could not be observed. However, this study did not necessarily contradict the previous results of Mc-Guire et al. (19), as the radiation could likely affect MMP-20 activity without considerably changing the total protein amount, which cannot be detected by conventional immunohistochemical techniques.

Regarding dentine metalloproteinases, one of the studies evaluated the effects of the immunolocalisation and gelatinolytic activity of MMP-2 and MMP-9 on teeth after radiation treatment and suggested that the gelatinases MMP-2 and MMP-9 were preserved in the components of post-radiation human teeth from patients with HNC, including the DEJ (9). During the later stage of teeth formation, MMP-2 and MMP-9 are more concentrated close to the DEJ and along the mantle dentine, whereas TIMP-1 and TIMP-2, which are natural inhibitors of MMPs, are variably distributed (32,34). For this reason, the gelatinases may be constantly inhibited in the DEJ and are not directly affected by radiation.

Additional studies with well-designed methodologies are necessary to investigate the effects of ionising radiation as an independent risk factor for physical and chemical changes of the DEJ, a pivotal dental topography for the onset and progression of RRC and enamel delamination.

Conclusions

This systematic review resulted in a small number of studies, which presented methodological heterogeneity that suggests that radiation therapy acts as an independent risk factor in causing direct radiogenic damage to the organic and inorganic components of the DEJ. Hence, well-designed methodologies, preferably longitudinal clinical studies, are necessary to identify the role of HNRT in the physicochemical properties of the DEJ in cancer patients and its potential impact on the aetiology

of RRC and enamel delamination.

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Conflict of interest:

We, authors of this manuscript, declare that there is none financial relationship with any commercial associations, current and within the past five years, that might pose a potential, perceived or real conflict of interest. These include grants, patent licensing arrangements, consultancies, stock or other equity ownership, advisory board memberships, or payments for conducting or publicizing our study. The authors also state the material is original, has not been published elsewhere, and is being submitted only to the Medicina Oral Patologia Oral y Cirugia Bucal Journal.

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Tables

Table 1. Characteristics of included studies.

Author/Year	Country	Study Design	Sample Size	Tooth	Control	Assays	Radiation Dose	Radiotherapy Modality	Storage solution	Time storage
Seyedmahmoud, et al., 2018, (18).	USA	*in vivo/ in vitro*	6	Posterior teeth	Yes	Microindentation/ Microhardness	70 GY	-	PBS, pH 7.4) + 0.002% sodium azide.	-
Gomes-Silva, et al., 2017[a] (9).	Brazil	*in vivo*	36	Anterior and posterior teeth	Yes	In situ zymography/ Immunohistochemistry	40- 70 GY	3D	Formalin	3 days
Thiagarajan et al., 2017 (24).	USA	*in vivo/ in vitro*	01	Molar	Yes	Finite element / Nano indentation	> 60 GY	-	-	-
Gomes-Silva, et al., 2017b (20).	Brazil	*in vivo*	36	Anterior and posterior teeth	Yes	Immunohistochemistry	40 - 70 GY	3D	Formalin	3 days
Reed, et al., 2015 (17).	USA	*in vitro*	07	Third molars	Not	Nanoindentation Raman microspectroscopy	70 GY	IMRT	Sodium Chloride (NaCl) - 0.09%	30 days
McGuire, et al., 2014c (19).	USA	*in vitro*	35	Anterior and posterior teeth	Not	Confocal Microscopy Western Blotting	> 60 GY	IMRT	0.9% (PBS) + 0.002% sodium Azide	-
McGuire, et al., 2014b (15).	USA	*in vivo*	-	-	Yes	Proteomic and Enzymatic analyses	70 GY	IMRT	PBS + 0.002% NaN3	14 days
Lee, et al., 2014 (25).	Brazil	*in vitro*	15	Third Molars	Yes	Optical Coherence Tomography	70 GY	-	Distilled water	-

Table 1 (continued)

Results	Main Conclusions
Middle and outer regions of enamel demonstrated a significant decrease in microhardness In irradiated group (p <0.05). The highest percentage of clean microindentation (65%) was observed in the *in vivo* irradiated group in the inner region of enamel near the dentin-enamel junction.	*In vitro* and *in vivo* irradiation alters enamel microhardness. Likewise, indentation pattern differences suggest that enamel may become more brittle following *in vitro* and *in vivo* irradiation.
No statistically significant differences were detected between groups in gelatinolytic activity or in MMP-2 expression levels (P > .05). Odontoblast MMP-9 expression was reduced in the irradiated group (P ¼ .02).	The study rejected the hypothesis that MMP-2 and MMP-9 would be overexpressed or more activated in the DEJ and dentin-pulp complex of irradiated teeth. Direct effects of radiation should not be regarded as an independent factor for explaining radiation-related caries onset and progression.
From the FE data, we observed an increase in the principal tensile stress within the inner enamel region of *in vivo* irradiated teeth (9.97 ± 1.32 MPa) as compared to control/non-irradiated teeth (8.44 ± 1.57 MPa).	This model predicts that failure occurs at the inner enamel/DEJ interface due to extremely high tensile and maximum shear stresses in *in vivo* irradiated teeth which could be a cause of enamel delamination due to radiotherapy.
No apparent damage to the DEJ microstructure or other dentin-pulp complex components was observed and no statistically significant differences were detected inMMP-20 expression (p > 0.05) between the irradiated and control groups.	This study rejected the hypothesis that MMP-20 is overexpressed in the DEJ, dentin-pulp complex components, and carious dentin of post-HNRT patients leading to detectable micromorphological changes.

The elastic modulus of enamel and dentin was significantly increased (P≤0.05) following radiation. Based on Raman spectroscopic analysis, there was a significant decrease in the protein to mineral ratio (2931/430 cm-1) following radiation at all sites tested except at D-500. Finally, phosphate peak width as measured by FWHM at 960 cm-1 significantly decreased at both D-30 and D-500 following radiation.	Simulated radiotherapy produced an increase in the stiffness of enamel and dentin near the DEJ. Such changes in mechanical properties and chemical composition could potentially contribute to DEJ biomechanical failure leading to enamel delamination that occurs post-radiotherapy.
Confocal microscopy revealed that immunostained type IV collagen was restricted to the 5- to 10-μm-wide optical DEJ, while collagenase treatment or previous *in vivo* tooth-level exposure to > 60 Gray irradiation severely reduced immunoreactivity. This assignment was confirmed by Western blotting with whole-tooth crown and enamel extracts. Compositionally, our results identify type IV collagen as the first macromolecular biomarker of the morphological DEJ of mature teeth.	Given its network structure and propensity to stabilize the dermal-epidermal junction, we propose that a collagen-IV-enriched DEJ may, in part, explain its well known fracture toughness, crack propagation resistance, and stability. In contrast, loss of type IV collagen may represent a biochemical rationale for the DEJ instability observed following oral cancer radiotherapy.
MMP-20 was composed of catalytically active forms at Mr=43, 41, 24 and 22 kDa and was immunolocalized predominantly to the morphological dentin enamel junction. The proportion of different sized MMP-20 forms changed with incubation and irradiation. Extracts of teeth from oral cancer patients who received >70 Gy radiation also contained relatively more 24 and 22 kDa MMP-20 than those of healthy age-related teeth.	MMP-20 is a radiation-resistant component of mature tooth crowns enriched in the dentin-enamel. We speculate that MMP-20 catalyzed degradation of organic matrix at this site could lead to enamel delamination associated with oral cancer radiotherapy.
Only the 20-day period of culture immersion for caries development resulted in significantly better lesion comparisons, by light microscopy. Of the three lesion dimensions analyzed, lesion depth (lD) differed statistically between groups A and B (p = 0.013). Analysis using OCT allowed the visualization of carious lesions without showing the carious layers.	The radiation treatment of sound teeth before a cariogenic challenge *in vitro* causes deeper carious lesions than in those teeth not subjected to radiation treatment.

Table 2. Risk of bias in individual studies. Fulfilled GRADE criteria (Pavan et al. 2015), (23).

Author/ Year	Study Limitation	Inconsistency	Indirectness	Imprecision	Publication Bias	Dose Effect	Overall Quality
Seyedmahmoud et al, 2018 (18)	√	√	√	√	√	√	++++
Gomes-Silva et al, 2017a (9)	√	√	√	√	√	√	++++
Thiagarajan et al. 2017 (24)	X	√	X	X	√	√	++
Gomes-Silva et al, 2017b (20)	√	√	√	√	√	√	++++
Reed et al, 2015(17)	√	√	√	√	√	√	++++
McGuire et al, 2014c (19)	√	√	√	√	√	√	++++
McGuire et al, 2014b (15)	X	√	√	X	√	√	+++
Lee et al, 2014 (25)	√	√	X	√	√	√	+++

Grade factors: √, No serious limitations; X, serious limitations; unclear, unable to rate item based on available information. For Overall Quality of Evidence: + very low; ++ low; +++ moderate; ++++ high.

Table 3. Synthesis of quantitative results.

Seyedmahmoud, et al., 2018 (18)	**Microhardness Vickers (GPa)**	**Control**	***In vitro***	***In vivo***	**Means difference**	***P* Value**
	Inner (50 μm from DEJ)	2,60	2,47	2,50	0,10	p > 0.05
	Middle (200 μm from DEJ)	3,46	3,02	3,05	0,41	p < 0.05
	Microidentation Vickers (%)	**Control**	***In vitro***	***In vivo***	**Means difference**	***P* Value**
	Inner (50 μm from DEJ)	30	59	65	35	p < 0.05
	Middle (200 μm from DEJ)	32	55	57	25	p < 0.05
Reed, et al., 2015 (17)	**Nanoindentation (GPa)**	**0 Gy**	**70 Gy**		**Means difference**	***P* Value**
	E-30	70.00	90.00	-	20.00	p≤0.05
	D-30	15.00	20.00	-	5.00	p≤0.05
	E-500	82.00	102.00	-	20.00	p≤0.05
	D-500	23.00	26.00	-	3.00	p≤0.05
Thiagarajan et al., 2017 (24)	**Finite Elements**	**Number of nodes**	**Number of elements**	**-**	**-**	**-**
	-	26,906	48,744	**-**	**-**	**-**
	Nanoidentation (GPa)	**Control**	***In vitro***	***In vivo***	**Means difference**	**Poisson's ratio**
	Dentin Enamel Junction	30	30	30	**-**	**0.3**
	Principal tensile stress(MPa)*	7.98 ± 1.46	9.72 ± 2.01	8.29 ± 1.19	**-**	**-**
	Maximum shear stress(MPa)	50.39 ± 1.59	52.66 ± 2.54	49.24 ± 2.11	**-**	**-**
Gomes-Silva, et al, 2017a(9)	**Immunohistochemistry**	Control		*In vivo*	**Means difference (%)**	***P* Value**
	MMP-2 (DEJ)	8/8 (100%)	-	8/8 (100%)	NA	NA
	MMP-9 (DEJ)	8/8 (100%)	-	8/8 (100%)	NA	NA
Gomes-Silva, et al, 2017b (20)	MMP-20 (DEJ)	8/8 (100%)	-	8/8 (100%)	NA	NA
Gomes-Silva, et al, 2017a (9)	**Zymography**	**Control**	**In situ**	***In vivo***	**Means difference (%)**	***P* Value**
	DEJ	0/7 (0%)	1/7 (14.2%)	-	14.2%	p = 0.31
Lee, et al, 2014 (25)	**OCT***	**Control**	***In vitro***	***In vivo***	**Means difference (%)**	***P* Value**
	Depth (μm) – 20 days	1.66	2.26	-	-0.6	p = 0.013

***OCT -** Optical Coherence Tomography

Figures

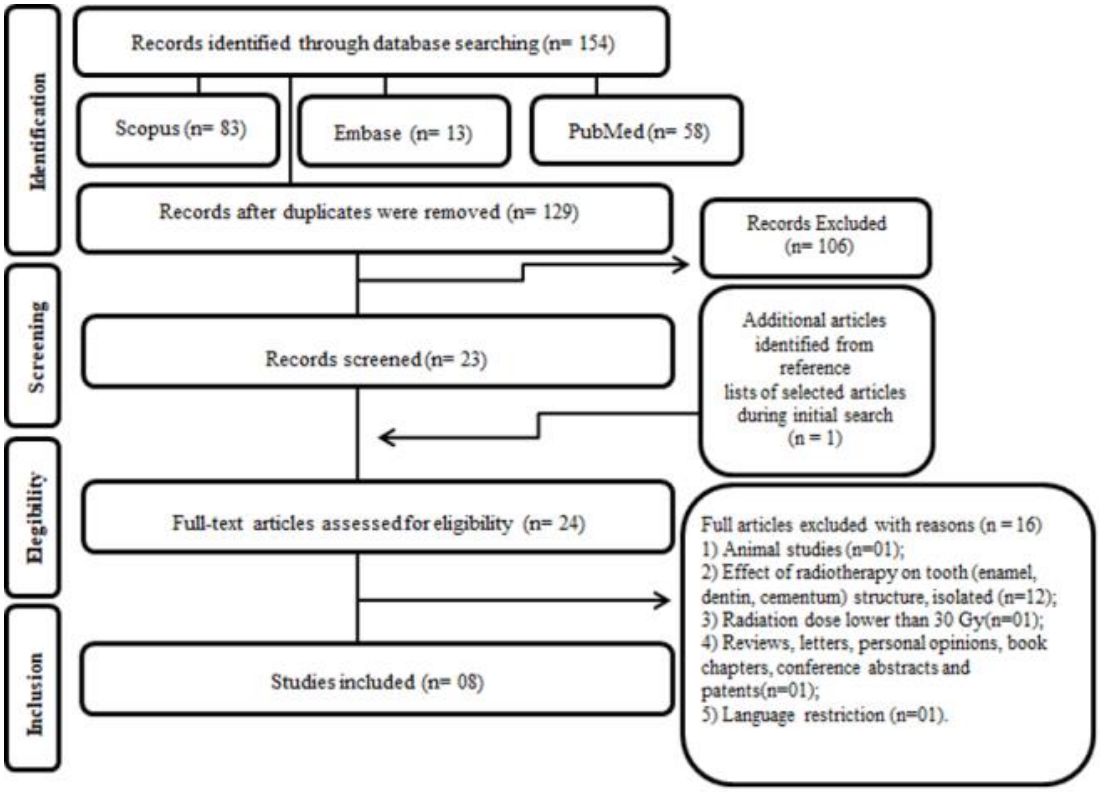

Figure 1. Flow Diagram of literature search and selection criteria adapted from PRISMA (Liberati, 2009), (21).

2.2 ARTIGO: Post-radiotherapy immunoexpression of type I collagen, BSP and BMP4 in the dentin-pulp complex of head and neck cancer patients.

Artigo submetido no periódico Brazilian Oral Research (Anexo 2).

Thematic area: Stomatology

Title: Post-radiotherapy immunoexpression of type I collagen, BSP and BMP4 in the dentin-pulp complex of head and neck cancer patients.

Running title: Immunoexpression of dentin-pulp complex proteins in head and neck cancer patients.

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Abstract

Objectives: This study tested the hypothesis that head and neck radiotherapy (HNRT) impacts the immunoexpression of type I collagen, bone sialoprotein (BSP) and bone morphogenetic protein 4 (BMP4), leading to micromorphological changes of the dentin-pulp complex (DPC) and favoring the onset and progression of radiation caries (RC). **Materials and Methods:** 22 demineralized sections of carious teeth extracted from cancer patients (11 irradiated group *versus* 11 control group) extracted from 19 head and neck cancer patients were analyzed by conventional optical microscopy and immunohistochemistry to investigate the micromorphology (cellular layers hierarchy, blood vessels, odontoblasts, fibroblasts, extracellular matrix, calcification, necrosis, reactionary dentin formation and chronic inflammation) and patterns of staining/immunolocalization of type I collagen, BSP and BMP4 in dental pulp of irradiated and control samples. **Results:** No significant differences were detected between both groups in micromorphology of the DPC that could be attributed to direct impact of radiotherapy. Also, the patterns of immunohistochemical staining and immunolocalization of the proteins studied did not differ between irradiated and control samples for type I collagen, BSP and BMP4. **Conclusions:** This study rejected the hypothesis that HNRT directly damages the dentition by changing the organic components and the microstructure of the DPC ultimately leading to RC. **Clinical Relevance:** This study failed to detect any microscopic evidence that radiotherapy could be directly injurious to the organic components and the microstructure of the dentin-pulp complex and impairing the response to radiation caries progression in cancer patients.

Keywords: radiation caries; type I collagen; bone sialoprotein; bone morphogenetic protein 4; immunohistochemistry.

Introduction

Radiotherapy is one of the main treatment choices of head and neck cancer. Although it is considered highly effective, head and head radiotherapy (HNRT) results in a number of acute and chronic toxicities to non-targeted tissues, including oral mucositis, hyposalivation, recurrent oral infections, trismus, RC and osteoradionecrosis, among others [1,2] .

Radiation caries (RC) is a chronic side effect that affects approximately 29% of patients who underwent HNRT with increased development risk of 37% within two years [3]. Usually presents a high potential for generalized dentition breakdown and clinical patterns of progression that differ from conventional caries, characterized by widespread cervical demineralization, incisal edges, cusp tips lesions and diffuse brownish to black discoloration in the enamel surface [4]. RC rapidly progresses causing enamel cracks, delamination, and amputation of teeth crowns, leading to teeth destruction, for the most severe cases. In addition, it can increase the risk for the development of osteoradionecrosis, and the negatively impact of the quality of life of cancer survivors [5,6]. The atypical clinical patterns of progression and lack of symptomatology associated with RC encourage research investigating its etiology and pathogenesis. Even though it is commonly attributed to the indirect effects of radiotherapy, such as mucositis and hyposalivation [7,8], *in vitro* studies proposed that the direct effects of HNRT on tooth-mineralized structures might also be a significant causal factor for RC [9,10].

The dentin-pulp complex (DPC) is a large and sophisticated component of the tooth structure [11] in which collagen and non-collagenous organic components, such as dentin sialoproteins, phosphoproteins, and proteoglycans, play an important role in the mechanical and structural integrity of the tooth tissue [12,13].

In this context, there are some proteins that participate in molecular events underlying the DPC responses to the progression of caries, such as the type I collagen that plays a decisive role in the dentin mineralization process, being the predominant collagen present in dentin [14]. Also, the bone sialoprotein (BSP) is synthesized by odontoblasts and preameloblasts, accounting for 5-8% of the dentin extracellular matrix [14,15]. Additionally, the bone morphogenetic protein 4 (BMP4), a growth factor related to tooth development and the type I collagen, seems to be found specifically in the dental pulp and may be related to the formation of dentin matrix [16,17].

Therefore, the present study aimed to test the hypothesis that HNRT impacts the immunoexpression of type I collagen, BSP and BMP4, leading to detectable micromorphological changes of the DPC with potential to contribute for the development of RC.

Materials and Methods

Patients and specimen collection

This study was approved by the local Ethics Committee (protocol number: CAAE 12837819.0.0000.5418) and was conducted in accordance with the Declaration of Helsinki.

Eleven irradiated teeth with RC (irradiated group) and eleven non-irradiated carious teeth (control group) extracted from 19 head and neck cancer patients were included in this study. Dental extractions were performed independently of the particulars of the study due to advanced caries or periodontal disease. Immediately after the extractions, teeth were identified, placed in plastic containers with 10% buffered formalin solution and fixed for at least 72 h at 4 °C [5].

For clinical characterization of the patients, the electronic medical record system of each patient was consulted and the following data were collected: age, gender, tumour location, alcohol consumption and smoking habit, tumour histological type, clinical cancer stage according to the American Joint Committee on Cancer [18], total radiation dose prescribed to tumour treatment and final dose delivered to each studied tooth (Gy) [19], anatomic origin of extracted teeth, and time between the completion of HNRT and teeth extraction.

Inclusion criteria

Teeth were extracted from patients with head and neck squamous cell carcinomas (SCC) subjected to clinical radiation protocols with tridimensional conformal RT (3DRT) in 6-mV linear accelerators on the Synergy Platform (Elekta AB, Stockholm, Sweden) with cumulative doses that ranged from 60 to 70 Gray (Gy) (2 Gy/day, five days per week). The 3DRT plan of the patients was retrieved from the CMS system XiO version 4.60 (Elekta CMS software, St. Louis, MS, USA) to study the radiation field and the total dose directed to the teeth [19].

Exclusion criteria

Teeth extracted from patients with SCCs located outside the head and neck region, who did not receive dental treatment prior to RT, who was submitted to radiotherapy regimens different from those included in the inclusion criteria or whose demographic and clinicopathological information were not fully available in the medical records.

Demineralization and histological preparation

Each sample was codified and classified according to the anatomic group of origin. All specimens were cleaned up with manual periodontal curettes to remove residual soft tissues and decalcified in Ana Morse's solution (equal volumes of 20% sodium citrate and 50% formic acid) at 4 °C for three weeks, with the solution being changed every two days. The decalcification was monitored and confirmed by weekly periapical radiographs. Subsequently, all specimens were radiographed in a standardized way. The teeth were placed individually on pieces of periapical radiographic film for adults (Kodak Ultra-speed; Eastman Kodak Company, Rochester, NY), with the crown facing the perforation of the film. An X-ray machine (Toshiba XR 6010 [127 V, 60 kV, 10 mA, and 60 Hz]) was used, with an exposure time of 0.3 seconds and focus-film distance of 15 cm. All radiographic films were processed in the same automatic processor. The samples were embedded in Paraplast Plus® (Leica Biosystems Richmond, Inc., Richmond, IL, USA) to produce 5-µm-thick sections on a microtome (Leica, Nussloch, Germany) in silanized slides for hematoxylin and eosin (H&E) morphological evaluation and immunohistochemical analysis [20].

Micromorphological analysis

An optical light microscope (OLM) (DM4000 B Leica, Wetzlar, Germany) was used for the micromorphological study. A previously calibrated oral pathologist analyzed three demineralized H&E-stained histological sections per sample in a descriptive way by covering all the extension of the samples included in each slide in 200 X and 400 X magnifications [21].

The microscopic analysis of DPC focused on the presence and morphologic preservation of the following parameters: dental pulp cellular layers hierarchy, blood vessels, odontoblasts, fibroblasts, dental pulp extracellular matrix components, calcification, necrosis, reactionary dentin formation and chronic inflammation [22].

Immunohistochemical preparation

The immunohistochemical reactions were conducted on 4-mm-thick histologic sections cut from the paraffin-embedded tissue blocks and mounted on silane-coated glass slides. Antigen retrieval was performed in a water bath for 20 minutes at 95ºC in the citrate buffer pH 6.0, and endogenous peroxidase activity was blocked using 5% hydrogen peroxide solution in methanol for 20 minutes. Protein blocking was performed with powdered skim milk diluted in 5% PBS for 20 minutes. All slides were incubated in a refrigerator at 2 to 8° C

overnight with primary antibodies and subsequently incubated with secondary antibody: Goat anti-rabbit IgG, peroxidase conjugated, Millipore, code: AP132P for 1 hour and 30 minutes at room temperature and visualization of the reaction was obtained with Liquid Dab (DAKO, K3468), according to the manufacturer's recommendations. The sections were counterstained using Mayer's hematoxylin and coverslips. Adequate positive control sections were used for each antibody, and the negative control was obtained by omitting the specific primary antibody[20]. The antibodies information, and protocol for dilution used are described in Supplementary Table 1.

Immunohistochemical analysis

Two calibrated observers evaluated the reactivity of each antibody. At the moment of observation, the oral pathologist had knowledge of the antibodies, but did not know which samples they were observing. The reactivity was qualitatively and semi-quantitatively evaluated for the five regions (dentin-enamel junction - DEJ, sound dentin, pre-dentin, dental pulp, and odontoblastic layer), as follows: 1 (positive staining in total area of each region evaluated) and 2 (absence of staining in total area of each region evaluated) [20].

Statistical analysis

Data was analyzed statistically using PASW Statistics 18 © 2015 SPSS Inc. All rights reserved, [SPSS (Hong Kong) Ltd, Rm 1804, 18/F, Westlands Centre,Westlands Road, Quarry Bay, Hong Kong] using the Fisher test, with the significance level set at $\alpha = 0.05$.

Results

Patients and specimen collection

Demographic features and clinicopathological data obtained from the 19 patients included in this study are described in Supplementary Table 2.

Eleven irradiated teeth samples were obtained from a total of 10 post-HNRT patients and consisted of 5 (45.5%) molars and 6 (54.5%) pre-molars. In the control group, 11 samples obtained from 9 non-irradiated head and neck cancer patients were included and consisted of 5 (45.5%) molars and 6 (54.5%) pre-molars.

Micromorphological analysis

The microscopic analysis revealed the presence and the preservation of the dental pulp cellular layers

hierarchy in 6 (54.5%) cases of the irradiated group and 6 (54.5%) cases of the control group (p=1.0) (Figure 1A, 1B). In the other samples of both groups, the presence and the preservation of the dental pulp cellular layers hierarchy were similarly and mildly changed because of the presence of small dental pulp calcification, diffuse chronic inflammation represented by mononuclear cells and necrosis associated to bacterial invasion. Blood vessels presence and vascular architectural preservation was observed in all (100%) samples of both groups.

Odontoblasts were detected in all samples in which the dental pulp cellular hierarchy layers were preserved of both groups 6 (54.5%) and were characterized by tall columnar cells arranged in palisade and located at the periphery of the dental pulp. Cell processes arising from the odontoblasts cell body could be observed penetrating into the dentin and in close contact between fibroblasts of all studied samples. Fibroblasts are preserved in all (100%) samples of both groups.

Superficial caries-infected dentin composed of disorganized dentin and bacterial colonies, as well as an inner demineralized layer with affected, but not disrupted, dentin was consistently observed in all studied specimens of both groups (Figure 1C, 1D).

Dental pulp extracellular matrix components were similarly detected among samples from both groups and characterized by focal areas of dental pulp calcification 6 (54.5%) among control cases vs. 5 (45.4%) irradiated cases (p=1.0); necrosis was detected in 5 (45.4%) control cases vs. 1 (9.0%) irradiated case (p= 0.14); chronic inflammation represented by mononuclear cells was reported in 2 (18.1%) control cases vs. 2 (18.1%) irradiated cases (p= 1.0) (Figure 1E, 1F); the presence of reactionary dentin was not observed in cases of the control group while 3 (27.2%) cases of the irradiated group demonstrated it (p=0.21).

No significant difference was encountered between irradiated and non-irradiated groups in any of the analyzed parameters. The summary of the histological analysis of specimens is presented in Table 1.

Immunohistochemical analysis

The type I collagen expression was positive along the DEJ in all 22 (100%) specimens (Figure 2A, 2B). In sound dentin the type I collagen expression was positive for both groups (n=22, 100%). The predentin region also demonstrated positivity for all specimens (n=22, 100%). In the odontoblastic layer no cases of any group was positive (n=0, 00%) (Figure 2C, 2D). In the extracellular matrix of the dental pulp 10 (90.9%) cases of the control group were positive vs. 7 (70%) cases of the irradiated group (p=0.31), (Figure 2E, 2F).

For BSP the immunoexpression in DEJ was negative for all the cases in control group (n=0,00%) and was positive for only 1 case in the irradiated group (20%), (p=0.33). In sound dentin any case was positive in

control group (n=0, 00%) vs. 1 (20%) in irradiated group (p=0.33). The predentin region demonstrated positivity for 4 (40%) cases of control group vs. 1 (20%) for irradiated group, (p=0.60). In odontoblastic layer 6 (60%) cases demonstrated positivity vs. 3 (60%) of irradiated group, (p=1.0), (Figure 3A, 3B). In the extracellular matrix of the dental pulp BSP immunoexpression was found to be positive for 7 (70%) cases of control group vs. 5 (100%) cases of irradiated group, (p=0.50), (Figure 3C, 3D).

For BMP4 protein, the immunoexpression in DEJ was negative for all the cases. In sound dentin 3 (27.2%) cases were positive in control group vs. 2 (20%) in irradiated group, (p= 1.0). In odontoblastic layer any cases of both groups were positivity (n=0,00%). The predentin region demonstrated positivity for 5 (45.4%) cases for control group vs. 5 (50%) for irradiated group, (p=1.0), (Figure 4A, 4B). In the extracellular matrix of the dental pulp was found to be positive for 7 (63.6%) cases of control group vs. 7 (70%) cases of irradiated group, (p=1.0), (Figure 4C, 4D).

There were no significant differences between the test and control groups for any of the features analyzed (Table 2).

Discussion

In this study, the histological (HE staining) and immunohistochemical analyses were utilized to determine the influence of the HNRT in the micromorphological response in DPC of conventional and irradiated carious teeth. Apparently, this is the first study to investigate, simultaneously, the distribution of the immunomarkers: type I collagen, BSP, and BMP4 in human teeth affected by RC compared to conventional caries.

The profile of patients in this study is in accordance with the traditional clinicopathological features of oral and oropharyngeal SCC patients observed worldwide[23]. The sample was mainly composed by elderly male individuals, smokers and drinkers with poor oral health status who were diagnosed at late stages of tumor progression [24].

Some authors suggest that the direct effects of radiation on the dental pulp would be able to negatively affect the metabolism of odontoblasts and damage the response of the DPC to the progression of radiation caries[2,25,26]. However, other studies disagree with the statement that radiation may affect the dental pulp, leading to the negative effect in the viability of odontoblasts and the repair capacity of dentin against caries progression, asserting that there is a cluster of oral symptoms such as hyposalivation, oral microbiota alterations, and insufficient fluoride, that can be an indirect causative factor for an increased risk of radiation caries[5,8,27,28].

Nevertheless, there is still a lack in the literature to confirm or refute this information.

Significant difference was not encountered between irradiated and non-irradiated groups in any of the histological analyzed parameters in this study, demonstrating preservation of the dentin structure and the dental pulp cellular layers hierarchy. This finding is in accordance with what is described in the literature[29,5], which characterizes the pulp cellular layers by a central region (rich in blood vessels, fibroblasts, and neural bundles), and the subodontoblastic region or Weil zone (the third most peripheral dental pulp with notable low cell density)[27,20]. Also, all specimens showed a normal extracellular matrix organization.

In addition, the immunoexpression of type I collagen, BSP and BMP4 were performed in a complementary way to validate the morphologic findings. One study demonstrated that the pattern of type I collagen distribution in carious dentin may be associated with mineral loss in dental caries progression[30]. They observed that carious specimens stained for collagen type I showed a typical breakdown of the organic matrix, presenting areas where the collagenous network was completely degraded, and the subsequent lack of staining was evident[30]. Unlike, the present study did not indicate differences in the immunoexpression of type I collagen, both in control and irradiated groups. This finding could be associated with the presence of more preserved epitopes for antibody recognition within the formic acid-demineralized tubules, where the arrangement of the collagen fibrils was preserved [13,30].

The type I collagen, which is an important constituent of the extracellular matrix of the dental pulp connective tissue, has also been shown to be preserved in the dental pulp of control and irradiated teeth on this study[31]. Some investigators suggest that this protein may be one of the factors involved in odontoblast differentiation; or it could be just a component of the predentin secreted by polarized odontoblasts[32,17]. This protein, just as the BSP, have been referred to as present in odontoblasts, predentin and extracellular matrix of the dental pulp with only slight differences in expression[33,34].

Another factor that could support the presence of BSP in mineralized tissues, such as sound dentin, cement and periodontal ligament, is that this protein was found not only in the cellular body of odontoblasts but also in the cytoplasmic processes located inside the dentin [35]. These findings strongly suggest that these cells secrete the proteins that ultimately compose the dentin matrix, probably participating in the organization and maintenance of mineralized dentin structure[36,37]. The presence of these proteins in the predentin fragments adjacent to the dental pulp of erupted teeth corroborates this hypothesis[14].

Regarding the BMP4, it might be said that this protein is expressed by odontoblasts and near the predentin region, suggesting that it plays an important role in dentin matrix formation [16,17]. In addition, BMP4 is

a growth factor related to tooth development, therefore it was chosen for evaluation in the present study. The current outcomes suggest that BMP4 expression is sensitive to the alterations induced by infection and inflammation[38]. Bottcher et al.[39] demonstrated that when dental pulp necrosis was induced, there was no BMP4 expression, indicating that inflammation of the apical periodontium arrests events associated with dentin formation. Similarly, this study sought for changes in the immunoexpression of BMP4 in DPC of the control and irradiated carious teeth, failing to find statistically significant differences between groups.

For all the foregoing, it can be concluded that HNRT is not able to impair the DPC metabolism by changing the immunoexpression of type I collagen, BSP or BMP4 of the *in vivo* human teeth affected by RC. Therefore, the hypothesis that radiotherapy is able to directly modify the micromorphological response of the DPC to RC progression was rejected.

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Tables

Table 1. Antibodies and protocol used in the immunohistochemical analysis.

Antibody	Clone	Dilution	Source
Type I Collagen	ab34710	1:400	Abcam
Bone Sialoprotein (BSP)	ab125227	1:200	Abcam
Bone Morphogenetic Protein 4 (BMP4)	ab39973	1:100	Abcam

Table 2. Clinicopathological profile of the patients included in the study

	Patient	Age	Tooth*	Gender	Tobacco	Alcohol	Site	T	N	M	CH	RT	Dose (Gy)	Dental Dose (Gy)
Irradiated Group	1	63	34	F	Yes	Yes	Tongue	1	2b	0	Yes	3D	60	59.42
	2	44	35	M	Yes	Yes	Tongue	4	2b	X	Yes	3D	60	59.42
	3	63	25	M	Yes	Yes	Oropharynx	3	2a	X	Yes	3D	70	51.81
	4	54	36	M	Yes	Yes	Tongue	3	3	X	Yes	3D	70	67.83
	5	62	15	M	Yes	Yes	Tongue	4	2c	X	Yes	3D	70	34.62
	6	49	36	F	No	N	Nasopharynx	4	2	X	Yes	IMRT	69.96	66.90
	7	-	15	M	Yes	Yes	Oropharynx	2	0	0	Yes	3D	70	51.81
	8	47	36,37	M	Yes	Yes	Tongue	3	2	0	Yes	3D	60	58.14
	9	56	37	F	No	No	Nasopharynx	4	1	X	Yes	IMRT	69.96	66.90
	10	61	25	M	Yes	Yes	Oropharynx	2	1	X	Yes	3D	70	51.81
Control Group	1	53	25	M	Yes	Yes	Oropharynx	3	2	X	No	-	-	
	2	56	45	M	Yes	Yes	Tongue	4a	3	X	No	-	-	
	3	56	25,34	M	Yes	Yes	Oral Cavity	4b	2b	X	Yes	-	-	
	4	52	26	M	Yes	Yes	Oral Cavity	4	2b	X	No	-	-	
	5	62	36	M	Yes	Yes	Pyriform sinus	3	3	X	Yes	-	-	
	6	57	36	F	No	No	Oral Cavity	3	0	X	No	-	-	
	7	46	27	M	No	No	Oropharynx	3	2a	X	Yes	-	-	
	8	57	26,27	M	Yes	Yes	Tongue	4a	3	X	No	-		
	9	46	34	M	Yes	No	Pyriform sinus	4a	3	X	Yes	-	-	

M: Male; F: Female; CH: Chemotherapy; RT: Radiotherapy; 3D: Tridimensional Conformal Radiotherapy; IMRT: Intensity Modulated Radiation Therapy. *According to FDI World Dental Federation notation. Dental dose: mean radiation dose delivered to each tooth according to Morais-Faria et al., 2015. [19]

Table 3. Micromorphological analysis of dentin-pulp complex components of the irradiated and control samples.

Morphological component	Control	Irradiated	*p*
Dental pulp hierarchy	6/11(54.5%)	6/11(54.5%)	1,0
Blood vessels	11/11(100%)	11/11(100%)	NA*
Odontoblastic layer	6/11(54.5%)	6/11(54.5%)	1,0
Fibroblasts	11/11(100%)	11/11(100%)	NA*
Extracellular Matrix	11/11(100%)	11/11(100%)	NA*
Calcification	6/11(54.5%)	5/11(45.4%)	1,0
Necrosis	5/11(45.4%)	1/11(9.09%)	0,14
Inflammation	2/11(18.1%)	2/11(18.1%)	1,00
Reactionary Dentin	0/11(00%)	3/11(27.2%)	0,21

NA*: not available. No statistics are computed because this variable is constant. Values in parentheses are percentages of positivity.

Table 4. Immunohistochemical analysis of type I collagen, BSP and BMP4 in the irradiated and control samples.

Type I collagen	Control	Irradiated	*p*
DEJ	11/11(100%)	10/10(100%)	NA*
Sound dentin	11/11(100%)	10/10(100%)	NA*
Pre-dentin	11/11(100%)	10/10(100%)	NA*
Dental pulp	10/11(90.9%)	7/10(70%)	0,31
Odontoblastic layer	0/11(00%)	0/10(00%)	NA*
BSP	**Control**	**Irradiated**	***p***
DEJ	0/10(00%)	1/5(20%)	0,33
Sound dentin	0/10(00%)	1/5(20%)	0,33
Pre-dentin	4/10(40%)	1/5(20%)	0,60
Dental pulp	7/10(70%)	5/5(100%)	0,50
Odontoblastic layer	6/10(60%)	3/5(60%)	1,0
BMP4	**Control**	**Irradiated**	***p***
DEJ	0/11(00%)	0/10(00%)	NA*
Sound Dentin	3/11(27.2%)	2/10(20%)	1,0
Pre-dentin	5/11(45.5%)	5/10(50%)	1,0
Dental pulp	7/11(63.6%)	7/10(70%)	1,0
Odontoblastic layer	0/11(00%)	0/10(00%)	NA*

NA*: not available. No statistics are computed because this variable is a constant. Values in parentheses are percentages of positivity. DEJ: dentin-enamel junction

Figures

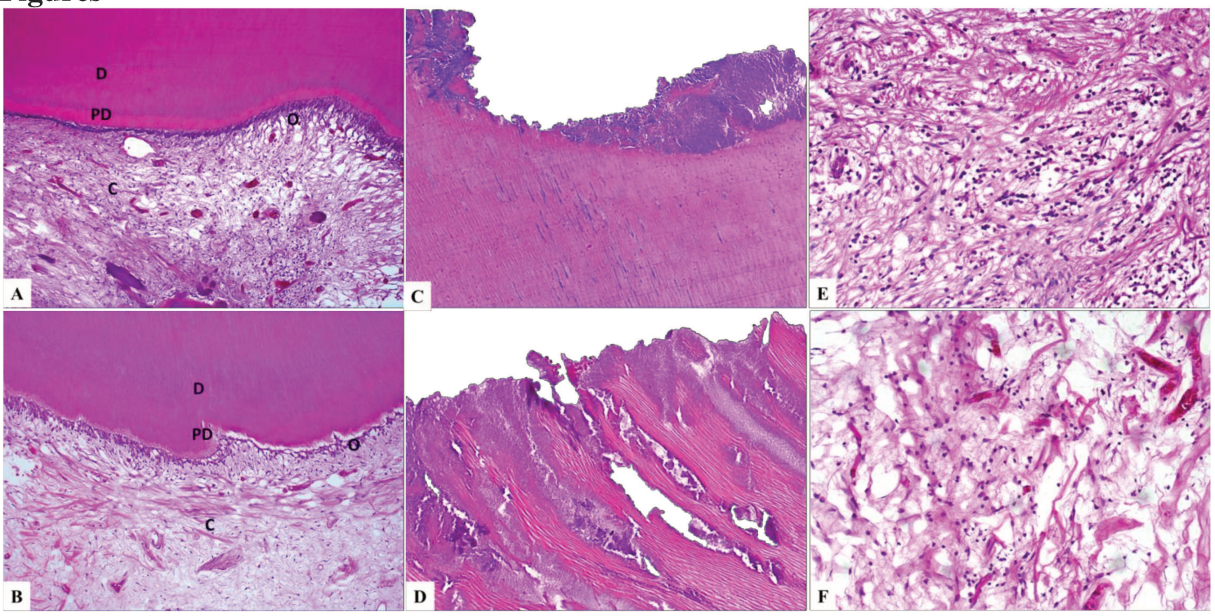

Figure 1. Microscopic overview of control and irradiated samples exhibiting preservation of the dental pulp layers hierarchy (Hematoxylin and Eosin-stained sections). **A. B. (X 200)** Dentin (D), predentin (PD), odontoblasts (O), dental pulp central region (C) in a control specimen and irradiated sample, respectively. **C. (X 200)** Inner demineralized layer with affected dentin showing normal patterns of bacterial invasion of the control dentin. **D. (X 200)** Caries-infected dentin composed of bacterial colonies and disorganized dentin in an irradiated sample. **E.F. (X 400)** Predominantly chronic inflammation represented by mononuclear cells affecting the dental pulp tissue of a control and irradiated specimen, respectively.

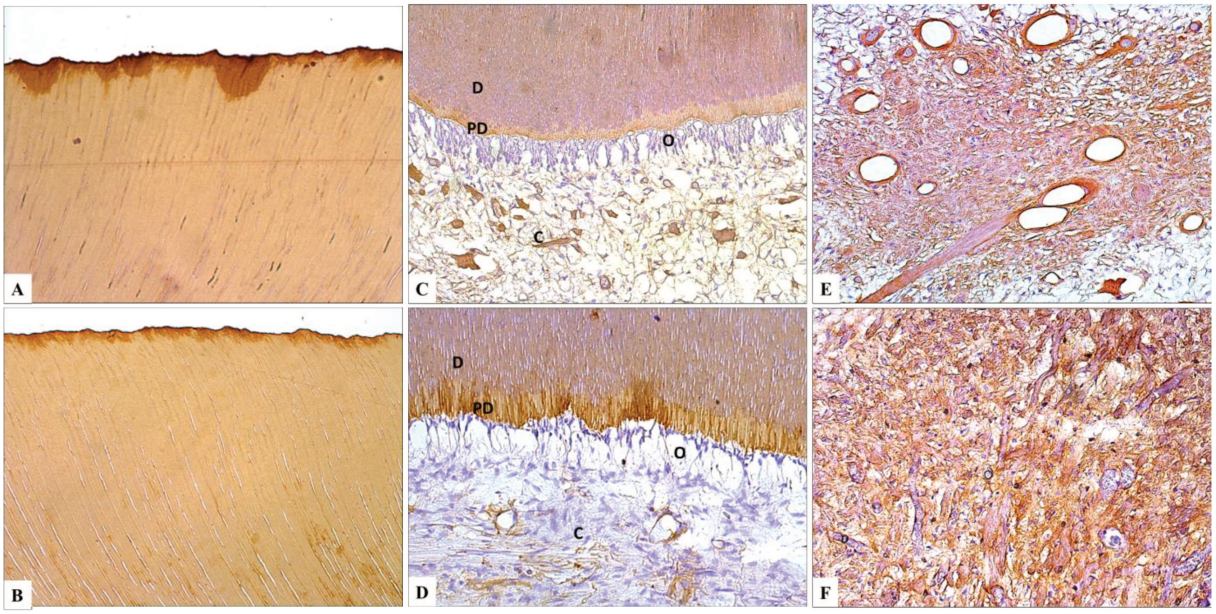

Figure 2. A. (X 200) Type I collagen expression along the dentin-enamel junction (DEJ) in a control specimen.

B. (X 200) The same pattern of positivity of the JAD in an irradiated tooth. **C. D. (X 200)** Dentin (D), pre-dentin (PD), odontoblastic layer (O) and dental pulp central region (C) expression of type I collagen in a similar pattern of immunostaining in the dentinal tubules, pre-dentin, fibroblasts and extracellular pulp matrix regions in a control and irradiated sample, respectively. **E.F. (X 400)** Immunoexpression of collagen type I in the central region of the dental pulp (extracellular matrix). Blood vessels of varying caliber and size showing discrete positivity for type I collagen in endothelial cell cytoplasm in a control and irradiated sample, respectively.

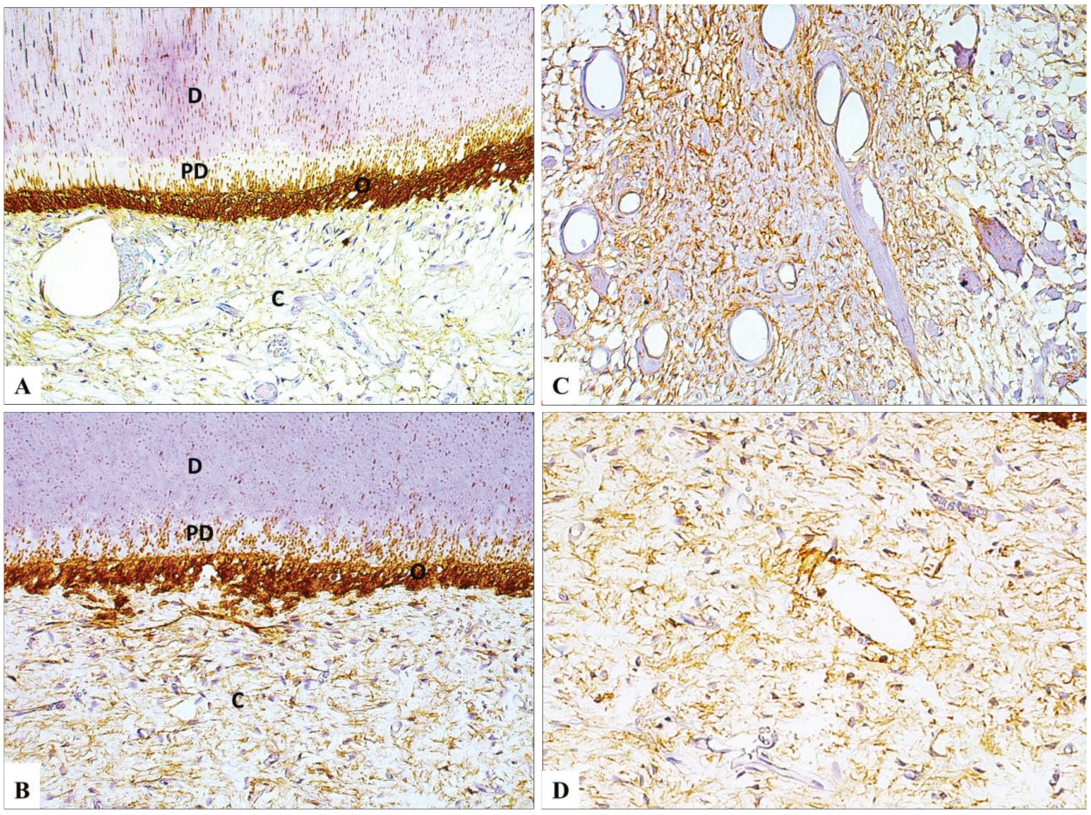

Figure 3. A.B. (X 200) Dentin (D), pre-dentin (PD), odontoblasts (O) and dental pulp central region (C). BSP immunoexpression in the odontoblastic layer and components of the extracellular pulp matrix. Discrete positivity in dentinal tubules in a control and irradiated specimen respectively. **C.D. (X 400)** Preservation of blood vessels of varying caliber and size and calcifications exhibiting a negative labeling pattern. Homogeneous immunostaining in the extracellular dental pulp matrix in a control and irradiated sample, respectively.

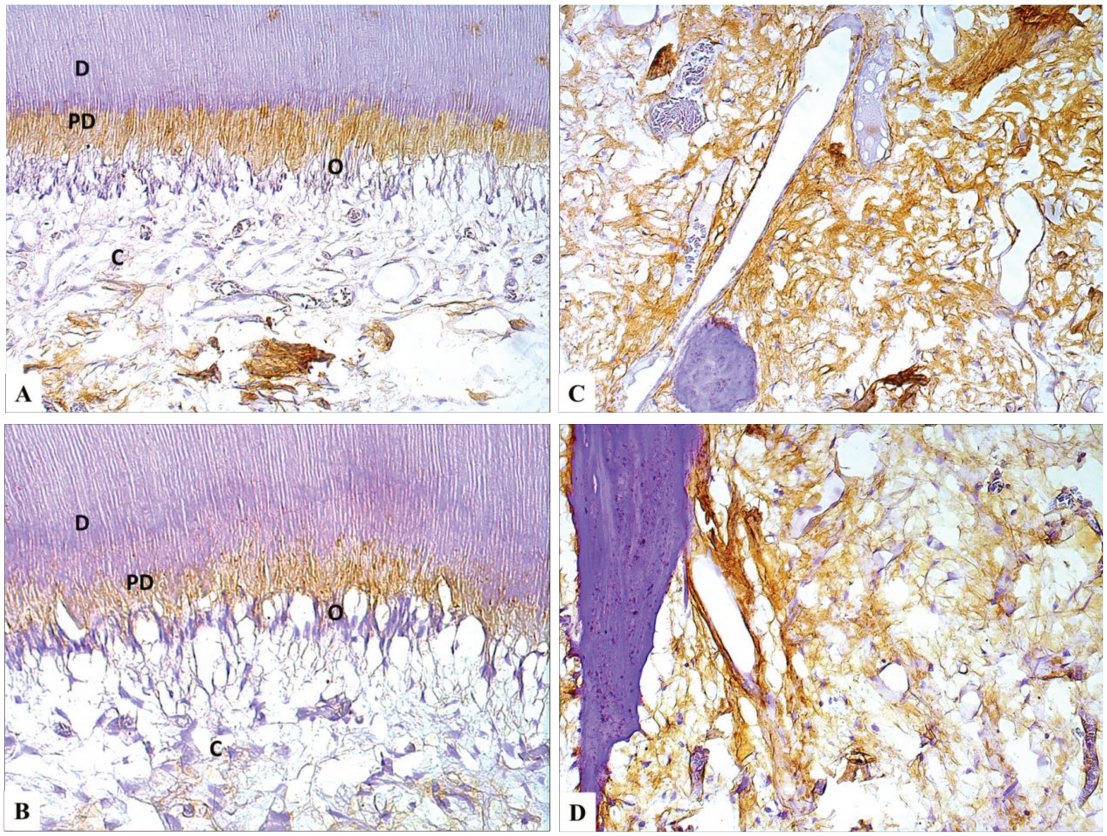

Figure 4. A.B. (X 200) Dentin (D), pre-dentin (DP), odontoblasts (O) and dental pulp central region (C). BMP4 immunostaining in the extracellular matrix of the pulp and pre-dentin similarly in a control and irradiated tooth, respectively. **C.D. (X 400)** Immunoexpression of BMP4 in the extracellular matrix of the pulp. Blood vessels of varying caliber and size showing discrete BMP4 immunopositivity in the endothelial cell cytoplasm in a control and irradiated specimen, respectively.

3 DISCUSSÃO

Neste estudo, foi realizada uma análise morfológica (coloração em HE) e imunoistoquímica em dentes com CR e CC, para determinar a ação da RDTCP na micromorfologia e composição orgânica do CDP. A distribuição das proteínas: COL I, SPO e PMO4, foi avaliada em dentes com CR em comparação à CC. De forma complementar, este trabalho inclui uma revisão sistemática reunindo estudos que avaliaram o impacto da RDT, especificamente na JAD.

O perfil clínico dos pacientes abordados na parte experimental deste estudo corresponde ao perfil de pacientes diagnosticados com câncer de cabeça e pescoço em todo o mundo (Rodrigues *et al*., 2014; Sloan et al., 2017), representado por indivíduos idosos, do sexo masculino, etilistas e tabagistas e com mau estado de saúde bucal (Jham *et al*., 2008; Neville *et al*., 2016).

Não houve diferença significativa entre os grupos irradiados e não irradiados em nenhum dos parâmetros histológicos analisados neste estudo, demonstrando preservação da morfologia dentinária e hierarquia das camadas do complexo dentino-pulpar, conforme descrito na literatura (região central - rica em vasos sanguíneos, fibroblastos e feixes neurais, região subodontoblástica ou zona de Weil, camada odontoblástica, pré-dentina e dentina) (Mjor *et al.,* 2001; Silva *et al.,* 2009).

Contudo, alguns estudos incluídos na revisão sistemática que compõe essa tese, sugerem que a RDT pode ser considerada um fator de risco para alterar a micromorfologia dentinária e gerar instabilidade na JAD. A exemplo do estudo de Seyedmahmoud *et al*., 2018, que relatou uma diminuição na microdureza do esmalte na região da JAD após irradiação *in vivo* e *in vitro* e de Reed *et al*., 2015 que encontrou um aumento significativo no módulo de elasticidade após a RDT simulada para câncer de cavidade oral em pontos do esmalte e dentina, envolvendo a região da JAD.

Para validar os achados morfológicos encontrados para o complexo dentino-pulpar neste estudo, foi avaliada a imunoexpressão das proteínas COL I, SPO e PMO4. Já foi demonstrado anteriormente que o padrão de distribuição do COL I na dentina cariada pode estar associado à perda mineral e à progressão da lesão de cárie, apresentando regiões onde a rede de colágeno estava completamente degradada e consequentemente, não havia imunoexpressão para o COL I (Stankoska *et al*., 2016). Contrariamente a este relato, o presente estudo não encontrou alterações na imunoexpressão do COL I, na dentina dos grupos controle e irradiados em dentes cariados. Isso pode estar associado à presença de epítopos

mais preservados na região dos túbulos desmineralizados com ácido fórmico, onde o arranjo radial das fibrilas de colágeno foi preservado (Bertassoni *et al*., 2015; Stankoska *et al*., 2016).

Adicionalmente, outro estudo mostrou que a intensidade da imunomarcação para o COL I, no CDP aumentou progressivamente de dentes não cariados para dentes com cárie a nível de esmalte e dentina, respectivamente, sugerindo que lesões cariosas podem estimular o complexo dentina-polpa a sintetizar proteínas como o COL I. Essa resposta a lesões de cárie provavelmente fornecerá uma base para formação de dentina reparadora e / ou reacionária (Lee *et al.,* 2006). Neste estudo todos os dentes incluídos apresentavam cárie, não sendo possível identificar essa diferença na intensidade de marcação entre dentes com lesões cariosas e sadios. Porém, um ponto importante é que a capacidade biológica de resposta à progressão de carie nos dentes irradiados está preservada, visto que, o padrão de imunomarcação da proteína COL I foi similar entre os especimes dos grupos irradiados e não irradiados.

A parte experimental deste estudo encontrou ainda, imunomarcação da proteína COL I na polpa dentária, pré-dentina e JAD dos dentes controles e irradiados, corroborando com Garcia *et al*., (2003), que descreveram esta proteína como constituinte mais importante da matriz extracelular do tecido conjuntivo da polpa dentária. Alguns pesquisadores sugerem ainda, que essa proteína pode ser um dos fatores envolvidos na diferenciação odontoblástica; ou poderia ser apenas um componente da predentina secretada por odontoblastos polarizados (Mao *et al*., 1990; Lee *et al.,* 2006).

Com o objetivo de compreender melhor o funcionamento da cascata de degradação do colágeno na estrutura dentária, um estudo (McGuire *et al*., 2014b) incluído na revisão sistemática deste trabalho, identificou (através de imunofluorescência e Western blot) o colágeno tipo IV como componente da JAD. McGuire et al. (2014b) levantaram a hipótese de que altas doses de radiação pode induzir a ativação de enzimas que degradam colágeno, a exemplo das metaloproteinases de matriz (MMPs), aumentando sua expressão e ativação em vários tecidos, como por exemplo a MMP-20, uma colagenase tipo IV localizada na JAD que pode ser superexpressa nos dentes pós-RDT. Alguns autores corroboram com esta hipótese (Imbeni *et al*., 2005; Strup-Perrot *et al.,* 2006), tendo demonstrado uma redução na imunocoloração do colágeno IV, na JAD de dentes irradiados *in vivo*, sugerindo que o colágeno tipo IV pode ser desestabilizado após a RDTCP, resultando na delaminação do esmalte seguida pela deterioração da dentina exposta (Väänänen *et al*., 2001; Imbeni *et al*., 2005).

Em contraste com essas conclusões, outro estudo incluído na revisão sistemática que

compõe essa tese (Gomes-Silva *et al.,* 2017), demonstrou, através de uma análise imunoistoquímica da MMP-20 em dentes pós-RDT, que não foi observada tal superexpressão. No entanto, este estudo não contradiz necessariamente os resultados anteriores de McGuire *et al*. (2014b), pois a radiação provavelmente poderia afetar a atividade da MMP-20 sem alterar consideravelmente a quantidade total de proteínas, o que não pode ser detectado pelas técnicas imunoistoquímicas convencionais.

Outra proteína estudada neste trabalho foi a SPO, que foi encontrada nas regiões de matriz extracelular da polpa, camada odontoblástica e pré-dentina, tendo sido pouco encontrada nos túbulos dentinários. Este achado está de acordo com alguns estudos anteriores, que relataram que a SPO é sintetizada por odontoblastos, algumas células pulpares e pré-ameloblastos (Bronckers *et al*., 1993; Butler *et al*., 2002) e que esta proteína é expressa em diferentes estágios da polpa, participando ativamente da formação de dentina (Oldberg *et al.,* 1988; Kawaguchi *et al*., 2001). Ganss *et al*. (1999) afirmaram que a imunomarcação para a SPO na dentina tubular é substancialmente mais baixa do que a observada na dentina do manto. O que sugere que a SPO atua de forma mais significativa nos estágios iniciais da formação e mineralização da matriz de dentina (Chen *et al.* 1998).

Contrariamente a esses estudos, não foi encontrada imunomarcação da proteína SPO na dentina do manto, para os espécimes incluídos neste estudo, sendo identificada somente nos túbulos dentinários. Considerando que estes espécimes foram provenientes de pacientes adultos com mais de 40 anos, pode-se explicar o fato de que o padrão de imunomarcação desta proteína não segue os padrões encontrados nos estágios iniciais de formação e mineralização da matriz dentinária.

Outros autores defendem ainda, que a SPO pode facilitar a rápida mineralização da dentina durante a formação reacionária ou reparadora, sendo mais intensamente imunoexpressa nos odontoblastos de dentes cariados a nível de esmalte e dentina, respectivamente. (Ogbureke e Fisher, 2004; Lee *et al.,* 2006). Esta afirmação corrobora com o achado de um padrão semelhante de imunomarcação da proteína SPO para os dentes com CR e CC incluídos neste estudo, reforçando a ideia de que existe uma preservação na capacidade de resposta a progressão de cárie nos dentes irradiados.

Por fim, este estudo identificou a PMO4 nas regiões de pré-dentina e matriz extracelular da polpa, estando raramente presente na parede de vasos sanguíneos, apoiando em partes os achados de Böttcher *et al.,* (2013) que detectou a imunoexpressão desta proteína na parede dos vasos sanguíneos, odontoblastos e camada subodontoblástica, próximo a pré-dentina, sugerindo sua atuação na formação da matriz dentinária (Hosoya *et al.* 2008;

Gluhak-Heinrich et al., 2010).

Böttcher *et al.,* (2013), demonstrou que a expressão de PMO4 é sensível às alterações induzidas por infecção e inflamação. Quando a necrose pulpar foi induzida, não houve expressão dessa proteína, indicando que este desafio impede eventos associados à formação de dentina. De forma oposta, este estudo buscou alterações na imunoexpressão da PMO4 no complexo dentina-polpa de dentes com CR e CC, encontrando um padrão de imunomarcação nas regiões de pré-dentina e matriz extracelular da polpa similar, mesmo em espécimes que apresentaram sinais de inflamação ou necrose. Porém não foi encontrada imunomarcação nos odontoblastos e camada subodontoblástica destes espécimes, o que pode ser justificado pelo desafio asociado às lesões cariosas presente nos mesmos.

4 CONCLUSÃO

A revisão sistemática apresentada nesta tese sugere que a RDT atua como um fator de risco independente para danificar de forma direta a composição orgânica e inorgânica da JAD. Entretanto, tal conclusão baseia-se em um pequeno grupo de estudos *in vitro*, os quais apresentaram grande heterogeneidade metodológica. Na tentativa de entender melhor o efeito RDT sobre o CDP foi desenvolvido um estudo imunoistoquímico baseado em amostras irradiadas *in vivo*, sugerindo que a RDTCP não foi capaz de prejudicar o metabolismo do CDP, pois não foram identificadas diferenças histopatológicas ou nos desfechos de imunoexpressão das proteínas COL I, SPO e PMO4 entre dentes com CR e CC.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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ANEXO 1

Comprovante de aceite do artigo 1

Journal section: Oral Medicine and Pathology
Publication Types: Review

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The impact of head and neck radiotherapy on the dentine-enamel junction: a systematic review

Jéssica Montenegro Fonseca [1], Cristhian Camilo Madrid Troconis [2], Natália Rangel Palmier [1], Wagner Gomes-Silva [3], Mariana de Pauli Paglioni [1], Anna Luiza Damaceno Araújo [1], Lady Paola Aristizábal Arboleda [1], Aljomar José Vechiato Filho [3], Wilfredo Alejandro González-Arriagada [4], Mario Fernando de Goes [2], Márcio Ajudarte Lopes [1], Thaís Bianca Brandão [3,5], Pablo Agustín Vargas [1], Ana Carolina Prado Ribeiro [1,3], Alan Roger Santos-Silva [1]

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ANEXO 2

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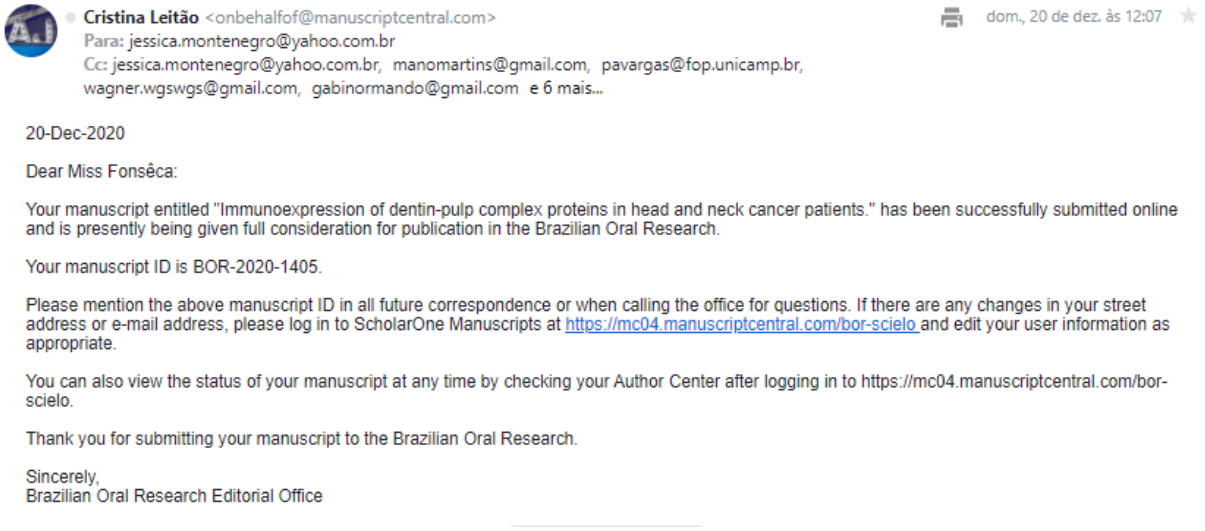

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Dear Miss Fonsêca:

Your manuscript entitled "Immunoexpression of dentin-pulp complex proteins in head and neck cancer patients." has been successfully submitted online and is presently being given full consideration for publication in the Brazilian Oral Research.

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ANEXO 3

Certificado do Comité de Ética em Pesquisa

COMITÊ DE ÉTICA EM PESQUISA
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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "Caracterização imunoistoquímica do complexo dentino-pulpar em pacientes com câncer de cabeça e pescoço submetidos a altas doses de radioterapia", CAAE 12837819.0.0000.5418, dos pesquisadores Jéssica Montenegro Fonsêca e Alan Roger dos Santos Silva, satisfaz as exigências das resoluções específicas sobre ética em pesquisa com seres humanos do Conselho Nacional de Saúde – Ministério da Saúde e foi aprovado por este comitê em 15/05/2019.

The Research Ethics Committee of the Piracicaba Dental School of the University of Campinas (FOP-UNICAMP) certifies that research project "Immunohistochemical caracterization of dentin-pulp complex in head and neck cancer patients undergoing radiotherapy", CAAE 12837819.0.0000.5418, of the researcher's Jéssica Montenegro Fonsêca and Alan Roger dos Santos Silva, meets the requirements of the specific resolutions on ethics in research with human beings of the National Health Council - Ministry of Health, and was approved by this committee on May, 15 2019.

Profa. Fernanda Miori Pascon
Vice Coordenador
CEP/FOP/UNICAMP

Prof. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo e a lista de autores aparecem como fornecidos pelos pesquisadores, sem qualquer edição.
Notice: The title and the list of researchers of the project appears as provided by the authors, without editing.

ANEXO 4

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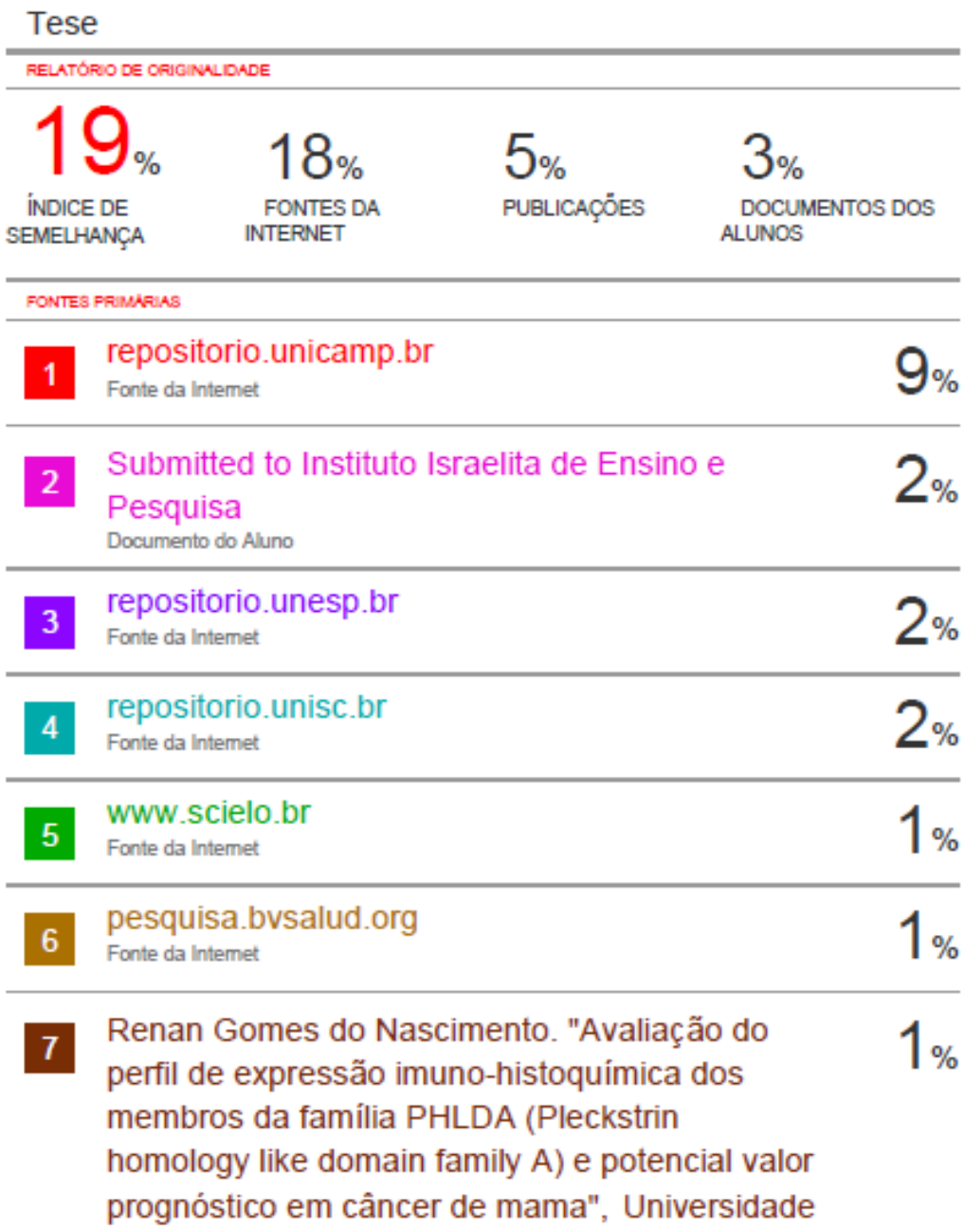

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