UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

FELIPE MARTINS SILVEIRA

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THE IMPACT OF PHOTOBIOMODULATION THERAPY ON HEAD AND NECK SQUAMOUS CELL CARCINOMA: SYSTEMATIC REVIEW AND EVALUATION IN A PATIENT-DERIVED XENOGRAFT ANIMAL MODEL

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

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 Pathology area.

Orientadora: Profª. Drª. Manoela Domingues Martins

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PROFª. DRª. MANOELA DOMINGUES MARTINS

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RESUMO

A terapia de fotobiomodulação (FBM) é considerada uma técnica efetiva para prevenção e tratamento da mucosite oral (MO) desenvolvida em pacientes com carcinoma espinocelular de cabeça e pescoço (CECP) submetidos à terapia antineoplásica. Entretanto, os potenciais efeitos da FBM em células de carcinoma espinocelular (CEC) ainda não são bem estabelecidos. O objetivo principal desta tese foi estudar o impacto da FBM no CECP. Para isso, primeiramente foi realizada uma revisão sistemática da literatura (RS) incluindo estudos *in vitro* e *in vivo* que avaliavam o efeito da FBM no CECP. A busca foi conduzida em quatro bases eletrônicas de dados, das quais quinze artigos foram incluídos (13 *in vitro* e dois *in vivo*). A análise dos estudos incluídos demonstrou importante heterogeneidade nas metodologias, nos protocolos de irradiação e nos resultados encontrados. Proliferação e viabilidade celular foram os desfechos primários principalmente avaliados nos estudos *in vitro*. Dos 13 estudos *in vitro*, sete mostraram inibição em células de CECP enquanto seis demonstraram que a FBM apresentava efeitos de proliferação. Dos estudos *in* vivo, um reportou redução na progressão dos CECP irradiados e outro demonstrou um aumento da indiferenciação no padrão histológico das lesões irradiadas. Por meio desta RS, não foi possível definir uma clara conclusão sobre os efeitos da FBM no CECP, reforçando a necessidade emergente de estudos pré-clínicos padronizados para melhor estabelecimento científico acerca do tema. Baseado nisso, foi desenvolvido um segundo estudo para avaliar o impacto da FBM no CEC oral (CECO) em modelos animais de xenoenxertos derivados de pacientes (do inglês, *patient-derived xenograft* - PDX). O estudo foi realizado em camundongos nude BALB/c com modelos de PDX de CECO (PDX-CECO) desenvolvidos a partir de fragmentos tumorais de dois pacientes com CECO em bordo lateral de língua. Os animais foram divididos em três grupos experimentais: Controle (C), sem FBM; Irradiação imediata (IIr), receberam FBM desde uma semana após implantação dos PDX-CECO; e Irradiação tardia (LIr), receberam FBM após os tumores atingirem um volume mínimo de 200mm^3. Os modelos de PDX-CECO foram irradiados diariamente (660nm; 100mW; 6J/cm^2; 0,2J/ponto, 0.03cm^2) durante 12 semanas e medidos uma vez por semana com um paquímetro digital. Os PDX-CECO foram então coletados e submetidos às análises de volume tumoral, graduação histopatológica, imunohistoquímica (anti-Ki-67, anti-H3K9ac, anti-BMI1) e de fases de ciclo celular por citometria de fluxo. Não foram observadas diferenças estatísticas entre os volumes tumorais dos PDX-CECO dos diferentes grupos avaliados (p=0.89). A graduação histopatológica não mostrou diferença estatística entre os padrões morfológicos dos grupos C, IIr e LIr (p>0.05). Também não houve diferença estatisticamente significativa entre os grupos na análise imunohistoquímica dos anticorpos Ki-67 (p=0.9661), H3K9ac (p=0.3794) e BMI1 (p=0.5182). A avaliação das fases do ciclo celular dos PDX-CECO por citometria de fluxo mostrou um pico da fase G1 seguido por uma menor expressão da fase G2, também sem diferença estatística entre os grupos irradiados e não irradiado (p>0.05). Neste estudo, a FBM não alterou o comportamento biológico dos modelos de PDX-CECO. Este é um importante resultado pré-clínico relacionado com questões de segurança no uso da FBM.

Palavras-chave: Terapia de fotobiomodulação; Carcinoma espinocelular de cabeça e pescoço; Câncer oral; Mucosite oral.

ABSTRACT

Photobiomodulation therapy (PBMT) is an effective method for prevention and management of oral mucositis (OM) developed in patients with head and neck squamous cell carcinoma (HNSCC) submitted to antineoplastic therapy. However, the potential effects of PBMT on squamous cell carcinoma (SCC) cells eventually present in the irradiation area are still not well established. The purpose of this thesis was to study the impact of PBMT on HNSCC. Firstly, a systematic review (SR) was performed to select studies investigating the *in vitro* e *in vivo* outcomes of PBMT on HNSCC. The search strategy was conducted in four electronic databases from which fifteen studies were included (13 *in vitro* and two *in vivo*). Analyzing the included studies, it was observed an important heterogeneity in the study designs, in the irradiation protocols and in the outcomes. Proliferation and cell viability were the primarily assessed outcomes in the *in vitro* studies. From the 13 *in vitro* studies, seven concluded an inhibition in HNSCC cells, while six showed proliferation effects. Regarding the two included *in vivo* studies, one reported a decrease in the progression of irradiated HNSCC and the other a poorer differentiation of the histological pattern of the irradiated lesions. Based on this SR, it was not possible to define a clear conclusion regarding the effects of PBMT on HNSCC, reinforcing the emerging need for further preclinical standardized studies for a definitive scientific conclusion on the subject. Therefore, a second study was developed to evaluate the impact of PBMT on oral SCC (OSCC) in a patient-derived xenograft (PDX) animal model. BALB / c nude mice with OSCC-PDX models developed from the tumor fragments of two patients with OSCC border lateral tongue lesions were used. The animals were divided into three experimental groups: Control (C), without PBMT; Immediate irradiation (IIr), received PBMT since one week after the implantation of OSCC-PDX; and Late irradiation (LIr), received PBMT after the tumors reached a minimum volume of 200mm^3. The OSCC-PDX were daily irradiated (660nm; 100mW; 6J/cm^2; 0.2J/point, 0.03cm^2) for 12 weeks and measured once a week. The OSCC-PDX models were then collected and submitted to the following analysis of tumor volume, histopathological grading, immunohistochemistry (anti-Ki-67, anti-H3K9ac, anti-BMI1) and cell cycle phases (propidium-iodide) by flow cytometry. No statistical differences were observed between the tumor volumes of OSCC-PDX in the different groups evaluated (p=0.89). The histopathological grading showed no statistical difference between the morphological patterns of C, IIr and LIr groups ($p>0.05$). There was also no statistically significant difference between groups in the immunoexpression of Ki-67 (p=0.9661), H3K9ac (p=0.3794) and BMI1 (p=0.5182). The cell cycle phases evaluation by flow cytometry showed a peak of G1 followed by a lower expression of G2, also without statistical difference between the irradiated and non-irradiated groups ($p>0.05$). In this study, PBMT did not impact the biological behavior of the OSCC-PDX models. This is an important preclinical result related to the safety issues in the use of PBMT.

Keywords: Photobiomodulation therapy; Head and neck squamous cell carcinoma; Oral cancer; Oral mucositis.

SUMÁRIO

1 INTRODUÇÃO

As estimativas mundiais a respeito dos cânceres de lábio e de cavidade oral reportam cerca de 355.000 novos casos com mais de 177.000 mortes relacionadas a esse tipo de neoplasia (Bray et al., 2018; Miranda-Filho e Bray, 2020). Dentre os tipos de cânceres que afetam a cavidade oral, o carcinoma espinocelular oral (CEC) (CECO) constitui mais de 90% dos casos, apresentando altas taxas de morbimortalidade (Panarese et al., 2018; Miranda-Filho e Bray, 2020). O tratamento do CECO engloba três modalidades principais: cirurgia, radioterapia (RT) e quimioterapia (QT), as quais podem ser realizadas individualmente ou em associação dependendo da localização e do estágio clínico da neoplasia (Chen et al., 2017; Thomson, 2018). Dentre os possíveis efeitos adversos relacionados às terapias antineoplásicas, a mucosite oral (MO) é um efeito agudo altamente debilitante, especialmente em pacientes com câncer de cabeça e pescoço (CCP) submetidos à RT e quimioradioterapia (QRT) (Villa e Sonis, 2015). Estima-se que cerca de 80% até 90% dos pacientes com CCP submetidos à RT desenvolvem algum grau de MO, com até 70% destes apresentando graus severos (Maria et al., 2017; Minhas et al., 2017; Chen et al., 2020).

Os mecanismos celulares e moleculares envolvidos na etiopatogenia da MO ainda não estão completamente estabelecidos, entretanto sabe-se que o processo resulta de efeitos citotóxicos inespecíficos não somente nas células epiteliais, mas também da associação de múltiplos eventos biológicos e do acometimento de células conjuntivas (Bockel et al., 2018). A MO desenvolve-se por meio das seguintes fases: (1) fase de iniciação, na qual ocorrem danos iniciais ao DNA das células tanto epiteliais como conjuntivas; (2) fase de resposta ao dano primário, na qual ocorrem as ativações de fatores de transcrição, citocinas e enzimas envolvidas no estresse oxidativo, resultando em apoptose das células endoteliais e danos ao tecido conjuntivo; (3) fase de amplificação do sinal, na qual citocinas pró-inflamatórias ativam e amplificam o processo inflamatório, resultando clinicamente em eritema e edema; (4) fase de ulceração, na qual surgem as lesões ulceradas como consequência da morte e da falta de reposição de células epiteliais; e (5) fase de reparação, onde ocorre a cicatrização das ulcerações (Sonis, 2007; Bockel et al., 2018). Dessa forma, a MO pode se manifestar clinicamente como eritema e edema da mucosa até ulcerações de diferentes graus, potencialmente causando dor,

disfagia, desidratação e perda de peso (Russo et al., 2008; Sonis, 2009). Consequentemente, o desenvolvimento de quadros severos de MO pode representar um fator diretamente relacionado com a interrupção temporária ou permanente do tratamento antineoplásico, aumentando os custos gerais da terapia e gerando efeitos negativos significativos na qualidade de vida dos pacientes (Bezinelli et al., 2014; Amadori et al., 2016; Antunes et al., 2017; Cinaseuro et al., 2017). O tratamento sintomático é o principal objetivo dos protocolos sugeridos para manejo da MO (Raber-Durlacher e Scully, 2012). Nas últimas duas décadas, diversos estudos tem avaliado modalidades preventivas e terapêuticas para a MO, sendo que, atualmente, as principais sugeridas são: uso de anti-inflamatórios, de analgésicos e de agentes antimicrobianos, crioterapia, adequação bucal, terapias alternativas, como o uso de geleia real, e terapia de fotobiomodulação (FBM) (Sorensen et al., 2008; Kashiwasaki et al., 2012; Erdem e Güngörmüş, 2014; Rastogi et al., 2017; Daugelaite et al., 2019; Saunders et al., 2020). Destacando a FBM, fortes evidências tem apontado a sua excelente efetividade para prevenção e manejo da MO (Lalla et al., 2014; Anders et al., 2015; Zecha et al., 2016; Zadik et al., 2019; Lima et al., 2020).

A FBM é conceituada como uma forma de terapia não térmica que utiliza fontes de luzes não ionizantes no espectro visível e infravermelho, incluindo lasers (do inglês, *Light Amplification by Stimulated Emission of Radiation*), LEDs (do inglês, *Light Emitting Diode*) e luz de banda larga. O mecanismo de ação da FBM não está completamente bem esclarecido. Quando aplicada, sabe-se que a FBM inicia um processo que envolve a ativação de cromóforos endógenos provocando eventos fotofísicos e fotoquímicos com consequentes desfechos terapêuticos, os quais podem incluir a modulação do processo inflamatório (alívio da dor, diminuição do edema e vermelhidão tecidual) e a aceleração do reparo de diferentes tecidos (aumento da proliferação e da migração celulares e da síntese de colágeno) (Anders et al., 2015). Sugere-se que a irradiação do laser seja absorvida pelo citocromo-C oxidase na mitocôndria, fotoexcitando este cromóforo e gerando, consequentemente, um aumento na produção de adenosina trifosfato (ATP) (Karu et al., 1995). A luz é absorvida por moléculas do tecido alvo (cromóforos ou fotorreceptores) que possuam afinidade por determinado comprimento de onda e a absorção luminosa ocorre em âmbito atômico, onde os elétrons captam essa energia luminosa e partem para um estado excitado de energia. Esta energia é utilizada

pelas células nas suas funções metabólicas e a energia luminosa é transformada em outro tipo de energia, a qual a célula é capaz de reconhecer e responder. Após as reações primárias de fotorrecepção, é desencadeada uma cascata de reações secundárias que ocorrem na ausência de luz e possuem papel amplificador das reações primárias (Karu, 1989; Labbe et al., 1990).

Nesse contexto, estudos mostram que a FBM acelera a migração de queratinócitos e estimula a síntese de diferentes proteínas, acelerando o processo de reparo tecidual e qualificando propriedades biomecânicas teciduais (Souza et al., 2011; Pellicioli et al., 2014; Wagner et al., 2016). Também tem sido demonstrado que a FBM é capaz de biomodular a expressão de cicloxigenase-2 (COX-2), fatores de necrose tumoral alfa (TNF-α), prostaglandina E2 (PG2) e interleucina 1-beta (IL-1β), portanto afetando a resposta inflamatória em modelos animais (de Jesus et al., 2015; Oliveira et al., 2017; Neves et al., 2018; Pigatto et al., 2019). Além disso, a FBM está relacionada a efeitos positivos no manejo da MO ao promover o reparo tecidual e desencadear efeitos anti-inflamatórios (Lopes et al., 2009; Lopes et al., 2010; Curra et al., 2015). Ensaios clínicos em humanos também tem apresentado inúmeros resultados positivos do uso da FBM, tanto na prevenção quanto no tratamento da MO (Schubert et al., 2007; Oton-Leite, 2015; Ferreira et al., 2016). Baseado nos diversos benefícios terapêuticos da FBM, a *Multinational Association of Supportive Care in Cancer* (MASCC) e a *International Society of Oral Oncology* (ISOO) recomendam a FBM como um método adjuvante eficaz para prevenção da MO em populações específicas de pacientes oncológicos (Zadik et al, 2019), dentre eles pacientes com CECO submetidos a RT e QRT.

A FBM representa, portanto, uma abordagem inovadora, não invasiva e não farmacológica. Com relação a segurança do uso do laser, um estudo do nosso grupo demonstrou que a irradiação com laser em baixa densidade de energia pode ser uma terapia segura para o aceleramento do reparo tecidual por não induzir dano no DNA e instabilidade genômica de células epiteliais humanas (Dillenburg et al., 2014). No entanto, os potenciais efeitos da FBM em células tumorais de CEC eventualmente presentes no campo de irradiação ainda não estão totalmente elucidados, com investigações acerca do tema apresentando resultados limitados e contraditórios (Schaffer et al., 1997; Sroka et al., 1999; Schartinger et al., 2012; Monteiro et al., 2011; Myakishev-Rempel et al., 2012; Liang et al., 2015; Rhee et al., 2016; Martins et al., 2020). Considerando que muitos pacientes com CECO

submetidos à terapia antineoplásica desenvolvem algum grau de MO, é crucial estabelecer dados científicos que definam a segurança do uso da FBM principalmente quando irradiada em regiões adjacentes ao tumor ou no tumor propriamente dito (Sonis et al., 2016). Estudos clínicos importantes apresentam resultados interessantes acerca deste tópico, demonstrando falta de impactos clínicos negativos ao avaliar pacientes com CCP submetidos à protocolos de FBM para MO (Antunes et al., 2017; Brandão et al., 2018; Genot-Klastersky et al., 2020). No estudo de Antunes et al. (2017), os autores avaliaram retrospectivamente pacientes com CCP tratados com QRT e submetidos a um protocolo de FBM. Os autores concluíram, a partir dos resultados de avaliação de sobrevida global e livre de doença, que a FBM pode melhorar a sobrevida dos pacientes avaliados. No estudo de Brandão et al. (2018), os autores avaliaram resultados relacionados com a terapia antineoplásica e com a recorrência tumoral em pacientes com CECO submetidos à FBM para MO. Os autores demonstraram resultados similares em termos de características clinicopatológicas e desfechos de sobrevivência nestes pacientes submetidos à FBM e pacientes de outros estudos prévios sem protocolo de FBM associado. Com isso, o estudo concluiu que o uso profilático da FBM não parece apresentar impactos negativos nos desfechos avaliados. Ainda nesse mesmo sentido, Genot-Klastersky et al. (2020) também concluíram falta de evidência de efeito negativo da associação terapêutica da FBM na avaliação da sobrevida global, do tempo de recorrência local e da sobrevivência livre de doença em pacientes com CCP submetidos à RT com ou sem QT. Finalmente, os resultados de uma revisão sistemática robusta realizada pelo nosso grupo também sugerem que a FBM não está relacionada ao desenvolvimento de desfechos negativos (Paglioni et al., 2019). Entretanto, faltam pesquisas pré-clínicas que avaliem os eventuais efeitos celulares e moleculares da FBM no CEC propriamente dito. Para isso, o uso de estudos animais com modelos tumorais de CEC é uma alternativa considerada para avaliar a questão.

No contexto de estudos em modelos animais e as diferentes possibilidades metodológicas, os modelos de xenoenxertos derivados de pacientes (PDX, do inglês *patient-derived xenograft*) são atualmente uma ferramenta importante para diversas avaliações pré-clínicas de tumores humanos (Lai et al., 2017). Os modelos de PDX são baseados no implante direto de fragmentos de tecido tumoral humano fresco em camundongos imunodeficientes, apontando como um modelo confiável para

pesquisas pré-clínicas, principalmente devido à retenção da heterogeneidade celular, arquitetura e características moleculares do tumor original (Garber, 2009; Tentler et al., 2012). Em um estudo de PDX com CEC, Pearson et al. (2016) demonstraram que a histopatologia dos tumores alteraram conforme as múltiplas passagens realizadas, mas as principais características morfológicas da neoplasia se mantiveram especialmente nas passagens mais iniciais. Nesse sentido, a literatura tem mostrado que os modelos de PDX representam uma ferramenta válida para pesquisa translacional em uma ampla variedade de tipos de tumores (DeRose et al., 2011; Wang et al., 2017; Yegodayev et al., 2020). Diante de todo o exposto, esta tese possui os seguintes objetivos principais: (1) avaliar o impacto da FBM no CECP por meio de uma revisão sistemática da literatura (RS) de estudos *in vitro* e *in vivo*; e (2) avaliar o impacto da FBM no CECO em um modelo animal de PDX.

2 ARTIGOS

2.1 Artigo: Examining tumor modulating effects of photobiomodulation therapy on head and neck squamous cell carcinomas.

Artigo publicado no periódico *Photochemical & Photobiological Sciences* (2019, 10; 18(7):1621-1637, doi: 10.1039/c9pp00120d) **(Anexo 1).**

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ABSTRACT

Photobiomodulation (PBM) therapy is an effective method for preventing and managing oral mucositis (OM) in head and neck squamous cell carcinoma (HNSCC) patients undergoing radiotherapy alone or in combination with chemotherapy. However, the potential effects of PBM therapy on premalignant and malignant cells eventually present in the treatment site are yet unknown. The aim of this systematic review was to analyze the effects of PBM therapy on HNSCC. A literature search was conducted in four indexed databases as follows: MEDLINE/PubMed, EMBASE, Web of Science, and Scopus. The databases were reviewed for papers published up to and including in October 2018. *In vitro* and *in vivo* studies that investigated the effects of PBM therapy on HNSCC were selected. From the 852 initially gathered studies, 15 met the inclusion criteria (13 *in vitro* and 2 *in vivo*). Only three *in vitro* studies were noted to have a low risk of bias. The included data demonstrated wide variations of study designs, PBM therapy protocols, and study outcomes. Cell proliferation and viability were the primary evaluation outcome in the *in vitro* studies. Of the 13 *in vitro* studies, seven noted a positive effect of PBM therapy on inhibiting or preventing an effect on HNSCC tumor cells, while six studies saw increased proliferation. One *in vivo* study reported increased oral SCC (OSCC) progression, while the other observed reduced tumor progression. Overall, the data from the studies included in the present systematic review do not support a clear conclusion about the effects of PBM therapy on HNSCC cells.

Keywords: Low-level light therapy; Squamous cell carcinoma of head and neck; Safety; Oral cancer; Oral mucositis; Systematic review.

INTRODUCTION

Head and neck squamous cell carcinoma (SCC) (HNSCC) treatment encompasses three modalities (surgery, radiotherapy, and chemotherapy) that can be administered exclusively or concomitantly with one another depending on the site of the cancer and the stage of the disease.[1,2] Use of the radiotherapy approach often leads to acute toxicities such as oral mucositis (OM), which is clinically characterized by painful ulcerations in the oral mucosa.[3,4] OM limits adequate nutritional intake, increasing the risk of malnutrition and poor quality of life, with the possibility of being a factor that increases overall treatment costs and negatively impacts cancer prognosis due to pain, bacteremia, and treatment interruptions.[5–7]

Photobiomodulation (PBM) therapy, also known as low-level light therapy, is one of the therapeutic approaches for OM management.[8–10] At this time, there are three well-described mechanisms of PBM therapy.[11] The first, involves an intracellular chromophore, cytochrome-C oxidase in the mitochondria; the second, cell membrane light-sensitive receptors such as opsins and TRPV1; and, the third, an extracellular latent growth factor, TGF-β1. In animal studies, PBM therapy has demonstrated positive effects on the management of OM by promoting tissue repair and anti-inflammatory effects.[12–15] Human clinical trials have also demonstrated positive results with the use of PBM therapy for preventing and managing OM.[16–18] Based on these therapeutic benefits of PBM, the Multinational Association of Supportive Care in Cancer (MASCC) and the International Society of Oral Oncology (ISOO) had designated PBM therapy as an effective adjunctive treatment for managing OM in 2013 (currently under revision).[8]

PBM therapy offers an innovative, noninvasive, and nonpharmacological approach for OM management. There have been no reports of any side effects and it is well-tolerated by tissues. However, the effects of PBM therapy on pre-transformed or residual primary tumor cells present in the laser treatment field are still being debated. Investigations regarding the effects of PBM therapy on neoplastic cells have yielded contradictory results.[19–25] Therefore, the safety of PBM therapy in HNSCC patients remains of major concern once laser treatments can be applied to tissues within, or contiguous to, a tumor site.[26] The aim of the present systematic review was to analyze studies that investigated the effects of PBM therapy on HNSCC cells.

MATERIALS AND METHODS

Protocol registration and focused question

This review was registered with the National Institute for Health Research's International Prospective Register of Systematic Reviews (*http://www.crd.york.ac.uk/PROSPERO* #CRD42017079588). We followed the Preferred Reporting Items for Systematic Reviews (PRISMA) guidelines.[27] The specific question for this review was: "*What are the effects of photobiomodulation therapy in head and neck squamous cell carcinoma*?"

Search strategy

The research was constructed according to the Populations, Interventions, Comparison, Outcomes, and Study Design (PICOS) principle. Individual search strategies were designed for each of the following bibliographic databases: MEDLINE/PubMed, EMBASE, Web of Science and Scopus. The four named electronic databases were searched to identify relevant articles published up to and including in October 2018. All publications included were in the English language only, with no restrictions on year of publication. All publications presented in these databases contained a combination of controlled predefined Medical Subject Heading (MeSH) terms and free terms related to PBM therapy in HNSCC, using Boolean operators (i.e., OR, AND) to combine searches. Previously defined terms were adapted to the rules of syntax of each bibliographic database and included (((((((“tumor cells, cultured” [MeSH terms]) OR “neoplastic stem cells” [MeSH Terms]) OR “tumor stem cells”) OR “neoplasms”) OR “tumor”)) AND (((((((“low-level light therapy” [MeSH Terms]) OR “low-level laser therapy”) OR “laser therapies, low-level”) OR “irradiation, low-power laser” OR “laser phototherapy”) OR “therapies, photobiomodulation”) OR “phototherapy, laser”)) AND ((((“cell proliferation” [MeSH terms]) OR “cell growth number”) OR “tumor growth”) OR “stimulatory effect”). Additionally, a manual search of bibliographies and reference lists of all included studies were performed to identify any publications not previously retrieved as part of the primary database searches.

Eligibility criteria

Inclusion criteria. This systematic review was based only on the contents of original research studies investigating the effects of PBM therapy on HNSCC. Study inclusion criteria were as follows: those that contained (1) study population(s) with HNSCC neoplastic cells and/or HNSCC tumors; (2) PBM therapy as an intervention; (3) no treatment as a comparison group; and (4) effects of PBM therapy on the treated population as outcomes.

Exclusion criteria. Review papers, letters to the editor, monographs, conference papers, book chapters, unpublished data, and studies published in a language other than English were all excluded. Separately, original research studies were excluded when: (1) PBM therapy was used along with other types of cancer treatments; (2) light therapy was performed with the use of external cromophores, such as in photodynamic therapy; (3) PBM therapy was not used as a treatment; and/or (4) the population(s) assessed were not HNSCC-related.

Study selection and data extraction

Titles and abstracts of all studies were reviewed and, based on the eligibility criteria, full texts were retrieved for complete review. Two reviewers (F. M. S. and M. D. M.) reviewed all of the papers independently and any disagreements were discussed with a third reviewer (A. R. S. S) for concordance. The following relevant information from eligible studies was collected: (1) publication details (first author and year); (2) samples [cell line(s) or animal model(s)]; (3) samples' characteristics (for *in vitro* studies: number of cells, darkness, distance between wells, reproducibility, growth medium; and for *in vivo* studies: environmental conditions, tumor induction, groups); (4) types and methods of evaluations; (5) main outcomes; and (6) major conclusions. Specific attention was focused on laser treatment parameters, as follows: (1) active medium; (2) application procedure; (3) wavelength (in nanometers or nm); (4) energy density (also called fluence, in Joules per square centimeters or J/cm^2); (5) power (in milliwatts or mW); (6) power density (also called irradiance, in mW/cm^2); irradiation time (in seconds); (7) spot size (in cm^2); (8) energy per point (in J); (9) schedule of irradiation; and (10) total energy (in J). These laser parameters analyzed were based on the consensus agreement of the design and the conduct of studies recommended by the World Association for Photobiomodulation Therapy (WALT). All the papers selected were organized using EndNote (Clarivate Analytics, Philadelphia, PA, USA).

Risk of bias assessment

For the assessment of bias, included studies were separated into *in vitro* or *in vivo* investigations. For the methodological quality of each *in vitro* study, criteria based on the parameters for developing cell culture studies were adopted.[28] The included articles were evaluated according to the following descriptions: (1) condition of cell culture; (2) description of methodology to evaluate outcomes; (3) reproducibility; (4) methods for preventing unintentional light scattering during laser application; (5) description of laser treatment parameters according to WALT recommendations; and (6) concurrence of conclusions based on the results obtained. The information was classified as *Yes* when it was possible to find the information or *No* if the information was not described, respectively. The publications were classified according to their risk of bias as "high" (one or two items classified as *Yes*), "medium" (three or four items classified as *Yes*), or "low" (five or six items classified as *Yes*). Regarding *in vivo* studies, the Systematic Review Centre for Laboratory Animal Experimentation's (SYRCLE) risk of bias tool was used to assess the quality of available evidence.[29] The items here were scored as *Yes*, *No*, or *Unclear*.

Statistical analysis

Due to a lack of methodological uniformity in the included studies, a meta-analysis of the obtained results was not feasible. Therefore, the results are instead descriptively summarized in this review.

RESULTS

Study selection

A total of 852 potentially relevant records were identified from the databases and further processed as per the PRISMA statement (**Figure 1**).[27] After the removal of duplicates, 581 records were further examined based on their titles and abstracts and 505 studies were excluded, as they did not meet the specific eligibility criteria for this study. A total of 76 full-text articles were finally evaluated and 64 were subsequently excluded for the following reasons: (1) PBM therapy was investigated in cell lines or tumors other than HNSCC (44 papers); (2) PBM therapy was associated with another type of treatment such chemotherapy or photodynamic

therapy (16 papers); (3) SCC cell line was irradiated with CO_2 laser (one paper); or (4) the paper was a review paper (three papers). Three studies were further included through a manual search of the bibliographies of included studies. A total of 15 studies fulfilled the selection criteria of the present review and were included for qualitative analysis.[19–23, 30–39]

General characteristics of included studies

Of the 15 included studies, 13 were restricted to *in vitro* analyses, while two studies performed *in vivo* studies. The relevant studies were conducted in various centers around the world such as Brazil, Germany, Austria, Italy, and Taiwan and were published between 1997 and 2018 (**Figure 2**). The general descriptions of the included studies are summarized (**Tables 1a,** *in vitro* studies and **Table 1b,** *in vivo* studies). The 13 *in vitro* studies evaluated various HNSCC cell lines arising at specific anatomical sites such gingival mucosa (ZMK and ZMK1), larynx (HEp-2), KB (human papillomavirus–infected), SCC9 (tongue), SCC25 (tongue), and OC2 (buccal mucosa). Among these, 12 (92.3%) reported the number of cells used in the experiments, three (23.0%) reported ambient light conditions during PBM therapy treatments, five (38.4%) reported the distance between irradiated wells, seven (53.8%) reported replicates, and 12 (92.3%) described the growth medium used. Several methods were employed to assess tumor cell proliferation or viability from dye exclusion utilizing Trypan blue or neutral red to BrdU incorporation as well as enzymatic substrate cleavage such as MTT, WST-1, or AlamarBlue assays. Some studies also assessed the mitotic index via orcein staining and microscopy or propidium iodide staining and FACS. Tumor cell death was assessed using annexin staining and FACS, TdT-mediated dUTP nick-end labeling (TUNEL) staining, or caspase-3 activity. The ability of tumor cells to migrate or invade was assessed with a scratch-wound assay or Transwell chambers, respectively. One study further examined invasiveness by investigating the osteoclastogenic response using TRAP activity, interleukin-11 (IL-11), and parathyroid hormone-related protein (PTHrP) gene expression, respectively.[37] Another study assessed reactive oxygen species (ROS) production by tumor cells using FACS.[23] Both included *in vivo* studies utilized carcinogen-induced tumor models with 7,12-dimethylbenz[a]anthracene (DMBA) in golden Syrian hamsters or 4-nitroquinoline-1-oxide (4-NQO) in mice. These studies examined the effects of PBM therapy on induction. The detailed description of animal

studies that were conducted was provided only in one of the research papers.[38] These studies used histopathology and immunohistochemistry for tumor assessment.

PBM treatment parameters used in these studies

There is still an incomplete understanding of the critical PBM treatment characteristics for effective therapeutic clinical dosing. All of the parameters used in the included 15 studies are summarized and outlined below (**Table 2**).

***Laser source*:** The laser source was specifically reported in six (40%) studies as gallium–aluminum–arsenide diode. One study employed an argon laser. The remaining studies did not explicitly specify the laser source(s) they used; however, the wavelengths reported suggested they all incorporated diode lasers.

***Wavelength*:** Five studies used only one wavelength within the visible red spectrum (630–670 nm) and three used the wavelength of 660 nm. Two studies compared the effects of application at 635 nm and 670 nm. Near-infrared wavelengths (780, 808, 830, and 850 nm) were also investigated either by themselves or in combination with visible red wavelengths.

***Beam characteristics*:** Nine studies (60%) used the laser in continuous-wave (CW) mode, while the rest did not report on this parameter. Five studies reported the distance between the laser source and the cells or tissue (two treatments were in contact mode, whereas the other three used noncontact mode. In the latter studies, distance between the laser source and cells varied from 0.5–2 cm).

***Power, spot size and power density (irradiance)*:** The maximal laser power was reported in 12 studies and varied from 5 to 25 W. Power density is the effective power output at a given surface area and is reported in mW/cm^2. This parameter was reported in nine studies and varied from 0.39 to 1,000 mW/cm^2. The actual illuminated surface is deemed as the spot size and can be as small as the size of the laser probe tip itself when used in contact mode. Alternatively, in non-contact mode, the spot size were calculated as the effective illuminated treatment area. Seven of 15 studies reported spot sizes varying from 0.039 to 0.8 cm^2.

***Treatment time and schedule*:** Although the time is a critical factor in dose estimation, surprisingly, only eight of the 15 studies reported this parameter, with findings varying from 8.2 to 450 seconds. Also, nine of the 15 studies informed on the number of treatment sessions and intervals, which ranged from one to seven days of consecutive treatments.

***Energy, energy per point, total energy, and energy density (fluence)*:** Two studies mentioned total energy, while only one reported on energy per point. All, but one, study reported energy density that varied from 0 to 60 J/cm^2.

Risk of Bias

Among the 15 included studies, five *in vitro* studies were classified as having a high risk of bias, while five had a medium risk and three had a low risk (**Table 3a**). The two *in vivo* studies appeared to have a medium risk of bias as per the SYRCLE's risk of bias assessment criteria (**Table 3b**).

Major outcomes regarding HNSCC following PBM treatments

Of the 13 *in vitro* studies, seven noted a positive effect of PBM therapy on inhibiting or preventing an effect on HNSCC tumor cells, while six studies saw increased proliferation. One *in vivo* study reported increased oral SCC (OSCC) progression, while the other observed reduced tumor progression (Fig. 3). The following sections presents further details and relevant descriptions of outcomes of individual studies grouped by their overall outcomes.

***In vitro* studies with positive impacts of PBM therapy on HNSCC cells**

Schaffer et *al.* and Sroka et *al.* analyzed the effects of PBM therapy on human SCC cells of the gingival mucosa (ZMK and ZMK1).[19,20] Based on the outcomes of Orcein and BrdU staining, both studies did not observe any significant change in the mitotic index of tumor cells when PBM treatments (630, 635 and 805 nm lasers; 50-150mW/cm^2; 20 J/cm^2) were compared with nontreated controls. Schartinger et *al.* investigated the effects of 660-nm PBM therapy on proliferation, cell cycle distribution, and apoptosis in human oral carcinoma cells (SCC25), a nonmalignant bronchial epithelial cell line, and periodontal-derived normal fibroblasts.[21] This study observed that PBM treatments at irradiances of 0.39 to 63.7 mW/cm^2 for 15 minutes resulted in fibroblast proliferation but reduced the cell viability of epithelial and SCC cells, as observed with the MTT assay. Examining the cell cycle with propidium iodide and FACS analyses, the authors reported an increased percentage of S-phase SCC cells that also demonstrated increased apoptosis with annexin V staining. Sperandio et *al.* separately examined the effects of 660- and 780-nm PBM therapy on three cell lines, specifically dysplastic oral keratinocytes, SCC9, and SCC25.[35]

These investigators showed that PBM therapy (40 mW; 2.05, 3.07, or 6.15 J/cm^2) reduced cell viability in all three cell lines. They also noted that the expressions of Akt, HSP70, S6, and cyclin D1 were significantly modulated by PBM treatments and correlated with reduced overall survival and increased apoptosis of tumor cells.

Another study by Liang et *al.* examined the effects of another popular near-infrared PBM wavelength (810 nm) on a human oral cancer cell line (OC2) and normal human gingival fibroblast cells.[23] PBM therapy was performed at varying fluences from 0 to 60 J/cm^2 and tumor cells were noted to have reduced viability along with an increased cell count in the G_1 and sub-G_1 cell cycle phases. Further, ROS production, reduced matrix metalloproteinase (MMP), and increased caspase-3–mediated apoptosis were evident in PBM-treated OC2 cells. In subsequent studies by Schalch et *al.* similar effects were observed with 660- and 780-nm PBM treatments on SCC9 cells at 4J/cm^2.[37,39] These investigations also noted the existence of reduced tumor cell viability, increased apoptosis, and reduced cell migration. Further, Schalch et *al.* reported the ability of PBM-treated SCC9 cells to lower tartare-resistant acid phosphatase (TRAP), positive osteoclastic activity via reduced IL-11, and PTHrP concentration.[37]

***In vitro* studies with negative impacts of PBM therapy on HNSCC cells**

Two studies by Pinheiro et *al.* and one by Werneck et *al.* used the HEp-2 SCC cell line and analyzed cell proliferation using the MTT assay. [30,31,34] These studies reported the occurrence of a significant increase in cell proliferation following treatments with 635, 670, 685, and 830 nm as compared with non-treated controls. A similar response was reported by Kreisler et *al.* who used PBM therapy with 809-nm laser treatments on human larynx carcinoma cells and assessed proliferation using the AlamarBlue assay.[32] Interestingly, Castro et *al.* assessed the proliferation of oral KB carcinoma cells treated with 685 and 830 nm and noted increased tumor cell proliferation with the MTT assay.[33] Finally, Henriques et *al.* used PBM therapy with application of 660 nm of energy on a tongue SCC cell line (SCC25) and observed an increased proliferation of tumor cells.[36] They performed cell cycle analyses where a predominant cell subpopulation in the S and G2/M phases was observed. This correlated with findings of increased cyclin D1 and β-catenin and decreased MMP-9 expression that correlated with an increased invasive potential of these tumor cells.

In vivo studies using PBM therapy on HNSCC tumors

Two *in vivo* studies examined the effects of PBM therapy on OSCC tumors in animal models. Monteiro et *al.* evaluated the effect of 660-nm PBM therapy on chemically induced cancer [9,10-dimethyl-1,2-benzanthracene and DMBA three times a week for eight weeks] of the oral mucosa of golden Syrian hamsters.[22] At the end of eight weeks of cancer induction, one group of animals was sacrificed and examined histologically using a World Health Organization (WHO) grading system. All studied animals had developed well-differentiated SCC. Two additional groups of animals were then either observed for four weeks or treated with PBM therapy with irradiance at 424 mW/cm^2 for 133 seconds every other day for four weeks, for a total fluence value of 56.4 J/cm^2 per treatment session. While the PBM-treated groups demonstrated 40% well-differentiated SCC, 20% moderately-differentiated SCC, and 40% poorly-differentiated-SCC findings, the non-PBM–treated group demonstrated 80% well-differentiated-SCC and 20% moderately-differentiated-SCC outcomes, with a statistically significant difference. Based on these observations, the authors concluded that PBM therapy might promote a progression of the severity of SCC *in vivo*.

In contrast, Ottaviani et *al.* explored the effects of PBM therapy in various cultured cells and *in vivo* models of cancer.[38] Among them, one of their studies involved a 4NQO-induced (16 weeks in drinking water) OSCC model in C57BL/6 female mice. One group of these animals was treated with PBM therapy (970 nm; 200 mW/cm^2 for 30 seconds four times a week for four weeks; 6 J/cm^2). Histopathological examination demonstrated that PBM therapy significantly reduced the incidence of dysplastic lesions as well as the number and percentage of both *in situ* and invasive SCCs in these animals in comparison with among non-PBM–treated controls. Immunohistochemical analyses of tumor samples using CD31 and αSMA staining noted more regular and structured tumor vasculature patterns. Based on these observations, the authors concluded that PBM therapy inhibits OSCC tumor progression.

DISCUSSION

There is strong evidence supporting the use of PBM therapy as an effective treatment in OM management associated with oncotherapy for head and neck cancer (HNC) or other malignances.[8,40,41] However, due to the reported stimulatory

biological effects of PBM therapy on various tissues, the safety of PBM therapy appears to stlll being debated.[26,42] Two recent clinical studies analyzed the impact of PBM therapy used for the prevention of OM through different tumor outcomes in HNC patients.[6,43] Antunes et *al.* retrospectively evaluated the overall, disease-, and progression-free survival of 94 HNC patients submitted to PBM therapy (λ = 660 nm, 100 mW, 1 J, 4 J/cm^2) to prevent OM.[6] Their study demonstrated that patients receiving PBM therapy had a statistically better treatment response, displayed increase in progression-free survival, and a tendency for better overall survival when compared with the placebo group. Brandão et *al.* examined outcomes of cancer therapy and the incidence of tumor recurrence in locally advanced OSCC patients treated with PBM therapy (λ = 660 nm, 40 mW, 0.4 J, 10 J/cm^2) for OM.[43] The authors concluded that the prophylactic PBM therapy did not impact treatment outcomes of the primary cancer, recurrence, new primary tumors, or survival of the patients. Specifically, the effects of PBM therapy on residual or dormant tumor cells in cancer patients remain a concern. This fact motivated the current systematic review to examine the literature regarding the effects of PBM therapy on HNSCC. A total of 13 *in vitro* and two *in vivo* studies were finally included. Unfortunately, the analysis of the 13 *in vitro* studies revealed significant variations in cell lines, culture conditions, methodological designs, PBM parameters, and evaluation methods. Moreover, five studies presented high risks of bias, another five were noted to have a medium risk of bias, and only three studies demonstrated low risk of bias. The two *in vivo* studies appeared to have a medium risk of bias due to the lack of adequately reported study parameters. Therefore, the overall conclusions of this review acknowledge important methodological limitations that compromise the reliability and direct significance of the data analyzed.

Summary of *in vitro* results

Our analyses showed that investigators used a broad range of HNSCC cell lines and methods to examine the effects of PBM therapy. Overall, six studies reported increased tumor cell proliferation and five studies noted tumor cell inhibition following PBM therapy. There appears to be some evidence that tumor cell viability could be enhanced or diminished depending on the precise culture conditions and laser treatment parameters used (**Table 4**)**.** Tumor cells from various anatomical niches and various transformation processes including spontaneous (oral tumors),

chemically induced (DMBA or 4NQO), and virally induced (EBV) were used in these studies, which might contribute to variations in PBM responses. Also, several studies reported opposite responses of normal versus tumor cell types in their results, which could be attributed to their underlying differences in basal transcriptional and pathophysiological statuses.[44] Schalch et *al.* observed that cell seeding density appeared to be a major factor in determining precise tumor cell response, as they noted SCC9 plated in lower (10^3 cells/well) densities showed consistent increased cell proliferation, while plating cells at higher (10^4 cells/well) densities resulted in a lowered viability of tumor cells post-PBM treatments as compared with non-PBM–treated cells.[37]

Another important aspect to consider in these studies is timing of both the outcomes analyses as well as the repetition of PBM treatments. One of the best-understood processes of PBM therapy is the direct absorption of light by the mitochondrial chromophore cytochrome-C oxidase, which results in increased ROS and adenosine triphosphate (ATP) levels. This induces concerted signaling and transcriptional pathways over a period of several hours to days that are capable of modulating tumor cell functions.[45] Moreover, differences in tumor metabolism, oxidative stress status, time of treatments, and repetitions of treatments would all be expected to result in discrete differences in PBM responses assessed by various assays.

Several outcomes methodologies were employed in these studies to assess tumor cell viability, proliferation, and apoptosis. It must be emphasized that, while these cellular characteristics are intimately related, they are discrete from each other and so must be evaluated individually. For example, the study by Schalch et *al.* used three discrete methods namely, MTT assay (mitochondrial activity), neutral red assay (cell viability), and incorporation of BrdU (proliferation) to assess SCC9 cells following PBM therapy.[39] They observed that PBM therapy reduced mitochondrial activity and cell viability but did not interfere with cell proliferation.

Other cellular response mechanisms evaluated in these studies include apoptosis, migration, and invasion. Four studies examined tumor cell apoptosis using activated caspase-3, annexin V, or TUNEL. All studies reported the finding of increased tumor cell apoptosis following PBM treatments. A recent review suggested that the increased ATP within the cancer cells might also promote energy-dependent cell death pathways.[44,46] Three studies examined the effects of PBM therapy on

tumor cell migration and, while two studies reported no differences, one study did observe increased invasion. Interestingly, two of these divergent studies, by Henriques et *al.* and Schalch et *al.* respectively, used comparable PBM parameters (660 nm, CW, ~30 mW and ~ 16 s) for all but one aspect and also examined other markers of tumor cell invasion such as E-cadherin, MMP, IL-11, and PTHrP expression that correlated with their invasive phenotypes.[36,37] A major difference between these two studies, however, is their use of two different OSCC cell lines (SCC25 versus SCC9). Also, the positively correlated study used higher doses (> 1 J/cm^2) as compared with the other. However, this minimal difference in these two studies does not allow for firm conclusions to be made; more comparable studies are needed.

Perhaps a more thorough understanding of the cellular response to PBM treatment could be particularly gleaned by cell-cycle phase analyses that would outline all three cellular responses—namely, viability, proliferation, and apoptosis. Henriques et *al.*[36] reported increased proportions of cells in the S and G2/M phases at all time points with 660-nm PBM treatments (30 mW and 1 J/cm^2) as compared with controls and PBM treatments at 0.5 J/cm^2. On the other hand, Liang et *al.*[23] found that PBM therapy with 810 nm (1000 mW/cm^2 and 60 J/cm^2) resulted in G_1 arrest and increased cell death in human oral cancer cells. While their use of different PBM wavelengths and doses is a clear confounder, making it difficult to extrapolate these observations, these authors also used two distinct cell lines (SCC25 versus OC2) with varying growth characteristics, further increasing the difficulty of applying their observations broadly. We attempted to compare the most similar studies in terms of cell lines (SCC25) and PBM treatment parameters (660 nm, ~30 mW/cm^2) that reported opposite effects. We noted several differences in initial cell seeding, media supplements (presence of dexamethasone), treatment repetitions, and outcomes assessments that could all account for variations in tumor cell responses to PBM therapy. Hence, there is a significant value of using *in vitro* systems to analyze tumor cell responses to PBM therapy, but more attention is necessary for elucidating appropriate biological and PBM treatment parameters for appropriate interpretations.

Summary of in vivo results

Cell cultures have been extensively used since the early 1900s and human cancer-derived cell lines are among the most widely used models to study cancer biology. However, conventional two-dimensional cell cultures poor mimic pathophysiological conditions within living organisms and have limited heterotypic cellular interactions.[47] Thus, the application of standardized preclinical *in vivo* models in animals is necessary to circumvent limitations of such *in vitro* approaches. Of note, for this review, we could only find two studies examining the effects of PBM therapy on OSCC in animal models.[22,38] Strikingly, both studies appear to show opposite outcomes. While both studies used chemically-induced oral carcinogenesis models, they employed different animal models (hamsters versus mice) and chemical carcinogens (DMBA versus 4-NQO). Further, PBM treatments were performed with different wavelengths (660 versus 970 nm), irradiances (424 versus 200 mW/cm^2), treatment times (133 versus 30 seconds), and repetitions (every two days versus every day) for four weeks. Unfortunately, these significant differences do not allow for a rigorous comparison of the contrasting effects of PBM therapy on tumor cells and, hence, more standardized studies are necessary.

Significant variations in PBM parameters

Clinically effective PBM therapies have been noted to have a few key characteristics that can be broadly divided into the categories of device parameters and treatment delivery parameters.[11] Device parameters include wavelength, mode (continuous-wave or pulsing), polarization, power density, treatment time, and energy density. PBM treatment delivery parameters include probe–target distance (also called treatment surface irradiance) and stationary or probe-scanning movements.[48] PBM treatments of normal cells have been known to follow the Arndt–Schulz law, where low doses do not cause any effect but optimal doses within a therapeutic window generate a therapeutic response and high doses reverse these beneficial effects, respectively. The complexity of the light-biological tissue interactions has prevented comprehensive description of PBM dose variables. Hence, the current consensus is to document and report as many treatment variables as feasible.[49,50] It would seem reasonable to expect tumor cells may not follow this dose-response and therefore careful attention is necessary for PBM treatments of premalignant and tumor tissues.[44] A common error noted in several of these studies is the use of a normal cell line as a control, but of varying (unmatched) anatomical origin. There is

growing evidence that cells of distinct lineages require specific PBM doses to evoke therapeutic responses.[51]

Unfortunately, as evident in this review (**Table 2**), there is a significant lack of attention when reporting on PBM treatment parameters. The most commonly reported parameters included wavelength, power or power density, treatment time, and energy or energy density. The wavelength refers to the physical distance between two successive photonic waves and determines several key PBM characteristics such as absorption by specific biological chromophores and depth of laser penetration. The PBM wavelength was reported by all studies and ranged from visible (red, 630 nm) to near-infrared (970 nm) spectrum. There appears to be significant variations in power density (50 to 1000 mW/cm^2), treatment times (8.4 to 900 seconds), and energy density (0.04 to 60 J/cm^2). It is prudent to emphasize that some of the treatments were performed only once, while some were repeated on alternate days or every day for up to four weeks. These variations were not explained in any of the included studies. Therefore, the data do not allow for the elaboration of consistent parameters that might provide insights into PBM treatment effects on tumor cells. Nonetheless, these reported parameters are within the MASCC and ISOO recommendations of using the wavelengths of 633 to 685 nm or 780 to 830 nm with power outputs of between 10 and 150 mW and energy densities of 2 to 4 J/cm^2 (but no more than 6 J/cm^2).[8,52] In summary, future studies should pay close attention to promoting standardization and on detailed reporting of the parameters used for PBM treatments.

Clinical Implications and conclusions

These analyses clearly demonstrate it is imperative to perform better *in vitro* and *in vivo* studies in relevant animal models to examine the effects of PBM therapy on HNSCC. Not only is this critical for our understanding of fundamental tumor mechanisms but also it is practically relevant to clinical safety for the increasingly popular use of PBM therapy in OM prevention and/or treatment in HNSCC patients. There are tantalizing early reports on the use of PBM therapy in the prevention and management of malignancy-related comorbidities as indicated in two recent publications.[6,43] Both studies noted a positive correlation of PBM therapy in reducing OM incidence with no significant adverse events. Further, somewhat surprisingly, a statistically significant improvement in treatment responses represented by an

increase in progression-free survival and a tendency for better overall survival was observed as compared with the control groups.

In conclusion, this review clearly noted a lack of uniformity in experimental protocols and PBM treatment parameters that indicate that the current effects of PBM therapy on tumor cells remain equivoc*al.* While the clinical safety of PBM therapy remains debatable, the available clinical evidence for its use as an adjunctive supportive therapy for OM and other treatment complications must be taken with caution. Thus, clinicians should remain aware of the risks when treating HNSCC patients and should avoid direct PBM treatment of suspicious malignant sites or frank tumors. It is strongly suggested that well-delineated studies, mainly based on *in vivo* models followed by human clinical trials, must be pursued to better evaluate the effects of PBM therapy on HNSCC patients.

CONFLICT OF INTEREST

There are no conflicts to declare.

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TABLE LEGENDS

Table 1. Description of experimental details of *in vitro* (a) and *in vivo* (b) studies included in this review.

Table 2. Parameters used for PBM treatments in studies included in this review.

Table 3. Criteria used to assess risk of bias in *in vitro* (a) and *in vivo* (b) studies.

Table 4. Outline of key parameters identified in this review that could contribute to the variances in observed results for effects of PBM therapy on tumor cells.

FIGURE LEGENDS

Figure 1. PRISMA flow diagram for systematic search and studies selection strategy.

Figure 2. Distribution of the included studies according to its respective countries.

Figure 3. Summary of the results from the included studies.

Table 1a. Description of experimental details of in vitro studies included in this review.

Study; Year	**Samples** *Cell line / Animal model*	**Study design** ***1.****Number of cells;* ***2.****Darkness;* ***3.****Distance between wells;* ***4.****Reproducibility;* ***5.****Growth medium.*	**Type of evaluation / Method**	**Main outcomes**	**Main conclusions**
Schaffer et al., 1997 [19]	Human SCC of the gingival mucosa (**ZMK**)	**1**. Not mentioned **2**. Not mentioned **3**. Not mentioned **4**. Yes **5**. Not mentioned	Mitotic index by Orcein staining DNA-synthesis by BrdU-test	ZMK tumor cells showed a non-significant decrease of the mitotic index compared to control in different irradiances The irradiation had no influence on the DNA synthesis rate in all groups	PBM therapy promoted an inhibition of human SCC tumor cells
Sroka et al., 1999 [20]	Human SCC of the gingival mucosa (**ZMK1**)	**1**. Yes. **2**. Not mentioned. **3**. Not mentioned. **4**. Yes. **5**. Yes.	Cell proliferation by BrdU-test Rate of mitosis by Orcein-staining	Cell treatment with λ= 805 nm had no influence on the DNA-synthesis rate ZMK1 cells exhibited similar results using λ = 630 nm, λ = 635 nm, λ = 805 nm. Decrease in the mitotic rate when exposed to light with 2-8 J/cm^2 and remained stable up to 20 J/cm^2. There was no change in the mitotic rate in dependency of the irradiance	PBM therapy resulted in an inhibition of human SCC tumor cells
Pinheiro et al.[a], 2002 [30]	SCC of the larynx (**H.Ep.2 cells**)	**1.** Yes **2.** Not mentioned **3.** Yes **4.** Not mentioned **5.** Yes	Cell proliferation by MTT method	The 670nm group showed a tendency to increase cell proliferation when compared to control *(p=0.014*) and 635nm group (*p=0.004)*. Control and 635nm groups were similar *(p=0.455*)	Cell proliferation increases in H.Ep.2 cells irradiated with 670nm. Dose and wavelength may affect cell proliferation
Pinheiro et al.[b], 2002 [31]	SCC of the larynx (**H.Ep.2 cells**)	**1.** Yes **2.** Not mentioned **3.** Yes **4.** Not mentioned **5.** Yes	Cell proliferation by MTT method	*S*ignificant differences in the proliferation were observed between the two concentrations of FBS (*p*=0.002) and between irradiated cultures and controls. Influence of the nutritional status of the culture of both 670nm and 635nm irradiated cultures was significantly different. The effect of the wavelength was also demonstrated at the same %FCS (*p*=0.000)	Irradiation with 670nm applied at doses from 0.04 J/cm^2 results in an increased cell proliferation
Kreisler et al., 2003 [32]	Human larynx carcinoma cells	**1.** Yes **2.** Not mentioned **3.** Not mentioned **4.** Not mentioned **5.** Yes	Proliferation activity by Alamar Blue Assay	After 24h and 72h, the irradiated cell cultures showed a higher proliferation activity compared to controls in all irradiation regimens	809nm PBM therapy had a considerable stimulatory effect on the cell proliferation
Castro et al., 2005 [33]	Oral carcinoma cells, strain KB	**1.** Yes **2.** Not mentioned **3.** Yes **4.** Not mentioned **5.** Yes	Cell proliferation by MTT method	Cultures irradiated with λ = 830nm exhibited increased proliferation than control (from 24h until 72h). The results demonstrated that time influenced significantly both controls and cultures irradiates with λ = 685nm and λ = 830nm	Positive biomodulatory effect of PBM therapy on the proliferation of KB cells. It was influenced by the wavelength

Werneck et al., 2005 [34]	SCC of the larynx (**H.Ep.2 cells**)	**1.** Yes **2.** Not mentioned **3.** Yes **4.** Not mentioned **5.** Yes	Cell proliferation by MTT method	Cultures irradiated with λ = 685- or λ = 830 nm wavelengths had increased cellular proliferation compared to non irradiated controls. Time had a significant effect on the proliferation of samples irradiated by λ = 685 nm	Positive biomodulatory effect of PBM on H.Ep.2 cells irradiated by λ = 685- and λ = 830 nm lasers compared with controls non irradiated samples
Schartinger et al., 2012 [21]	Human oral SCC cell line (**SCC25**).	**1.** Yes **2.** Not mentioned **3.** Not mentioned **4.** Yes **5.** Yes	Cell proliferation by MTT method	Lower absorbance was observed after PBMT treatment of SCC-25 than the sham controls (*p<0.001*). PBMT induced a significant 0.8-fold decrease in the level of proliferation in SCC-25 cells	No proliferative or antiapoptotic effects of PBM on SCC cells were observed
			Cell cycle analysis by FACS analysis (PI DNA staining)	PBM induced an increase in the percentage of S-phase in SCC-25 (*p<0.001*). The increase in S-phase cells paralleled the decrease in G1-phase	
			Apoptosis assay by FACS analysis (Annexin V-FITC)	In SCC-25 cells, the relative amount of Annexin V^+ cells was higher in laser treated cultures than in the controls (*p=0.02*)	
Sperandio et al., 2013 [35]	Oral SCC cell lines (**SCC9** and **SCC25**).	**1.** Yes **2.** Not mentioned **3.** Not mentioned **4.** Not mentioned **5.** Yes	Cellular viability by MTS assay	SCC9 lines presented general enhanced cell viability (λ = 780 nm) and pronounced inhibition of growth (λ = 660 nm). SCC25 lines showed growth stimulation at some fluences (λ = 660 nm and λ = 780 nm). SCC9 and SCC25 had a tendency to show lower levels of cell viability at the latest evaluation time point (72h)	PBM therapy can modify SCC9 and SCC25 cell lines growth by modulating the Akt/mTOR/CyclinD1 signaling pathway. PBM significanty modified the expression of proteins related to progression and invasion in all the cell lines and could aggravate oral cancer cellular behavior. Apoptosis was detected for SCC25
			Protein analysis by western blot and immunofluorescence	The Akt, pAkt, Hsp90, S6, CyclinD1, β-actin were influenced by PBM. PBM increased the expression of pAkt, pS6 and cyclin D1 and produced an aggressive isoform of Hsp90	
			Apoptosis assay by TUNEL	Apoptosis was only detected in SCC25 cell line irradiated with λ = 780 nm, 6.15 J/cm^2 at 48h and 3.07 J/cm^2 at 72h	

Henriques et al., 2014 [36]	Human tongue SCC (**SCC25**)	**1.** Yes **2.** Yes[a] **3.** Yes **4.** Yes **5.** Yes	Cell growth by Trypan blue	After 24h, SCC25 cells irradiated with 1.0J/cm^2 showed the highest proliferation when compared to the control and the group irradiated with 0.5J/cm^2 (p=0.019)	PBM therapy stimulated the proliferation and invasion of SCC25 cells in a dose- and time-dependent manner, influencing the expression of cyclin D1, B-catenin, E-cadherin and MMP-9
			Cell cycle by flow cytometry (PI)	After 24h, all groups showed a reduction in the number of cells in the G0/G1 phase with an increase in the S and G2/M phases. $L_{1.0}$ demonstrated a more pronounced difference (*p=0.027*) and the control group the lowest proportion of cells in the S and G2/M phases (p = 0.027). Laser-irradiated groups showed generally constant or slightly higher proportion of cells in the S and G2/M phases compared to control. $L_{1.0}$ presented the highest proportion of cells in the G2/M phase throughout the experiment (p = 0.027)	
			Protein analysis (cyclin D1, β-catenin, E-cadherin, MMP-9) by immunofluorescence and flow cytometry	PBMT influenced the expression of cyclin D1, β-catenin, E-cadherin and MMP-9. Cyclin D1 and nuclear β-catenin demonstrated an increased expression. PBMT at 1.0 J/cm^2 significantly reduced E-cadherin and induced MMP-9 expression	
			Invasion assay by transwell chamber	A significantly higher invasion potential was observed for $L_{1.0}$ when compared to control and $L_{0.5}$ group ($p < 0.001$)	
Liang et al., 2015 [23]	Human oral cancer OC2 cells (**OC2**)	**1.** Yes **2.** Not mentioned **3.** Not mentioned **4.** Yes **5.** Yes	Cell viability assay by WST-1	PBMT significantly diminished cell viability of OC2 cells	PBM therapy induced apoptosis in human oral cancer cells, possible mediated by ROS production and the loss of MMP
			Cell cycle by FACScan flow cytometer (PI)	PBMT increased the number of OC2 cells in G_1 and subG_1 phases	
			ROS production measurement by FACScan flow cytometer (H_2DCFDA)	The production of ROS was significantly elevated in irradiated OC2 cells	
			MMP detection by FACScan flow cytometer (CCCP)	MMP was lost in irradiated OC2 cells	
			Apoptosis analysis by FACScan flow cytometer (Annexinv V$^+$)	PBM increased the number of apoptotic cells in OC2 cells	
Schalch et al., 2016 [37]	Human lingual SCC (**SCC9**)	**1.** Yes **2.** Yes[b] **3.** Not mentioned	Viability/proliferation of SCC9 cells by MTT assay	10^3 SCC9/cm^2 showed increases in cell proliferation compared to non-irradiated. 10^4 SCC9/cm^2 showed cell proliferation slightly lower	PBM irradiation with an energy density of 4J/cm^2 decreased the pro-osteoclastogenic potential of SCC9

		4. Yes **5.** Yes		(day 1) or similar/higher (longer periods) than the non-irradiated	cells
			Characterization of the osteoclastogenic response by TRAP (*p*NPP) hydrolysis assay), actin rings and expressing vitronectin and calcitonin receptors	Co-cultures with 10^3 SCC9/cm^2 showed no significant differences until day 14. At day 21, it was observed sharp TRAP decreases of ~60% (660nm-4J/cm^2-40mW) and ~90% (780nm-4J/cm^2-70mW) compared with SCC9 non-irradiated cells. With 10^4 SCC9/cm^2, it was observed a slight TRAP decrease at day 14 and a sharp TRAP decrease at day 21 (4 J/cm^2). The 70mW output power caused the highest TRAP decrease	
			Characterization of the osteoclastogenic response by intracelular signaling pathways (MEK, p38, NFkB, JNK)	For monocultured PBMC, TRAP activity partially decreased in the presence of MEK and JNK, was abolished fo NFkB and was not significant for p38 For non-irradiated SCC9 + PBMC, no significant effects for TRAP activity was seen in the presence of MEK and p38 pathway inhibitors, decreasing in the presence of NFkB and JNK For irradiated SCC9 + PBMC, MEK and p38 pathways inhibitors significantly decreased TRAP activity. TRAP activity was also reduced for NFkB and JNK	
			IL-11 and PTHrP gene expression by RT-PCR method	Increased expression of IL-11 and PTHrP in irradiated cells with 780nm-4J/cm^2-40mW and 660nm-4J/cm^2-40mW at day 2. With 9 days, the molecules expressions in irradiated cultures were lower than in the non-irradiated ones	
Scalch et al., 2018 [39]	**SCC9** cell line	**1.** Yes **2.** Yes **3.** Not mentioned **4.** Yes **5.** Yes	Mitochondrial activity by MTT assay	PBMT significantly decreased the mitochondrial activity of irradiated SCC9 cells compared to control, except for the cells irradiated with 660nm-30mW-2J/cm^2	PBM therapy with 780nm-70mW and 40mW-4J/cm^2 demonstrated an induction on apoptosis and a reduction on cell viability and migration capacity of irradiated SCC9 cell line
			Apoptosis by caspase 3 activity	PBMT significantly increased caspase 3 activity of irradiated SCC9	
			Cell viability by neutral red assay	Cell viability of irradiated SCC9 cells was significantly decreased with 660nm-40mW-4J/cm^2 and 780nm-40mW and 70mW-4J/cm^2	
			Cell proliferation by BrdU assay	No differences in the number of BrDU-positive cells were found between irradiated and control cells	

			Migration by scratch-wound assay	Reduction in the migration capacity of the tumor cells	

[a], Partial darkness; [b], Dim lighting; c, Minimal ambient lighting

BrdU, 5-bromo-2-deoxyuridine; **λ**, wavelength; **nm**, nanometer; **PBM**, photobiomodulation; **SCC**, squamous cell carcinoma; **MTT**, 3-(4,5-dimethyl-2-thia-zolyl)-2, 5-diphenyl-2H-tetrazolium bromide; **FACS**, fluorescent-activated cell sorting; **PI**, propidium iodide; **FITC**, fluorescein isothiocyanate; **TUNEL**, TdT-mediated dUTP Nick-End Labeling; **MMP**, matrix metalloproteinases; **TRAP**, Tartrate-resistant acid phosphatase; ***p*NPP,** *para*-nitrophenyl phosphate; **NFkB**, nuclear factor kappa B; **IL-11**, interleukin 11; **PTHrP**, parathyroid hormone-related protein; **RT-PCR**, reverse transcription-polymerase chain reaction; **ROS**, reactive oxygen species

Table 1b. Description of experimental details of in vivo studies included in this review.

Study; Year	**Samples** *Cell line / Animal model*	**Study design** **1.** *Environmental conditions*; **2.** *Tumor induction;* **3.** Description of *groups.*	**Type of Evaluation / Method**	**Main outcomes**	**Main conclusions**
Monteiro et al., 2011 [22]	Oral chemical carcinogenesis (DMBA) on hamsters cheek pouch model	**1.** Not mentioned **2.** Yes **3.** Yes	Histological analysis by light microscopy	G1 showed 100% well-differentiated SCC. G2 showed 20% moderately differentiated and 80% well-differentiated SCC. G3 showed 40% well differentiated, 40% poorly differentiated, and 20% moderately differentiated SCC	PBM caused a significant progression of the severity of SCC in the oral cavity of hamsters
Ottaviani et al., 2016 [38]	Oral carcinogenesis (4NQO) mouse tongue model	**1.** Yes **2.** Yes **3.** Yes	Microscopic evaluation by histopathological analysis (diagnosis and grading), immunofluorescence (fluorescein isothiocyanate-labeled lectin) and immunohistochemistry (anti-CD31 and -αSMA)	αSMA^{+} arterioles were significant increased in laser treated lesions. A more regular and structured vessel pattern was showed by the perfusion of fluorescein-labeled lectin Laser treatment reduced the appearance of dysplastic lesions and was more effective in reducing the number and the percentage of both *in situ* and invasive carcinomas	PBM inhibited tumor progression and improved functional vessel maturation

Table 2. Parameters used for PBM treatments in studies included in this review.

Study; Year	Active medium	Application procedure	Wavelength (nm)	Energy density (J/cm^2)	Power (mW)	Power density (mW/cm^2)	Irradiation time	Spot size (cm^2)	Energy per point (J)	Schedule of irradiations	Total energy (J)
Schaffer et al.; 1997 [19]	-	-	805	2 to 20	-	50 and 150	-	-	-	-	-
Sroka et al.; 1999 [20]	Ar^+-pumped tunable dye laser GaAlAs	-	630 635 805	0 to 20	-	50 and 150	-	-	-	-	-
Pinheiro et al.[a]*;* 2002 [30]	-	CW	635 670	0.04 to 0.48	5	-	-	-	-	Seven consecutive days	-
Pinheiro et al.[b]*;* 2002 [31]	-	CW	635 670	0.04 to 4.8	5	-	-	-	-	Seven consecutive days	-
Kreisler et al.; 2003 [32]	GaAlAs	CW Noncontact	809	1.96 3.92 7.84	10	-	75.0s 150.0s 300.0s	-	-	-	-
Castro et al., 2005 [33]	-	-	685 830	4	31 34.5	-	-	0.8	-	48h intervals	-
Werneck et al., 2005 [34]	-	-	685 830	4	31 34.5	-	-	0.8	-	-	-
Monteiro et al., 2011 [22]	-	CW	660	56.4	30	424	133.0s	0.07	4	48h intervals	-
Schartinger et al., 2012 [21]	GaAlAs	Noncontact	660	-	350	0.39 to 63.7	15min	-	-	Three consecutive days for 15 min	-
Sperandio et al., 2013 [35]	GaAlAs	Contact	660 780	2.05 3.07 6.15	40	-	-	0.039	-	-	-
Henriques et al., 2014 [36]	InGaAlP	CW Noncontact	660	0.5 1.0	30	30	16.0s 33.0s	0.03	-	0 and 48h	0.48 0.99
Liang et al., 2015 [23]	-	CW	810	0 10 30 60	-	1000	00.0s 10.0s 30.0s 60.0s	-	-	.-	-
Schalch et al., 2016 [37]	-	CW	660	4	30	214.29	25.3s	0.14	-	1 session	0.76

		Contact	780	4 4	30.8 53.9	220 385	24.7s 14.1s				0.76 0.76
Ottaviani et al., 2016 [38]	GaAs + InGaAlAsP	CW	970	6	2500*	200	30.0s	-	-	Once a day for 4 consecutive days	-
Schalch et al., 2018** [39]	-	CW	660	1.4	22.5	160.7	8.4s	0.14	-	1 session	0.19
				2.7		160.7	16.9s				0.38
				2.7	30	214.3	12.7s				0.38
				5.4		214.3	25.3s				0.76
				8.1		214.3	38.0s				1.14
			780	1.4	23.1	165.0	8.2s				0.19
				2.7		165.0	16.5s				0.38
				2.7	30.8	220.0	12.3s				0.38
				5.4		220.0	24.7s				0.76
				8.1		220.0	37.0s				1.14
				5.4	53.9	385.0	14.1s				0.76

GaAlAs, alluminium-gallium-arsenide; **InGaAlP**, indium-gallium-alluminium phosphide; **GaAs**, gallium-arsenide; **InGaAlAsP**, indium-gallium-alluminium phosphide; **CW**, continuous wave

* the device was used in an unfocused manner

** the study used 11 combinations of dosimetric parameters; for cell viability (neutral red assay), cell proliferation (incorporation of BrdU assay) and migration (scratch-wound assay) only the parameters that used an energy density of 4 J/cm^2 were chosen

Table 3a. Criteria used to assess risk of bias in in vitro studies

	Schaffer et al., 1997	*Sroka et al.*, 1999	*Pinheiro et al.*, 2005[a]	*Pinheiro et al.*, 2005[b]	*Kreisler et al.*, 2003	*Castro et al.*, 2005	*Werneck et al.*, 2005	*Schartinger et al.*, 2012	*Sperandio et al.*, 2013	*Henriques et al.*, 2014	*Liang et al.*, 2015	*Schalch et al.*, 2016	*Schalch et al.*, 2018
Condition of cell culture	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Description of methodology to evaluate the outcome	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Reproducibility	Yes	Yes	No	No	No	No	No	Yes	No	Yes	Yes	Yes	Yes
Methods for preventing unintentional light scattering during laser application	No	No	Yes	No	Yes	No	No	No	No	Yes	No	Yes	Yes
Description of laser parameters according to WALT*	No	No	No	No	No	No	No	No	No	Yes	No	Yes	Yes
Conclusions according to outcomes description	No	No	No	No	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes
Risk classification	*High*	*High*	*Medium*	*High*	*Medium*	High	*High*	*Medium*	*Medium*	*Low*	*Medium*	*Low*	*Low*

* The studies were classified as *YES* only if all laser parameters were described

Table 3b. Criteria used to assess risk of bias in in vivo (b) studies (SYRCLE's RoB tool)

	Monteiro et al., 2011	*Ottaviani et al.,* 2016
Was the allocation sequence adequately generated and applied?	No	No
Were the groups similar at baseline or were they adjusted for confounders in the analysis?	Yes	Yes
Was the allocation to the different groups adequately concealed?	Unclear	Unclear
Were the animals randomly housed during the experiment?	Unclear	Unclear
Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?	Unclear	Unclear
Were animals selected at random for outcome assessment?	Unclear	Unclear
Was the outcome assessor blinded?	Unclear	Unclear
Were incomplete outcome data adequately addressed?	Unclear	Unclear
Are reports of the study free of selective outcome reporting?	Unclear	Unclear
Was the study apparently free of other problems that could result in high risk of bias?	Yes	Yes

Table 4. Outline of key parameters identified in this review that could contribute to the variances in observed results for effects of PBM therapy on tumor cells.

BIOLOGICAL	DEVICE	OUTCOMES
Cell origin - Lineage - Transfromation state	**Power** - Power output - Power density (irradiance) - Treatment surface irradiance	**Proliferation** *versus* **Apoptosis**
Cell density - Initial cell concentration - Plating surface area - Confluency at PBM treatments - Time outcome analyses	**Time** - Single session - Repetitions	**Cell viability** - Membrane integrity **Mitochondrial function** - Respiraroty and metabolic health
Culture conditions - Media and supplements - Serum concentration - Synchronication of cell subpopulations	**Energy** - Energy per point - Energy density - Total energy per session	**Cell Cycle analyses** - Synetheitc phases - Checkpoint arrest

Figure 1. PRISMA flow diagram for systematic search and studies selection strategy

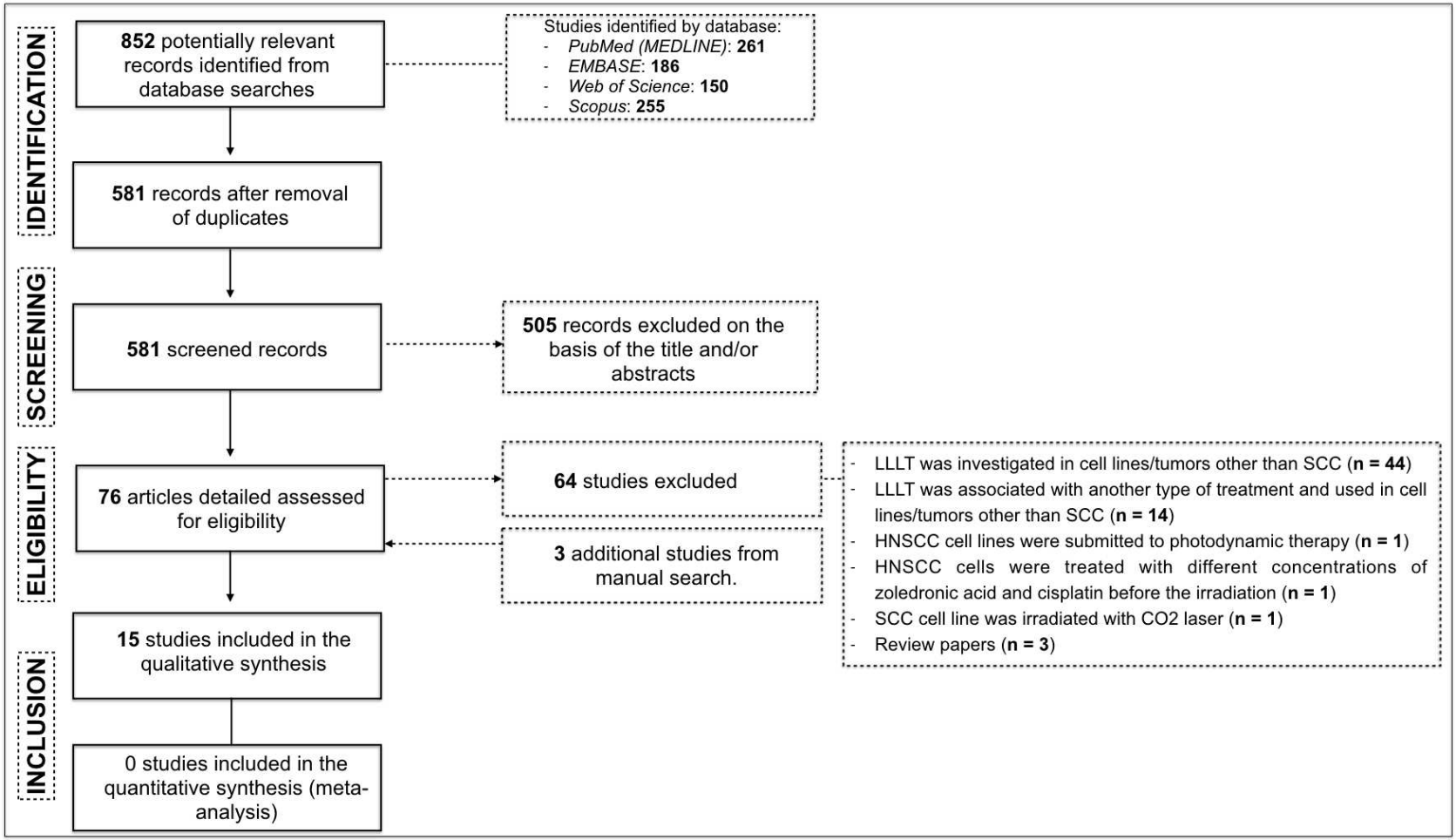

Figure 2. Distribution of the included studies according to its respective countries

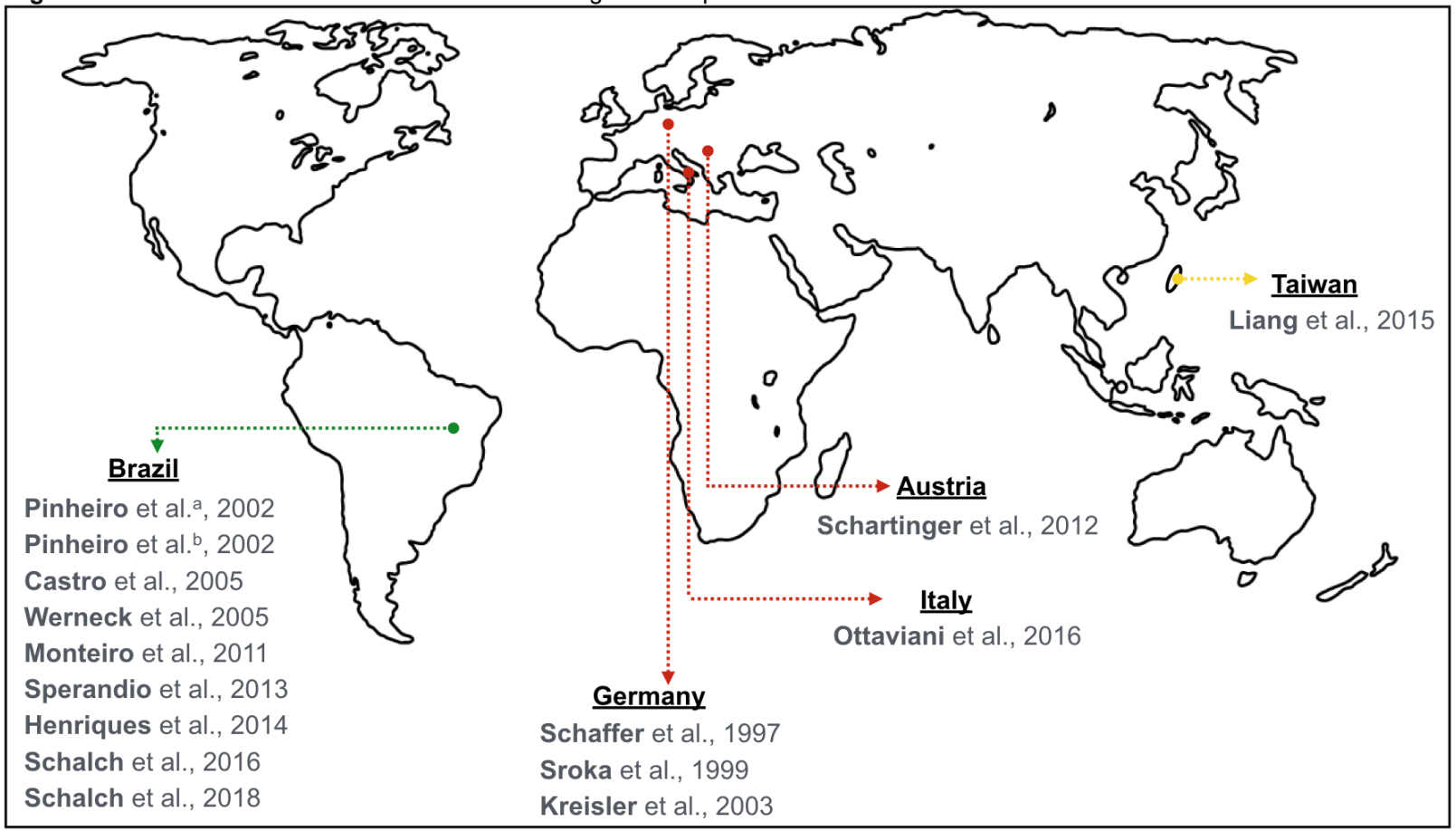

Figure 3. Summary of the results from the included studies.

A. *In vitro* studies

Author / Year	Wavelength (nm)	Dose (J/cm²)	Cell type	Overall Result	Risk of Bias
Schaffer et al., 1997	805	2 to 20	ZMK	Positive	High
Sroka et al., 1999	630 635 805	0 to 20	ZMK1	Positive	High
Pinheiro et al., 2002[a]	635 670	0.04 to 0.48	H.Ep.2	Negative	Medium
Pinheiro et al., 2002[b]	635 670	0.04 to 4.8	H.Ep.2	Negative	High
Kreisler et al., 2003	809	1.96 3.92 7.84	Larynx SCC	Negative	Medium
Castro et al., 2005	830	4.0	KB	Negative	High
Werneck et al., 2005	685 810	4.0	H.Ep.2	Negative	High
Schartinger et al., 2012	660	-	SCC25	Positive	Medium
Sperandio et al., 2013	660 780	2.05 3.07 6.15	SCC9 SCC25	Positive	Medium
Henriques et al., 2014	660	1.0	SCC25	Negative	Low
Liang et al., 2015	810	0 10 30 60	OC2	Positive	Medium
Schalch et al., 2016	660 780	4.0	SCC9	Positive	Low
Schalch et al., 2018	660 780	4.0	SCC9	Positive	Low

B. *In vivo* studies

Author / Year	Wavelength (nm)	Dose (J/cm²)	Animal model	Overall Result	Risk of bias
Monteiro et al., 2011	660	56.4	Hamsters cheek	Negative	Medium
Ottaviani et al., 2016	970	6.0	Mouse tongue	Positive	Medium

2.2 Artigo: **Photobiomodulation therapy does not impact the behavior of oral squamous cell carcinoma: evaluation in a patient-derived xenograft model**

Artigo formatado para submissão no periódico *The Federation of America Societies for Experimental Biology Journal (The FASEB Journal).*

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Running title: Photobiomodulation therapy did not affect oral cancer.

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Nonstandard abbreviations

1. OSCC-PDX: Oral squamous cell carcinoma patient-derived xenograft model;
2. IIr: Immediate irradiation group;
3. LIr: Late irradiation group.

Abstract

Background and aims: Photobiomodulation therapy (PBMT) is recommended for the prevention of oral mucositis (OM). However, the potential effects of PBMT on oral squamous cell carcinoma (OSCC) have not been fully elucidated. This is a pioneering study that aimed to evaluate the impact of PBMT on OSCC behavior in an OSCC-patient-derived xenograft (OSCC-PDX) model.

Materials and methods: BALB/c nude mice with OSCC-PDX models were divided into three groups: Control (C) - without PBMT; Immediate irradiation (IIr) - PBMT since one week after tumor implantation; and Late irradiation (LIr) - PBMT after tumors reached 200mm^3. OSCC-PDX were daily irradiated (660nm; 100mW; 6J/cm^2; 0,2J/point) for 12 weeks and the tumors were measured once a week. OSCC-PDX were collected for volumetric analysis, histological grading, immunohistochemistry (anti-Ki-67, anti-H3K9ac and anti-BMI1) and cell cycle analysis (propidium-iodide) by flow cytometry.

Results: No significant differences in OSCC-PDX volumetric measurements were detected between the C, IIr and LIr groups (p=0.89). The histopathological grade also did not differ between the non-irradiated *vs.* irradiated groups (p>0.05). No differences between groups were detected in the immunohistochemical analysis of Ki-67 (p=0.9661)**;** H3K9ac (p=0.3794); and BMI1 (p=0.5182). The evaluation of the cell cycle phases by flow cytometry showed a peak in G1 followed by a minor expression in G2, also without significant differences between groups (p>0.05).

Conclusions: PBMT did not impact the behavior of OSCC-PDX models. This is an important preclinical outcome regarding safety concerns of PBMT in cancer patients.

Keywords: Photobiomodulation Therapy; Oral Mucositis; Oral squamous Cell Carcinoma.

Introduction

Worldwide estimates of lip and oral cavity cancers reported about 355,000 new cases with more than 177,000 deaths related to this type of disease in 2018.[1,2] Among the different types of these tumors, oral squamous cell carcinoma (OSCC) constitutes more than 90% of all oral cavity cancers.[2,3] OSCC is highly morbid, with a 5-year survival rate of about 50%, and its treatment mainly involves surgery, radiotherapy and/or chemotherapy.[4] Oral mucositis (OM) is an acute side effect of antineoplastic therapy clinically characterized by mucosal ulcerations potentially causing pain, dysphagia, dehydration, and weight loss.[5] The deleterious effects of OM may cause temporary or cancer treatment interruptions.[6-8] The different therapeutic interventions for OM are mainly symptomatic, with specific limitations.[9]

In this context, photobiomodulation therapy (PBMT) is a well-established method for the prevention and treatment of OM using laser irradiation with specific parameters for defined oncological patient populations, reducing morbidity and hospital patient costs.[10-13] It is suggested that laser irradiation is absorbed by mitochondrial cytochrome *c* oxidase, causing photoexcitation and a change in electron flow with a consequent increase of adenosine triphosphate (ATP) production.[14,15] Our group has demonstrated that PBMT accelerates keratinocyte migration and stimulates the synthesis of different proteins and epigenetic events, improving the wound healing process and enriching the biomechanical properties of tissue.[16-20] We also demonstrated that laser irradiation does not induce DNA damage in normal oral keratinocytes.[21]

Despite the well-known positive effects of PBMT on tissue repair, discrepant information exists regarding the impact of laser irradiation on neoplastic cells.[22-26] Considering that OSCC patients submitted to antineoplastic therapy usually develop OM, it is crucial to define the safety of PBMT in the oncological setting specially when irradiated areas within the same region of active tumoral lesions. In a recent systematic review on this same subject, our group observed that the effects of PBMT on head and neck squamous cell carcinoma (HNSCC) cells were mainly evaluated using *in vitro* models, with important discrepancies in study designs, PBMT protocols and outcomes.[27] The results of that systematic review strongly reinforced the need of

further well-delineated *in vivo* studies in order to consistently evaluate the effects of PBMT on HNSCC.

Patient-derived xenograft (PDX) models are acknowledged as an important tool for preclinical screening of human tumors.[28] PDX models are based on the implantation of fresh cancer tissue fragments from patients directly into immunodeficient mice, representing a reliable model for preclinical research mainly due to the retaining of the cellular heterogeneity, architecture and molecular characteristics of the original tumor.[29,30] To our knowledge, despite the high translational potential of PDX methodology, no previous study has evaluated the effect of PBM on OSCC using this *in vivo* strategy. The present pioneer study aimed to evaluate the impact of PBMT on tumor growth, tumor morphology, tumor proliferation, epigenetic and stem cell profiles in an oral squamous cell carcinoma patient-derived xenograft (OSCC-PDX) model.

2. Materials and methods

2.1 OSCC-PDX models

2.1.1 Patients and tumor samples

Two fresh OSCC fragments were collected from the surgical specimens of two oncological patients undergoing surgery at the Otorhinolaryngology Service / Porto Alegre Clinics Hospital (HCPA) and at the Head and Neck Surgery Service / *Hospital Santa Rita de Porto Alegre*. The patients were selected according to the following criteria: (1) 45-70 years of age, (2) being a male and (3) a smoker, (4) with an OSCC lesion located in the posterior lateral border of tongue (histopathologically confirmed by a previous incisional biopsy), (5) with advanced clinical staging (stages III or IV, with the primary tumor being T3 or more), and (6) without previous radiotherapy and/or chemotherapy (***Figure 1***). The fresh fragments were excised from solid tumor tissue during the surgical procedure, avoiding necrosis or hemorrhage foci, maintained in Dulbecco's Modified Eagle Medium (DMEM) and immediately transferred at room temperature to the animal facility. The human participation in the present study was approved by the Research Ethics Committee (CAEE protocol 86434718.0.0000.5327) and the patients gave written informed consent to participate in the study and to donate their tumor samples.

2.1.2 Animals

Thirty-four 5-7-week-old BALB/c nude mice (20-25g) from the Animal Experimentation Unit - HCPA were used. The animals were maintained in a controlled environment under specific pathogen-free conditions and fed an autoclaved laboratory rodent diet *ad libitum*. All the experimental procedures and the use of animals were approved by the Institutional Committee for Animal Care and Use (GPPG/HCPA, protocol no. 2018-0134) according to Brazilian Law 11.794 and the Brazilian Guideline for the Care and Use of Animals of the National Council for Animal Experimentation Control. The animal manipulations were performed under sterile conditions using a laminar flow chamber and mice were anesthetized with isoflurane inhalation.

2.1.3 Surgical procedure for PDX

The protocol for tumor implantation was partially based on Acasigua et al.[31] and Pearson et al.[32]. Briefly, a tumor fragment sample from one patient, the sample was cut into 2 fragments of approximately 6mm^3. Each tumor fragment was firstly grafted into a subcutaneous area on the back of 2 animals for the OSCC-PDX – passage 0 (OSCC-PDX-0). OSCC-PDX-0 volumes were measured once a week by calculating the tumor maximum length x width^2 / 2 and each tumor was harvested when it reached the endpoint volume of 1000mm^3. Each final OSCC-PDX-0 tumor was then cut into multiples fragments of approximately 6mm^3 and immediately grafted into the next generation of another 15 animals from the experimental groups, constituting the OSSC-PDX - passage 1 (OSCC-PDX-1). The same protocol was performed for the tumor fragments from patient 2, ensuring an equal representation of the two tumors in the experimental groups (15 animals for each patient, with a final number of 30 animals with OSCC-PDX-1 models).

2.2 Experimental groups

OSCC-PDX-1 animals were randomly divided into 3 groups according to the treatment performed: (1) Control Group (C): OSCC-PDX-1 animals that received daily manipulation without tumor irradiation; (2) Immediate irradiation Group (IIr): OSCC-PDX-1 animals that received daily tumor irradiation starting one week after tumor implantation; and (3) Late irradiation Group (LIr): OSCC-PDX-1 animals that

received daily tumor irradiation after the tumor reached a volume of 200mm^3. The average time for OSCC-PDX-1 tumors to reach a volume of 200mm^3 was 7 weeks.

2.3 Photobiomodulation therapy

The PBMT was performed with an Indium Gallium Aluminum Phosphorus diode laser (*MMOptics, São Carlos, São Paulo, Brazil*) based on the following parameters:

- *Center wavelengths*: 660nm±10nm;
- *Operating mode*: continuous;
- *Peak power*: 0.01W;
- *Average power*: 100mW;
- *Spot size*: 0.03 cm^2;
- *Irradiance*: 3.333 mW/cm^2;
- *Fluence*: 6J/cm^2;
- *Exposure duration*: 2s;
- *Total energy/point*: 0.2J;
- *Application form*: contact;
- *Number of points irradiated*: 4 equidistant points, covering the entire tumor implantation area;
- *Frequency of sessions*: Once a day, 5 days per week (weekends excluded) for 12 weeks.

The power of the equipment was measured before each irradiation session with a power meter (*Laser Check, MM Optics Ltd., São Carlos, SP, Brazil*). C Group animals were manipulated in the same manner as the irradiated groups, but with the laser device turned off. IIr animals received daily PBMT sessions (24-hour interval, with irradiation always performed on the same day shift) starting one week after the surgical procedures for OSCC-PDX-1 until the tumor reached 1000 mm^3 (volume endpoint) or by the twelfth postoperative week (time endpoint). For LIr animals, PBMT was instituted after the OSCC-PDX-1 reached a minimum volume of 200 mm^3. The laser irradiation protocol was applied until OSCC-PDX-1 reached the volume endpoint or the time endpoint. When the animals reached an endpoint, they were euthanized by excess isoflurane inhalation and OSCC-PDX-1 tumors were collected and then cut into three fragments: the first piece was fixed in 10% buffered formalin

solution for histopathological and immunohistochemical studies; the second piece was immediately prepared for flow cytometry analysis; and the third one was frozen for later experiments.

2.4 Tumor growth analysis

The OSCC-PDX dimensions were measured in a standardized manner with a digital caliper and photographed with a digital camera once a week during the experimental period. All photographs were taken in the same environment. Volumetric measurements were performed using the following formula: Volume (mm^3) = Length x $Width^2$ / 2. The relative tumor increases from each group were also calculated using the following formula: relative tumor volume = T_{12} (mean tumor volume at week 12) x $100/T_6$ (mean tumor volume at week 6). Week 6 was selected since it was the week during which OSCC-PDX-1 models became established and started their development.

2.5 Histopathological analysis

Slides of the OSCC-PDX were stained with hematoxylin-eosin (HE) and submitted to histological grading based on the consensus of two blinded oral pathologists (F.M.S. and M.D.M.). The cases were graded using a light microscope according to an adaptation of the criteria described by Bryne et al.[33]

2.6 Immunohistochemistry

For immunohistochemical staining, the samples were cut into 4-µm sections and placed on silanized glass slides. The slides were deparaffinized in xylene and hydrated in decreasing ethanol series. Antigen retrieval was performed before incubation with the following primary antibodies: Ki-67 (clone 30-9, Roche, 1:100), H3K9ac (clone C5B11, Cell Signalling, 1:300) and BMI1 (clone 38295, Abcam, 1:100). The sections were then incubated with diaminobenzidine (DAB) tetrahydrochloride (Novocastra, Newcastle upon Tyne, UK) and counterstained with Mayer's hematoxylin. Negative controls were obtained by replacing the primary antibodies with non-immune serum. One oral pathologist (F.M.S.) analyzed the slides. Images of the selected fields were captured at a magnification of 400X using a conventional light microscope (CX41RF model; Olympus Latin America, Inc., Miami, FL, USA). The images were analyzed using the Image J program (NIH, Bethesda,

MD, USA). In each case, 1000 cells were counted, and the results were expressed as the percentage of positive cells. Nuclear labeling was classified as positive for Ki-67 and H3K9ac (proliferative labelling index) and cytoplasmic labelling as positive for BMI1.

2.7 Flow cytometry

Flow cytometry was immediately performed after collection and preparation of tumor fragments. Briefly, each tumor fragment was completely macerated with a scalpel blade in DMEM culture medium. After maceration, the culture medium was carefully pipetted and 2ml of collagenase was added. The macerated product in collagenase was transported to a water bath for 30 minutes and resuspended every 15 minutes. Next, the macerate was sieved through a cell strainer (50µm, Sigma Aldrich) and 3ml of fetal bovine serum (FBS) was added. The solution was centrifuged, (10min, 3000rpm) and the supernatant was removed; 3 ml of PBS (1X) was added and, for each sample, equal parts of 300µl of the final solution were mixed with 300µl of propidium iodide (PI) solution for the acquisition of 10.000 events with an Attune Flow Cytometer (ThermoFisher Scientific, USA), using a BL2 channel for the detection of PI passage in the flow cytometer. The percentage of cells in each cell cycle phase (G1, S and G2) was recorded for each sample.

2.8 Statistical analysis

Data are reported as mean ± standard error of the mean (SEM) and were compared by the Kruskal Wallis test followed by Dunn's post-hoc test for multiple comparisons. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant in all tests.

3. Results

3.1 Establishment of the OSCC-PDX models

The success of OSCC-PDX models was based on a stable tumor growth along the follow-up weeks, starting at least five weeks after implantation. For the OSCC-PDX-0 models, tumor fragments from five patients were used to obtain the two successful models (success rate = 40%). Three tumor fragments did not lead to successful OSCC-PDX-0 models due to the following reasons: (1) ulceration, (2)

PDX resorption, and (3) non-established tumor (no stable growth). The two successful OSCC-PDX-0 models reached the volume endpoint of 1000mm^3 within 8 and 10 weeks, respectively. OSCC-PDX-1 models were implanted in 30 animals, with a 66.6% successful rate (20 animals). The failures occurring in animals from the three experimental groups were due to the following similar reasons: (1) PDX resorption/no tumor (one C animal, two IIr animals, and two LIr animals); (2) ulceration (one IIr animal and one LIr animal); and (3) formation of a swelling in the PDX region (one C animal, one IIr animal, and one LIr animal). The animals with the tumoral swelling in the PDX region were excluded due to the impossibility of performing the volumetric analysis. Thus, 20 animals with successful OSCC-PDX-1 development were considered for this study: 8 C animals, 6 IIr animals and 6 LIr animals. We compared the histopathological OSCC-PDX-1 slides to the patient original slide and we confirmed that the morphological features of OSCC were maintained in the PDX models (***Figure 1***). The establishment and development of OSCC-PDX models designed in this study are schematically illustrated in ***Figure 2***.

3.2 PBMT does not impact tumor growth in the OSCC-PDX models

To evaluate the impact of PBMT on OSCC tumor growth, we measured the OSCC-PDX-1 volumes from each animal along the 12 weeks of the daily irradiation protocol. No tumor reached the volume endpoint before the twelfth week. The volumetric OSCC-PDX-1 values are presented in ***Table 1*** and ***Figure 3.*** All groups demonstrated a stable growth of OSCC-PDX-1 volume. The OSCC-PDX-1 models showed similar tumor relative increases from week 6 to week 12, of 486.66% in C, 488.46% in IIr, and 415% in LIr. Overall analysis of tumor volume (mm^3) throughout the experiment revealed no significant differences between groups (p=0.89). No significant difference was also observed when comparing the volumes of each group at specific time points (weeks 2, 4, 6, 8 and 10) including the final mean value (*C*: 664.35mm^3 ± 170.19; *IIr*: 803.86mm^3 ± 195.58; *LIr*: 649.32mm^3 ± 161.46) (p=0.7274). Taken together, the results indicate that PBMT did not impact tumor growth in this OSCC-PDX-1 model.

3.3 PBMT does not change the morphological pattern of the OSCC-PDX models from irradiated and non-irradiated groups

To compare the morphological patterns of the non-irradiated and irradiated groups, the OSCC-PDX-1 slides were graded according to the criteria of Bryne et al.[33] The mean values of each scored histological characteristic and the representative images from each group are illustrated in ***Figure 4***. No significant difference was observed between C, IIr and LIr groups for keratinization degree (p=0.6636), nuclear pleomorphism (p>0.999), pattern of invasion (p=0.7134), number of mitosis (p=0.6972), or lymphoplasmacytic infiltrate (p=0.8375), indicating that PBMT did not affect the histopathological pattern of the different OSCC-PDX-1 groups.

3.4 PBMT does not affect the proliferation rates or cell cycle phases of the OSCC-PDX models

Cell proliferation in the OSCC-PDX-1 models was evaluated based on the expression of the Ki-67 immunohistochemical marker. The cell cycle phases were analyzed by flow cytometry. A high expression of Ki-67 was observed in the C group (76.24 ± 3.16*)* as well as in the IIr (75.60 ± 8,77) and in LIr (77.93 ± 4.05) groups, with no statistically significant difference between the three groups (p=0.9661) (***Table 2; Figure 5A***). Analysis of the cell cycle phases by PI staining also showed a similar proliferative aspect with no significant difference between the three groups, with a peak in the G1 phase (*C*: 42.12 ± 6.9; *IIr*: 45.82 ± 7.69; *LIr*: 38.84 ± 13.64; p=0.7244) followed by the G2 phase (*C*: 30.23 ± 7.16; *IIr*: 26.36 ± 7,6; *LIr*: 23.19 ± 9.3; p=0.7914) (***Table 3; Figure 6****).*

3.5 PBMT does not influence histone acetylation or stem cell profiles in the OSCC-PDX models

The effects of PBMT on the epigenetic events of the OSCC-PDX-1 models were evaluated by the immunohistochemical expression of acetyl-histone 3 at lys9 (H3K9ac). A high mean expression of H3K9ac was observed in the C, IIr and LIr groups, with no significant difference between them (*C*: 90.60 ± 1.42; *IIr*: 91.36 ± 2.04; *LIr*: 86.66 ± 2.69; p=0.3794; ***Table 2***; ***Figure 5B***). The stemness profile of OSCC-PDX-1 following laser irradiation was assessed by BMI1 expression. A high mean value of BMI1-positive cells was observed in the C, IIr and LIr groups, with no significant difference between them (*C*: 98.03 ± 0.43; *IIr*: 96.43 ± 1.25; *LIr*: 9156 ± 5.07; p=0.5182*;* ***Table 2; Figure 5C***).

Discussion

In the present study we evaluated the impact of PBMT on OSCC-PDX models implanted in BALB/c nude mice. This is the first time the effects of laser irradiation were assessed using OSCC-PDX models. The PBMT parameters used in this study were based on the most recent update of the Multinational Association of Supportive Care in Cancer (MASCC) and International Society of Supportive Care in Cancer (ISOO) for the prevention of OM in HNC patients.[13] The well-established recommendation of PBMT for supportive cancer care in OM has not involved the evaluation of its safety when considering the eventual irradiation of residual neoplastic cells. For OSCC-associated OM, this issue is of major relevance since irradiation is applied to tissues close to the original tumor site. While controversial results have been reported regarding the outcomes of HNSCC cell irradiation *in vitro*[27], the present report is a pioneering preclinical study demonstrating that the robustness of the laser did not influence tumor growth, morphological patterns, cell proliferation outcomes, or epigenetic and stem cell profiles in the context of OSCC.

PDX is a robust methodology that allows the development of valuable tumor models for testing different outcomes, such as drug screening, biomarker identification, and tumor biological behavior.[34-36] Currently, different studies have demonstrated that PDX replicates the diversity of tumor biology and faithfully recapitulates its characteristics along passages.[32,37-39]. Herein, we elaborated OSCC-PDX models from resected specimens with a previous histopathological diagnosis of OSCC. The OSCC-PDX models were developed comprising 2 passages (from human to mice and from mice to mice), with a successful tumor establishment rate of 66.6%. Indeed, studies with PDX models have shown that not every sample successfully forms a graft, and similar engraftment successful rates have been described. Karamboulas et al.[40] developed a large collection of HNSCC-PDX into NOD/SCID/IL2R$_{\gamma}^{-/-}$ (NSG) mice resulting in 161 successfully formed xenografts from 243 surgically resected and subcutaneously implanted HPV- HNSCC (successful rate of approximately 66.2%).

In the present study, animals were randomly divided into irradiated and non-irradiated groups for comparison of different analyses. After 12 weeks of daily irradiation, volumetric analysis and histopathological grading of harvested tumors did not show statistically significant differences between the C, IIr and LIr groups. These

results imply that PBMT did not interfere with OSCC-related tumor growth or morphological pattern in an animal model with high translational value. The use of PDX models hampers the comparison of our results with the current literature since this is the first study to address the safety of PBMT in OSCC using this innovative methodology. Regarding *in vivo* studies with OSCC, Ottaviani et al.[41] aimed to explore the effects of three laser protocols on oral carcinogenesis using mouse models submitted to 4-NQO and showed a reduction in tumor progression. In addition, Barasch et al.[42] used an orthotopic mouse model with OSCC cells to evaluate the tumor effects of PBMT (660nm, 75mW, 18.4J/cm^2, 5.6J) applied before radiotherapy. The authors demonstrated that the orthotopic OSCC model exposed to PBMT exhibited the same response to radiotherapy as non-irradiated cancer cells. Although these cited studies involved considerably different methodologies, they did not demonstrate a negative impact of PBMT in OSCC, agreeing with the results of the present study.

In addition to tumor size and morphology, PBMT also did not impact OSCC-PDX models in terms of Ki-67 immunoexpression or cell cycle phase analysis, a fact that may allow inferring that laser light did not modify the proliferative profile of OSCC cells. PBMT has been used to accelerate repair processes due to its capacity to increase cell proliferation and migration by stimulating the respiratory chain in mitochondria, the production of ATP, the synthesis of proteins, and the modulation of distinctive biological processes.[20,43-49] However, in the context of irradiated neoplastic cells, the *in vitro* proliferation of OSCC cells has demonstrated inconsistent results.[50-54] Henriques et al.[55] irradiated (660nm, 0.5- and 1.0J/cm^2) SCC25 cells, showing that laser light at 1.0J/cm^2 induced a significant increase of cell proliferation (cell growth curves and cell cycle analysis) and invasion (Matrigel invasion assay). The authors concluded that PBMT had a stimulatory effect on SCC25 cells. On the other hand, Schartinger et al.[56] demonstrated that PBMT (660nm, 350mW, 0,39- to 63.7mW/cm^2) decreased SCC25 and BEAS-2B cell proliferation, while increasing the proliferation of fibroblasts also irradiated (MTT method). In addition, the authors demonstrated an increase in the percentage of SCC25 cells in the S-phase and a decrease in their percentage in the G1-phase, concluding that PBMT did not exhibit an *in vitro* promoting result in SCC25 cells. These controversial results are probably due to the application of different laser dose rates. In the present study, our data demonstrated no PBMT impact on tumor growth or cell proliferation, probably justified by the *in vitro*

concept that shows the stimulating capacity of laser light only in cells with low metabolism, different from the fast-growing physiology of oral cancer cells.[14,57] In this context, our group has recently demonstrated that PBMT with a similar protocol (660nm, 100mW, 0,24J) did not exacerbate the behavior of HNSCC stem cell lines (HN6 and HN13) in terms of cell proliferation, migration, survival or percentage of irradiated and non-irradiated groups.[26]

Epigenetic changes associated with chromatin acetylation that may impact tumor aggressiveness and H3K9ac immunoexpression have been previously correlated with OSCC malignancy, lymph node progression, advanced clinical stage, poor degree of differentiation and poor prognosis.[58-60] Our group showed an association between changes in H3K9ac and the aggressiveness/resistance to chemotherapy of HNSCC.[61] In parallel, we have recently shown that laser irradiation is capable of modulating H3K9ac in oral epithelial cells during wound healing.[20] Thus, we thought it would be important to understand if PBMT could induce epigenetic changes in OSCC. Analysis of the H3K9ac marker showed high mean of immunoexpression in all groups of OSCC-PDX-1 tumors, with no statistically significant difference between them. Again, our hypothesis is that laser effects depend on the cell type and its status at the time of irradiation. Thus, it is expected that normal oral keratinocytes under stress during wound healing would respond differently than neoplastic cells. One of the most important hallmarks of cancer cells is the ability to proliferate constantly, independent of proliferation signals and in the absence of external stimuli, consequently resulting in unlimited growth.[62] Our H3K9ac result also corroborates this aspect, suggesting that PBMT does not affect the behavior of cancer cells submitted to the external stimuli of laser irradiation.

The BMI1 is also a valuable marker since it is considered to be a stemness-related gene.[63] BMI1 has been associated with self-renewal of cancer stem cells (CSCs) in HNSCC, indicating a role in metastasis capacity.[64-66] In addition, BMI1 expression in HNSCC is also correlated with tumor formation, invasion and maintenance of CSCs properties.[67,68] Chen et al.[69] showed that BMI1 positive tumor cells represented tumor-initiating CSCs in HNSCC responsible for both tumor development and therapy resistance. Herein, the percentage of BMI1 positive cells did not differ between groups, suggesting that PBMT has no impact on the expression of this CSC-related gene.

Previous controversial and inconsistent results regarding the effects of PBMT in neoplastic cells may be attributed to several factors including the lack of uniformity in laser parameters and the *in vitro* variations in cell types or study conditions. The present *in vivo* results may be related to outcomes observed in important clinical investigations. Previous retrospective human studies assessing head and neck cancer (HNC) patients submitted to PBMT for OM found no clinical negative impact.[70-72] PBMT showed no harmful effect on overall survival, time to local recurrences, or disease-free survival of HNC patients.[72] Similarly, treatment outcomes, recurrence or new primary tumors, and survival time were not affected by PBMT in advanced OSCC patients.[71] In addition, Antunes et al.[70] concluded that PBMT might even improve the survival of chemoradiotherapy-treated HNC patients. Finally, results of a robust systematic review by our group also suggested that PBMT is not related to the development of negative tumor safety issues.[73]

In summary, the present study demonstrated that these established parameters of PBMT did not interfere with tumor growth, morphological pattern, proliferation outcomes, or epigenetic and stem cell profile of irradiated OSCC-PDX models. Within the limits of this study, it may be concluded that PBMT has no stimulatory or protective effects on OSCC. Further prospective clinical studies are suggested for supporting these demonstrated null effects of PBMT on OSCC.

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Conflict of interest

The authors declare no conflict of interest.

Authors contributions

F. M. Silveira and M. D. Martins designed and performed research, analyzed data and wrote the paper; V. P. Wagner and A.R. Santos-Silva designed research, analyzed data and wrote the paper; T. R. Schmidt and B. Neumann designed and performed research; C. Rosset, V. G. Zanella, G. S. Maahs, M. A. Lopes, M. A. T. Martins and P. Arany analyzed data and wrote the paper.

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Table legends

Table 1. Volumetric measurements in OSCC-PDX-1 models.

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Table 3. Flow cytometry analysis of cell cycle phases OSCC-PDX-1 models.

Figure legends

Figure 1. Clinical and histopathological characteristics of the OSCC lesion excised from patient 1. Clinical presentation of an ulcerated tumoral lesion (T3, N0, Mx) with approximately 5 months of evolution on the lateral border of the tongue from a 51-year-old male patient reporting smoking habit and alcoholism, without previous chemo- or radiotherapy **(A)**. Microscopic characteristics of an OSCC **(B)**.

Figure 2. Illustrative scheme for the development and establishment of OSCC-PDX models in this study. The successful OSCC-PDX models demonstrated maintenance of the morphological features from the original tumor of the patients.

Figure 3. Volumetric measurements of OSCC-PDX-1 models during 12 weeks of PBMT. PBMT did not promote significant differences in tumor growth in OSCC-PDX-1 models when comparing C **(C)**, IIr **(D)** and LIr **(E)** groups (p=0.89) **(A,B)**. All OSCC-PDX models showed a similar rate of tumor growth along the 12 weeks of PBMT, with an initial volume decrease until the week 6 and a progressive increase between weeks 6 and 12. From week 1 to 12, all animals showed tumor growth **(A)**. During week 1, there was no significant difference in the initial OSCC-PDX-1 volumetric measurement between the C, IIr and LIr groups (p=0.5381), while during week 12 there was also no significant difference in the final OSCC-PDX-1 volumetric measurements between the C, IIr and LIr (p=0.7274) groups **(B)**.

Figure 4. Histopathological grading of OSCC-PDX-1 models. C, IIr and LIr groups did not differ significantly in keratinization degree (p=0.6636), nuclear pleomorphism (p>0.999), pattern of invasion (p=0.7134), number of mitosis (p=0.6972) or lymphoplasmacytic infiltrate (p=0.8375) **(A)**. Representative images of the histological pattern from each group (H&E, 100X, 400X) **(B,C,D)**.

Figure 5. Immunohistochemistry analysis of Ki-67, H3K9ac and BMI1 markers in the OSCC-PDX-1 models. Ki-67, H3K9ac and BMI1 labeling was similarly highly expressed in the C, IIr and LIr groups. Graphs and representative images display high immunoreactivity with no significant differences between the C, IIr and LIr groups for Ki-67 (p=0.9661) **(A)**; H3K9ac (p=0.3794) **(B)**; and BMI1 (p=0.5182) **(C)**.

Figure 6. Flow cytometry analysis of cell cycle phases in OSCC-PDX-1 models. The analysis showed similar proportions in the three groups analyzed, demonstrating a peak in G1 followed by a minor expression in the G2 and S phases, with no statistically significant differences (p>0.05).

Table 1. Volumetric measurements in OSCC-PDX-1 models

	Tumor volume (*mm^3 ± SEM*)			
	Control *(n=8)*	**Immediate irradiation** *(n=6)*	**Late irradiation** *(n=6)*	*p value*
Week 1	147.14 ± 31.08	208.70 ± 57.08	131.36 ± 27.49	*0.5381*
Week 2	117.59 ± 23.56	97.62 ± 31.43	84.83 ± 24.55	*0.8055*
Week 4	83.00 ± 18.08	81.34 ± 26.49	129.29 ± 37.28	*0.3465*
Week 6	136.51 ± 25.26	164.57 ± 35.92	156.46 ± 27.70	*0.8696*
Week 8	267.34 ± 42.73	274.83 ± 52.64	336.27 ± 86.54	*0.8814*
Week 10	417.14 ± 85.40	453.92 ± 79.27	477.69 ± 117.79	*0.9054*
Week 12	664.35 ± 170.19	803.86 ± 195.58	649.32 ± 161.46	*0.7274*

Table 2. Ki-67, H3K9ac and BMI1 immunoexpression in the OSCC-PDX-1 models

	Positive cells *(%±SEM)*			
	Control *(n=8)*	**Immediate irradiation** *(n=6)*	**Late irradiation** *(n=6)*	*p value*
Ki-67	76.24 ± 3.16	75.60 ± 8.77	77.93 ± 4.05	*0.9661*
H3K9ac	90.60 ± 1.42	91.36 ± 2.05	86.66 ± 2.69	*0.3794*
BMI1	98.25 ± 0.43	96.43 ± 1.25	91.56 ± 5.07	*0.5182*

Table 3. Flow cytometry analysis of cell cycle phases OSCC-PDX-1 models

	Cell cycle phases *(%±SEM)*			
	Control *(n=6)*	**Immediate irradiation** *(n=5)*	**Late irradiation** *(n=4)*	*p value*
G1	42.12 ± 6.9	45.82 ± 7.69	38.84 ± 13.64	*0.7244*
S	4.32 ± 0.75	6.64 ± 2.33	4.89 ± 0.78	*0.6589*
G2	30.23 ± 7.16	26.36 ± 7.6	23.19 ± 9.3	*0.7914*

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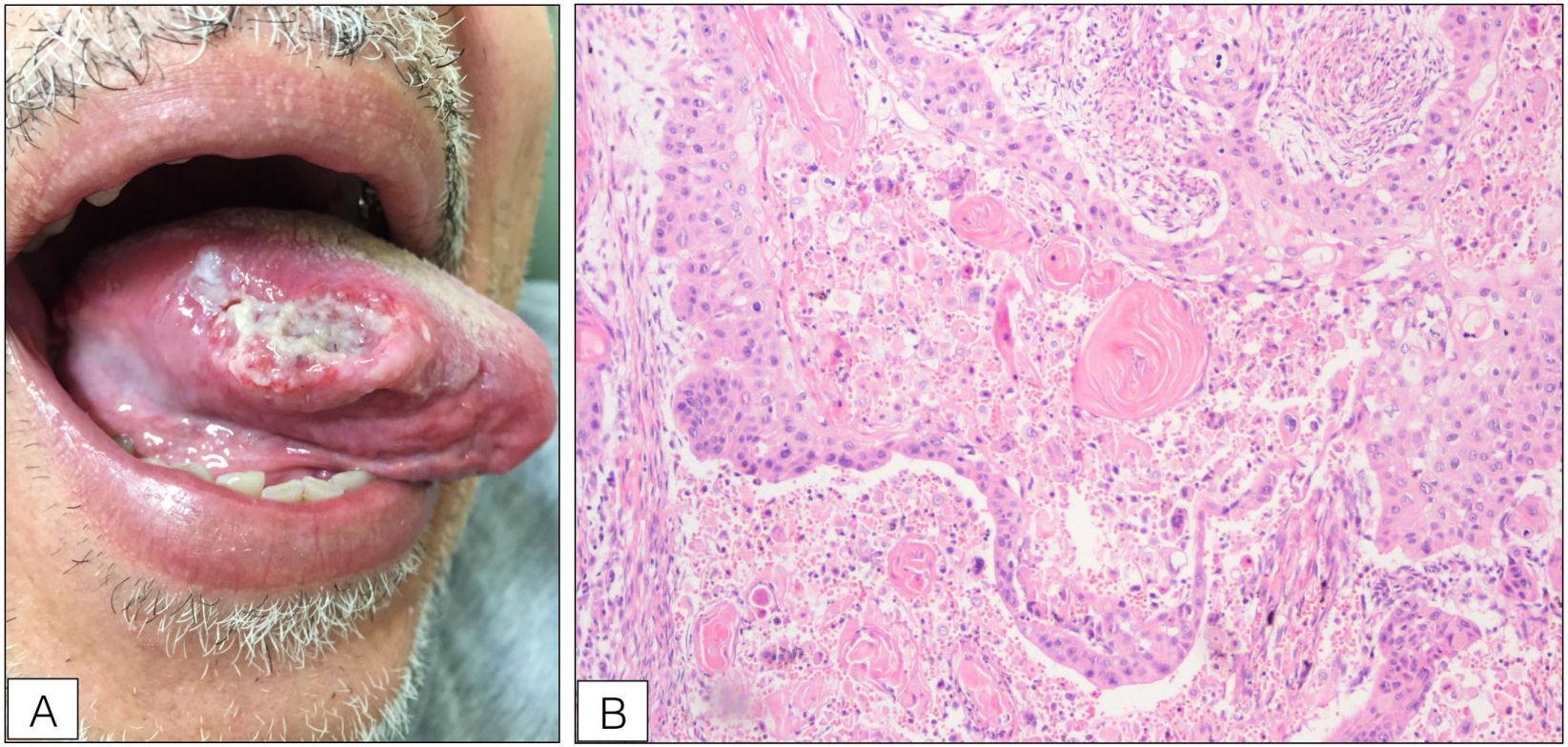

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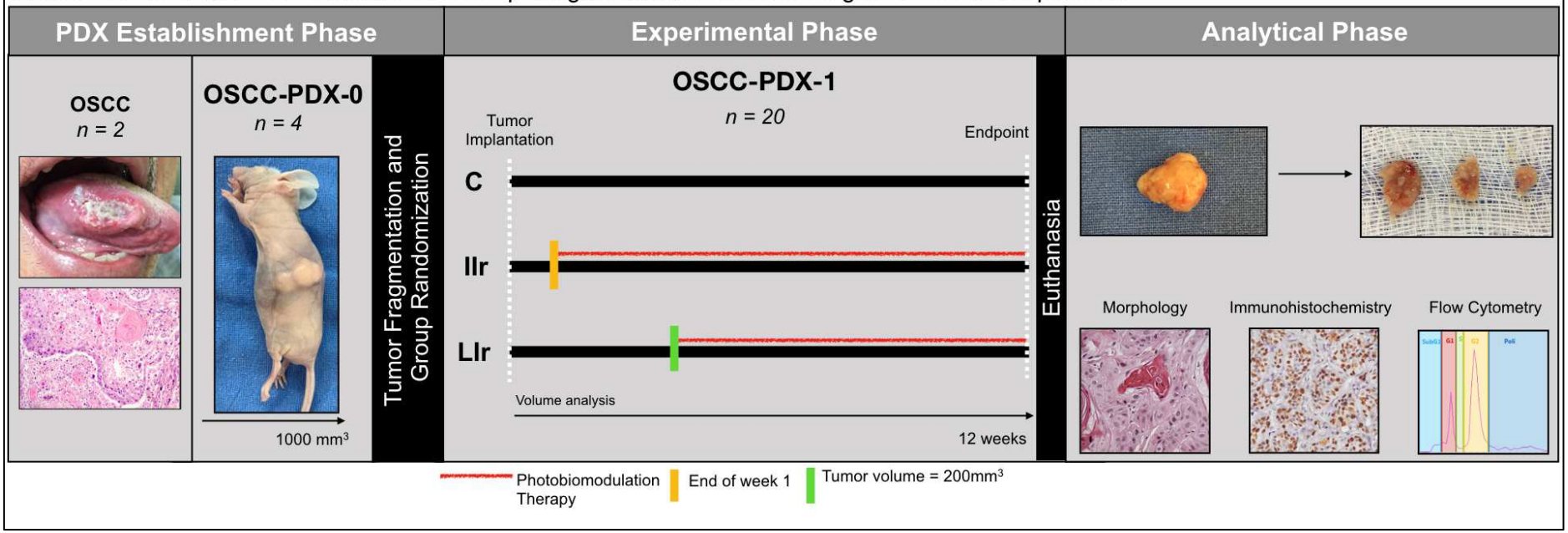

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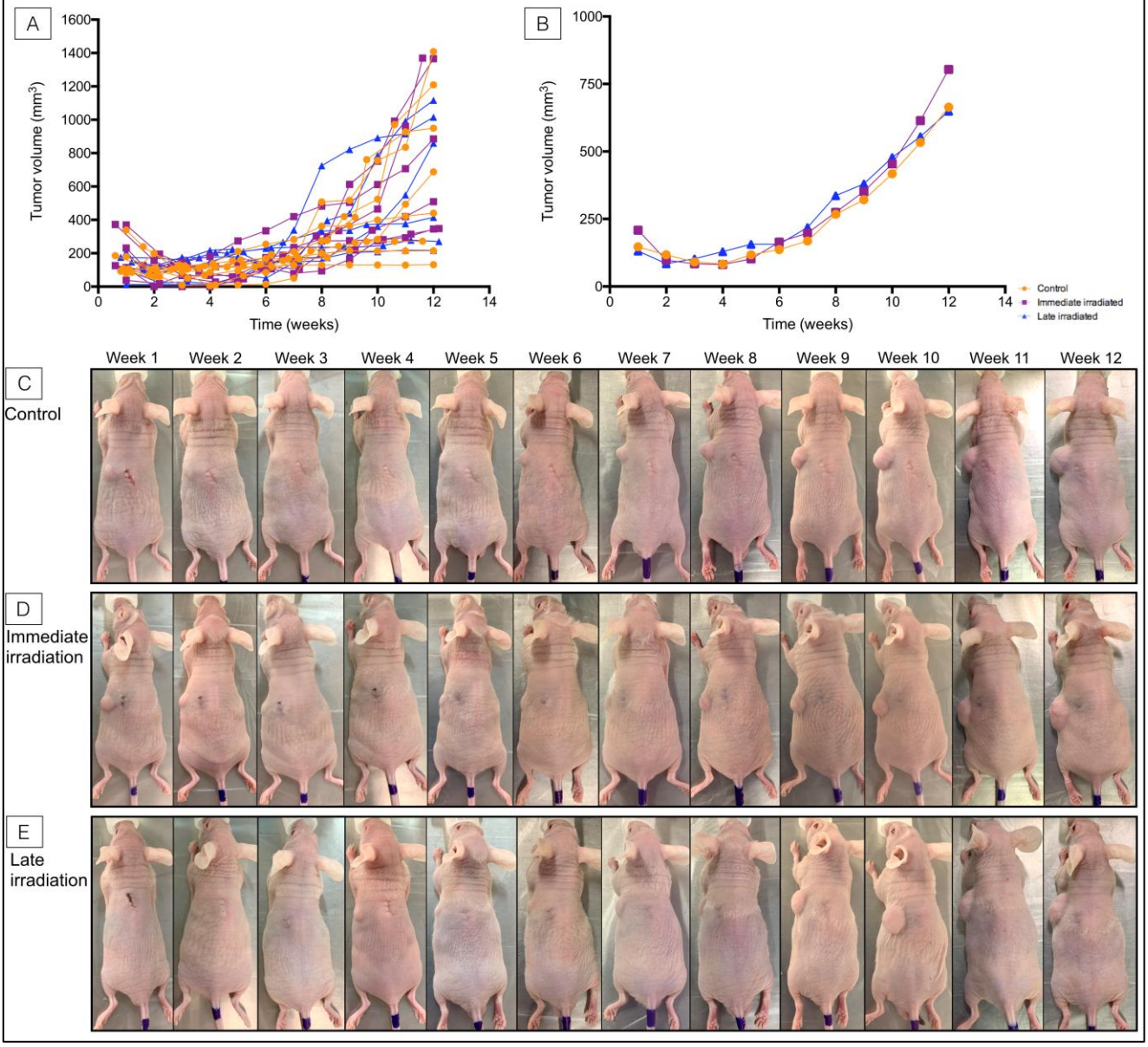

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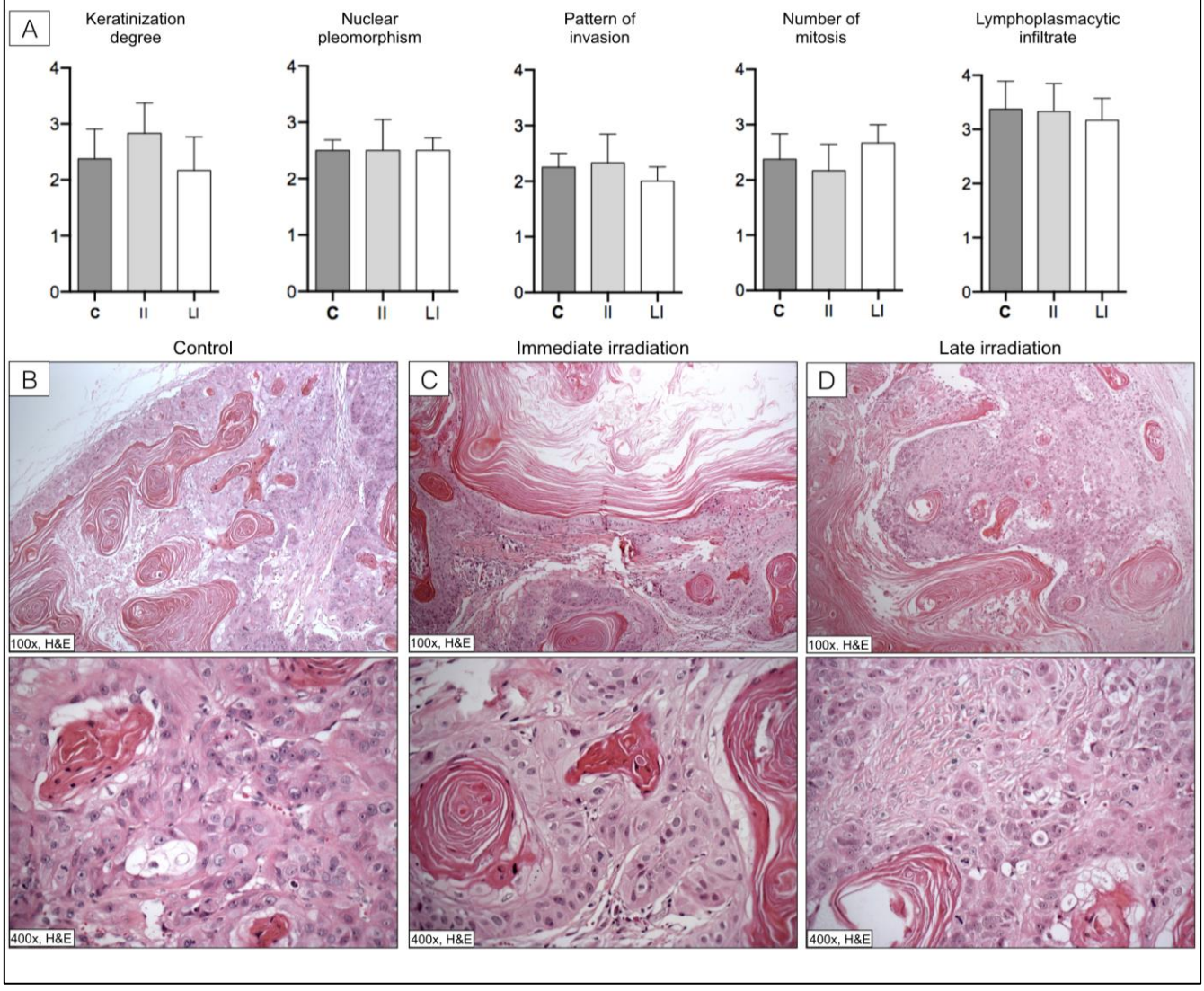

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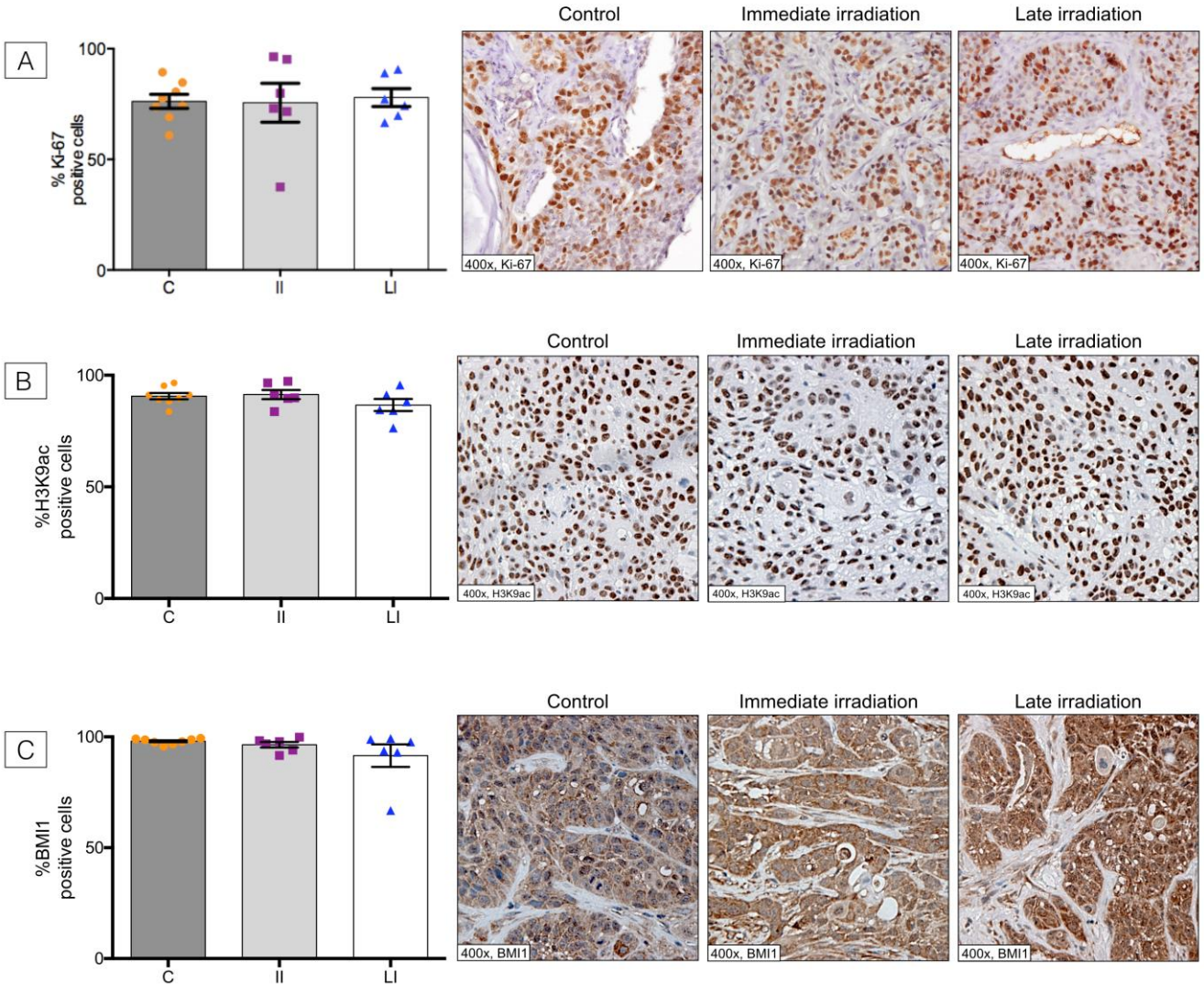

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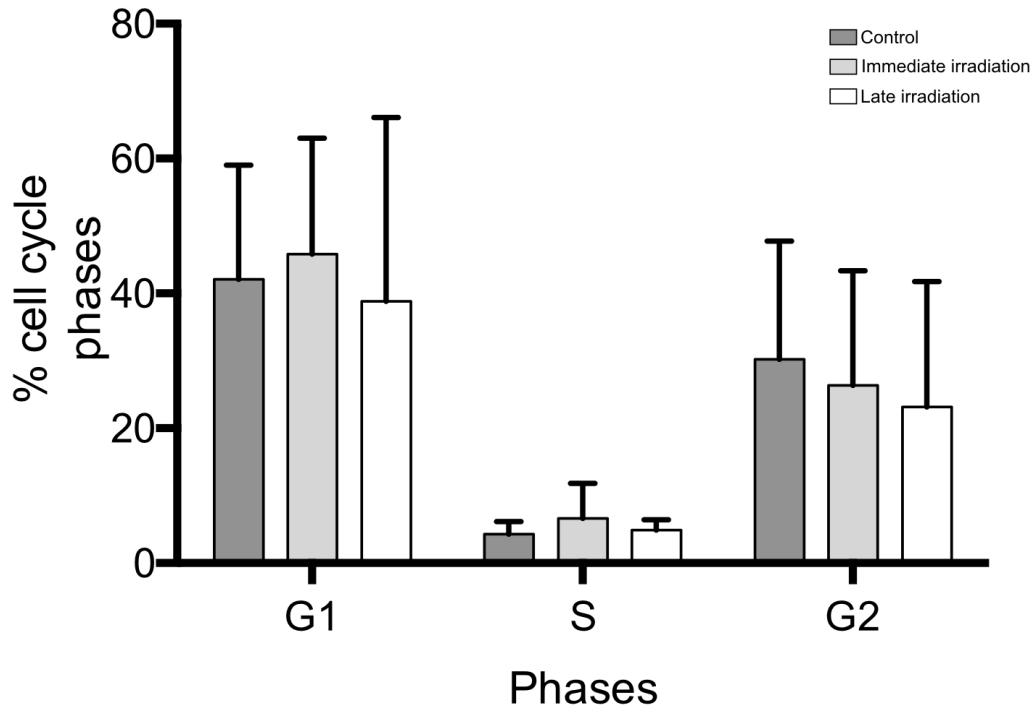

3 DISCUSSÃO

O uso da FBM como ferramenta para prevenção e manejo da MO tem sido suportado por diversas evidências científicas (Lalla et al., 2014; Bjordal et al., 2011; Zadik et al., 2019). A FBM tem sido amplamente utilizada para acelerar processos de reparo por conta da sua capacidade em aumentar proliferação / migração celulares por meio do estímulo da cadeia respiratória na mitocôndria celular, da produção de ATP, da síntese de diferentes proteínas e da modulação de processos biológicos distintos (Karu, 1999; Karu et al., 2004; Gao and Xing, 2009; Huang et al., 2009; Lopes et al., 2009; Lopes et al., 2010; Wagner et al., 2013; Wagner et al., 2016; Gabriel et al., 2019). Entretanto, conforme demonstrado, a segurança da sua utilização em locais com eventual presença de células tumorais ainda carece de maior avaliação, principalmente pelos efeitos proliferativos que a terapia ocasiona em alguns tipos de células não neoplásicas (AlGhamdi et al., 2012; Sonis et al., 2016). Nesse contexto, no primeiro estudo desta tese foi proposto o desenvolvimento de uma RS para avaliar e compilar os resultados de estudos que avaliem diferentes desfechos da FBM no CECP em modelos de cultivo celular e animal. A análise dos estudos incluídos permitiu concluir que os trabalhos acerca do tema apresentam uma importante heterogeneidade nas amostras utilizadas (linhagens celulares), nas condições e nos desenhos metodológicos, nos parâmetros de irradiação e nos métodos de avaliação. Além disso, a avaliação do risco de viés mostrou que apenas 3 dos 13 estudos *in vitro* apresentaram baixo risco. Em conjunto, tais fatores corroboram com a existência de importantes limitações metodológicas que podem comprometer o entendimento científico acerca dos possíveis impactos da FBM no CEC.

De acordo com a RS desenvolvida, observaram-se resultados discordantes ao avaliar os efeitos da irradiação em células de CEC. Essa discordância pode estar relacionada com alguns pontos, tais quais as diferentes condições de cultura celular em que as linhagens de células de CEC foram tratadas e com os distintos protocolos de irradiação instituídos em cada estudo. Nesse cenário, o estudo de Schalch et al. (2016) demonstrou que a densidade de plaqueamento celular apontou como um fator importante na determinação de resposta celular após irradiação. No estudo, observou-se que a linhagem SCC9 plaqueada em menores densidades (10^3

células/poço) apresentaram aumento na proliferação celular, enquanto que essa mesma linhagem plaqueada em maior densidade (10^4 células/poço) resultou em uma menor taxa de viabilidade após irradiação. Outros aspectos metodológicos importantes na interpretação destes resultados são o tempo após a FBM que os desfechos são analisados e as repetições nos protocolos de irradiação definidas em cada estudo. Hamblin (2018) menciona que o mecanismo de ação da FBM baseado na absorção direta da luz pelo cromóforo mitocondrial citocromo-C oxidase, que consequentemente resulta no aumento dos níveis de espécies reativas de oxigênio (EROs) e de ATP, ocorre após um período que varia entre horas até dias para finalmente modular a célula irradiada. Nesse sentido, as diferenças celulares metabólicas das linhagens avaliadas e os protocolos de irradiação heterogêneos são fatores maiores que devem influenciar nos resultados demonstrados através dos diferentes métodos de análises dos estudos.

Os estudos sistematicamente revisados utilizaram diferentes metodologias para avaliação dos seus desfechos. É importante enfatizar que, embora sejam metodologias relacionadas entre si, cada desfecho deve ser avaliado e interpretado separadamente. Uma compreensão mais completa da resposta de células tumorais irradiadas pode ser obtida por meio da análise das fases do ciclo celular, a qual pode abranger, em sua interpretação final, viabilidade, proliferação e apoptose das células estudadas. Henriques et al. (2014) relataram proporções aumentadas das fases S e G2/M em células SCC25 irradiadas utilizando um protocolo de irradiação com laser de 660 nm (30 mW e 1 J/cm^2) em comparação com o grupo controle e com outro grupo irradiado com dose de 0,5 J/cm^2. Por outro lado, Liang et al. (2015) demonstraram que a FBM com 810 nm (1000 mW/cm^2 e 60 J/cm^2) resultou na parada da fase G1 e no aumento da morte celular em linhagem de células cancerígenas orais humanas. Pode-se observar que os estudos avaliam as fases do ciclo celular a partir do uso de lasers com diferentes comprimentos de onda e doses associado a avaliação de linhagens celulares também distintas, dificultando a comparação dos resultados. Além desses, outros importantes pontos discordantes foram observados nos estudos incluídos na RS, tais quais as condições de cultura celular e os suplementos de meio de cultura utilizados (presença de dexametasona). Portanto, apesar do significativo valor de estudos *in vitro* para análise das respostas de células tumorais irradiadas, é necessário cautela na comparação dos resultados obtidos entre as diferentes pesquisas.

Outro ponto importante observado a partir dos estudos incluídos na RS é a falta de reporte completo dos parâmetros de irradiação utilizados. Os parâmetros mais descritos nos estudos foram comprimento de onda, potência, tempo e densidade de energia. O comprimento de onda foi reportado em todos os estudos, variando desde o espectro de luz visível até o infravermelho. Com relação aos demais parâmetros, observaram-se significativas variações na potência, na densidade de energia e no esquema de repetições de irradiação. Por meio da avaliação de todas as limitações extraídas a partir da RS desenvolvida, demonstrou-se de forma nítida a emergente necessidade de se realizar mais estudos *in vitro* padronizados e robustos. Baseado nisso e na tentativa de simular os parâmetros de irradiação mais indicados para uso em pacientes que necessitam de prevenção e/ou manejo da MO, nosso grupo demonstrou em um trabalho recente que a irradiação (660nm, 100mW, 0.24J) de linhagens celulares de CECP (HN3 e HN6) não as impactou em termos de migração celular, sobrevivência e porcentagem celular quando comparadas ao grupo controle (Martins et al., 2020). Com isso, por meio desse protocolo padronizado de irradiação, concluímos que a FBM não foi capaz de modificar o comportamento destas linhagens celulares. Considerando todo o exposto e percebendo a falta de estudos em modelos animais para avaliar os efeitos da FBM no CECP, desenvolvemos o segundo estudo desta tese para avaliar o impacto da FBM no CECO por meio de um modelo animal de PDX. Este foi um estudo pré-clínico pioneiro no tema, demonstrando que a irradiação não impactou no crescimento tumoral, no padrão morfológico, na proliferação celular e nos perfis epigenético e tronco-tumoral de modelos de PDX-CECO.

No segundo estudo desenvolvido, foram utilizados camundongos BALB/c nudes para estabelecimento e desenvolvimento de modelos de PDX-CECO. Para isso, utilizamos fragmentos de tumores provenientes de lesões em bordo lateral de língua com diagnóstico histopatológico prévio de CEC originários de dois pacientes do sexo masculino, com mais de 45 anos de idade e com histórico clínico de tabagismo e etilismo. As lesões possuíam estadiamento clínico III ou IV, com tumor em T3 ou mais. Os animais com esses modelos de PDX-CECO foram randomicamente divididos para posteriores análises entre grupos irradiados e não irradiado e conforme apresentado, após completar o protocolo de irradiação instituído, as avaliações de volume e morfologia tumorais, quantificações imunohistoquímicas (Ki-67, H3K9ac e BMI1) e análise de ciclo celular não

apresentaram diferenças estatisticamente significativas entre os grupos do estudo, sugerindo um impacto nulo da FBM nos modelos de PDX-CECO. Considerando que este foi o primeiro estudo avaliando o impacto da FBM em modelos de PDX-CECO, a comparação dos resultados com outros trabalhos torna-se um desafio. Os trabalhos em modelos animais com irradiação de CECP envolvem a indução química da carcinogênese com DMBA (Monteiro et al., 2011) e com 4NQO (Ottaviani et al., 2016). O estudo de Monteiro et al. (2011) induziu CEC em hamsters e os dividiu em grupo controle e irradiado (660nm, 424mW/cm^2, 56.4J/cm^2), demonstrando ao final do estudo que os animais irradiados apresentaram CEC com padrão mais indiferenciado quando em comparação ao grupo controle. Baseado nisso, os autores concluíram que a FBM promoveu uma progressão na severidade dos CEC irradiados. Entretanto, apesar desse resultado de casos de CEC mais pobremente diferenciados após irradiação, o número de CEC não parece ter mudado entre os diferentes grupos. Isso sugere que a irradiação não levou a um desenvolvimento de mais casos de CEC. Já no estudo de Ottaviani et al. (2016), a irradiação (970nm, 200mW/cm^2, 6J/cm^2) dos CECO desenvolvidos ocasionou uma redução na incidência de displasia e no número de carcinomas *in situ* e invasivos quando em comparação com as lesões não irradiadas. Além disso, uma maior organização do padrão vascular foi observada, permitindo que os autores concluíssem uma inibição na progressão tumoral após irradiação. Observam-se, mais uma vez, resultados contraditórios entre si e baseados em metodologias distintas, dificultando uma conclusão definitiva acerca dos efeitos da FBM no CEC.

Além dos resultados demonstrando falta de impacto da FBM no crescimento, na morfologia e na proliferação dos tumores, outro desfecho avaliado nos modelos de PDX-CECO do segundo estudo desta tese foi com relação a alterações epigenéticas por meio da imunomarcação com o anticorpo H3K9ac. Alterações epigenéticas associadas à acetilação da cromatina podem afetar a agressividade do tumor e a imunoexpressão do H3K9ac já foi previamente correlacionada com grau de malignidade, progressão linfonodal, estágio clínico avançado, baixo grau de diferenciação e mau prognóstico de CEC (Song et al., 2012; Noguchi et al., 2013 ; Webber et al., 2017). Nosso grupo de pesquisa mostrou associações entre alterações no H3K9ac e a agressividade / resistência à quimioterapia de CECO (Almeida et al., 2013). No artigo 2, a análise do anticorpo H3K9ac mostrou médias elevadas de imunoexpressão em todos os grupos avaliados, sem diferença

estatisticamente significativa entre eles. Com isso, a hipótese levantada é a de que os efeitos celulares do laser devem depender do status da célula no momento da irradiação. Além da imunomarcação do H3K9ac, a porcentagem de células positivas para o anticorpo BMI1 também não diferiu entre os grupos com modelos de PDX-CECO irradiados e não irradiado. Este resultado permite sugerir que a FBM não teve impacto no perfil tronco-tumoral dos modelos de PDX-CECO. A expressão de BMI1 em CECO tem sido correlacionada com a auto renovação de células-tronco tumorais, indicando um papel na capacidade metastática da neoplasia (Prince et al., 2007; Yang et al., 2010; Siddique e Salem, 2012). Em outros estudos, a imunoexpressão de BMI1 também foi relacionada com os eventos de desenvolvimento e capacidade invasiva de tumores e manutenção das propriedades de células-tronco tumorais de CECO (Song et al., 2006; Cao et al., 2011).

Estes resultados demonstrando falta de impacto em células neoplásicas irradiadas de CECO, tanto do segundo artigo desta tese quanto do trabalho recente de Martins et al. (2020) aqui discutido, podem estar relacionados com o conceito de que a irradiação com laser apresenta capacidade estimulatória somente em células em situações de baixo metabolismo (Karu, 1989; Peplow et al., 2010), o que não ocorre em células neoplásicas. Outra consideração importante de ser levantada na discussão destes resultados é o conhecimento de que um importante *hallmark* das células neoplásicas é a sua habilidade de proliferar constantemente e na ausência de fatores externos, o que consequentemente resulta em crescimento tumoral ilimitado e independente (Hanahan e Weinberg, 2011). Portanto, a influência externa da irradiação do laser em células neoplásicas pode realmente não ser suficiente para impactar o comportamento tumoral do CEC, configurando uma importante evidência científica relacionada com a segurança do uso da FBM especialmente em casos onde o campo de irradiação se encontra contíguo à regiões tumorais.

4 CONCLUSÃO

Baseado nos resultados dos estudos apresentados nesta tese, pode-se concluir que:

- Existe uma alta heterogeneidade metodológica nos estudos *in vitro* e *in vivo* que avaliam os efeitos da terapia de fotobiomodulação em células tumorais de carcinoma espinocelular de cabeça e pescoço;
- A avaliação da irradiação de carcinoma espinocelular oral em modelos de xenoenxertos derivados de pacientes não demonstrou impacto no volume tumoral e nos perfis morfológico, proliferativo, epigenético e tronco-tumoral dos tumores. Em conjunto, estes resultados permitem concluir que a terapia de fotobiomodulação nos parâmetros definidos não apresentou efeitos inibitórios, protetivos ou estimulatórios nos modelos de carcinoma espinocelular oral desenvolvido neste modelo animal.

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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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ANEXOS

Anexo 1 – Comprovante de publicação do Artigo 1.

Photochemical & Photobiological Sciences

PERSPECTIVE

View Article Online
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Cite this: DOI: 10.1039/c9pp00120d

Examining tumor modulating effects of photobiomodulation therapy on head and neck squamous cell carcinomas

Felipe Martins Silveira,[a] Mariana de Pauli Paglioni,[a] Márcia Martins Marques,[b] Alan Roger Santos-Silva,[a] Cesar Augusto Migliorati,[c] Praveen Arany[d] and Manoela Domingues Martins[a,e]

Photobiomodulation (PBM) therapy is an effective method for preventing and managing oral mucositis (OM) in head and neck squamous cell carcinoma (HNSCC) patients undergoing radiotherapy alone or in combination with chemotherapy. However, the potential effects of PBM therapy on premalignant and malignant cells eventually present in the treatment site are yet unknown. The aim of this systematic review was to analyze the effects of PBM therapy on HNSCC. A literature search was conducted in four indexed databases as follows: MEDLINE/PubMed, EMBASE, Web of Science, and Scopus. The databases were reviewed for papers published up to and including in October 2018. *In vitro* and *in vivo* studies that investigated the effects of PBM therapy on HNSCC were selected. From the 852 initially gathered studies, 15 met the inclusion criteria (13 *in vitro* and 2 *in vivo*). Only three *in vitro* studies were noted to have a low risk of bias. The included data demonstrated wide variations of study designs, PBM therapy protocols, and study outcomes. Cell proliferation and viability were the primary evaluation outcome in the *in vitro* studies. Of the 13 *in vitro* studies, seven noted a positive effect of PBM therapy on inhibiting or preventing an effect on HNSCC tumor cells, while six studies saw increased proliferation. One *in vivo* study reported increased oral SCC (OSCC) progression, while the other observed reduced tumor progression. Overall, the data from the studies included in the present systematic review do not support a clear conclusion about the effects of PBM therapy on HNSCC cells.

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Accepted 9th June 2019

DOI: 10.1039/c9pp00120d

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Introduction

Head and neck squamous cell carcinoma (SCC) (HNSCC) treatment encompasses three modalities (surgery, radiotherapy, and chemotherapy) that can be administered exclusively or concomitantly with one another depending on the site of the cancer and the stage of the disease.[1,2] Use of the radiotherapy approach often leads to acute toxicities such as oral mucositis (OM), which is clinically characterized by painful ulcerations in the oral mucosa.[3,4] OM limits adequate nutritional intake, increasing the risk of malnutrition and poor quality of life, with the possibility of being a factor that increases overall treatment costs and negatively impacts cancer prognosis due to pain, bacteremia, and treatment interruptions.[5–7]

Photobiomodulation (PBM) therapy, also known as low-level light therapy, is one of the therapeutic approaches for OM management.[8–10] At this time, there are three well-described mechanisms of PBM therapy.[11] The first, involves an intracellular chromophore, cytochrome-C oxidase in the mitochondria; the second, cell membrane light-sensitive receptors such as opsins and TRPV1; and, the third, an extracellular latent growth factor, TGF-β1. In animal studies, PBM therapy has demonstrated positive effects on the management of OM by promoting tissue repair and anti-inflammatory effects.[12–15] Human clinical trials have also demonstrated positive results with the use of PBM therapy for preventing and managing OM.[16–18] Based on these therapeutic benefits of PBM therapy, the Multinational Association of Supportive Care in Cancer (MASCC) and the International Society of Oral Oncology (ISOO) had designated PBM therapy as an effective adjunctive treatment for managing OM in 2013 (currently under

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Anexo 2 - Certificados de aprovação do Comitê de Ética em Pesquisa (Hospital de Clínicas de Porto Alegre)

UFRGS - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE DA UNIVERSIDADE FEDERAL

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ESTUDO DO IMPACTO DA FOTOBIOMODULAÇÃO NA PROGRESSÃO DO CARCINOMA ESPINOCELULAR ORAL EM MODELO DE XENOENXERTO DERIVADO DE PACIENTE

Pesquisador: Manoela Domingues Martins

Área Temática:

Versão: 2

CAAE: 86434718.0.0000.5327

Instituição Proponente: Hospital de Clínicas de Porto Alegre

Patrocinador Principal: Hospital de Clínicas de Porto Alegre

DADOS DO PARECER

Número do Parecer: 2.676.935

Apresentação do Projeto:

O carcinoma espinocelular (CEC) é o tipo mais comum de neoplasia maligna da cavidade oral. O tratamento desta condição varia entre abordagem cirúrgica, radioterapia e quimioterapia, isoladas ou em associações. A radioterapia de cabeça e pescoço e a quimioterapia podem acarretar inúmeras sequelas ao paciente, sendo a mucosite oral (MO) uma das mais frequentes e graves. Clinicamente, a MO se manifesta como lesões que variam de áreas eritematosas a ulcerações. Essas lesões podem causar dor, restringir a alimentação, serem sítios agravadores de infecção secundária, aumentar o custo do tratamento e tempo de hospitalização do paciente, interferir no curso do tratamento antineoplásico e, consequentemente, na sobrevida do paciente. O uso da terapia de fotobiomodulação (PBMT do inglês photobiomodulation therapy) para prevenir ou tratar a MO está bem estabelecido na literatura. Porém, os efeitos da PBMT em eventuais células tumorais residuais presentes na área de aplicação ainda não estão completamente elucidados. O presente projeto de pesquisa objetiva avaliar in vivo os efeitos da PBMT em células de CEC humano. Para isso, será estabelecido um modelo de xenoenxerto derivado de paciente (patient-derived xenograft – PDX) por meio da coleta de tumores de CEC de língua provenientes de dois pacientes. Cada tumor será dividido em três fragmentos (n = 6 fragmentos). Um fragmento de cada tumor será submetido à análise histopatológica e os outros 4 fragmentos serão implantados no dorso de quatro camundongos BALB/c nude machos e fêmeas, com 5-7 semanas de idade,

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Continuação do Parecer: 2.676.935

Informamos que obrigatoriamente a versão do TCLE a ser utilizada deverá corresponder na integra à versão vigente aprovada.

e) Deverão ser encaminhados ao CEP relatórios semestrais e um relatório final do projeto.

f) A comunicação de eventos adversos classificados como sérios e inesperados, ocorridos com pacientes incluídos no centro HCPA, assim como os desvios de protocolo quando envolver diretamente estes pacientes, deverá ser realizada através do Sistema GEO (Gestão Estratégica Operacional) disponível na intranet do HCPA.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1046753.pdf	09/05/2018 02:36:03		Aceito
Outros	cartacep.docx	09/05/2018 02:34:26	Manoela Domingues Martins	Aceito
Orçamento	orcamento.pdf	09/05/2018 02:31:42	Manoela Domingues Martins	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	tcle.pdf	09/05/2018 02:31:16	Manoela Domingues Martins	Aceito
Cronograma	cronograma.pdf	09/05/2018 02:30:44	Manoela Domingues Martins	Aceito
Projeto Detalhado / Brochura Investigador	projeto.pdf	09/05/2018 02:29:06	Manoela Domingues Martins	Aceito
Declaração de Pesquisadores	delegacaopesquisadores2.PDF	28/03/2018 12:04:23	Manoela Domingues Martins	Aceito
Declaração de Pesquisadores	declaracao.pdf	15/03/2018 13:39:09	Manoela Domingues Martins	Aceito
Folha de Rosto	folhaderosto.pdf	15/03/2018 13:37:20	Manoela Domingues Martins	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

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HOSPITAL DE CLÍNICAS DE PORTO ALEGRE

Grupo de Pesquisa e Pós Graduação

Carta de Aprovação

Certificamos que o projeto abaixo, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) e pelas áreas de apoio indicadas pelo pesquisador.

Projeto: 2018/0134

Título: ESTUDO DO IMPACTO DA FOTOBIOMODULAÇÃO NA PROGRESSÃO DO CARCINOMA ESPINOCELULAR ORAL EM MODELO DE XENOENXERTO DERIVADO DE PACIENTE

Pesquisador Responsável: **MANOELA DOMINGUES MARTINS**

Equipe de Pesquisa: GERSON SCHULZ MAAHS; FELIPE MARTINS SILVEIRA; MARCO ANTONIO TREVIZANI MARTINS; PATRICIA LUCIANA DA COSTA LOPEZ;

Data de Aprovação:

Data de Término: 31/12/2019

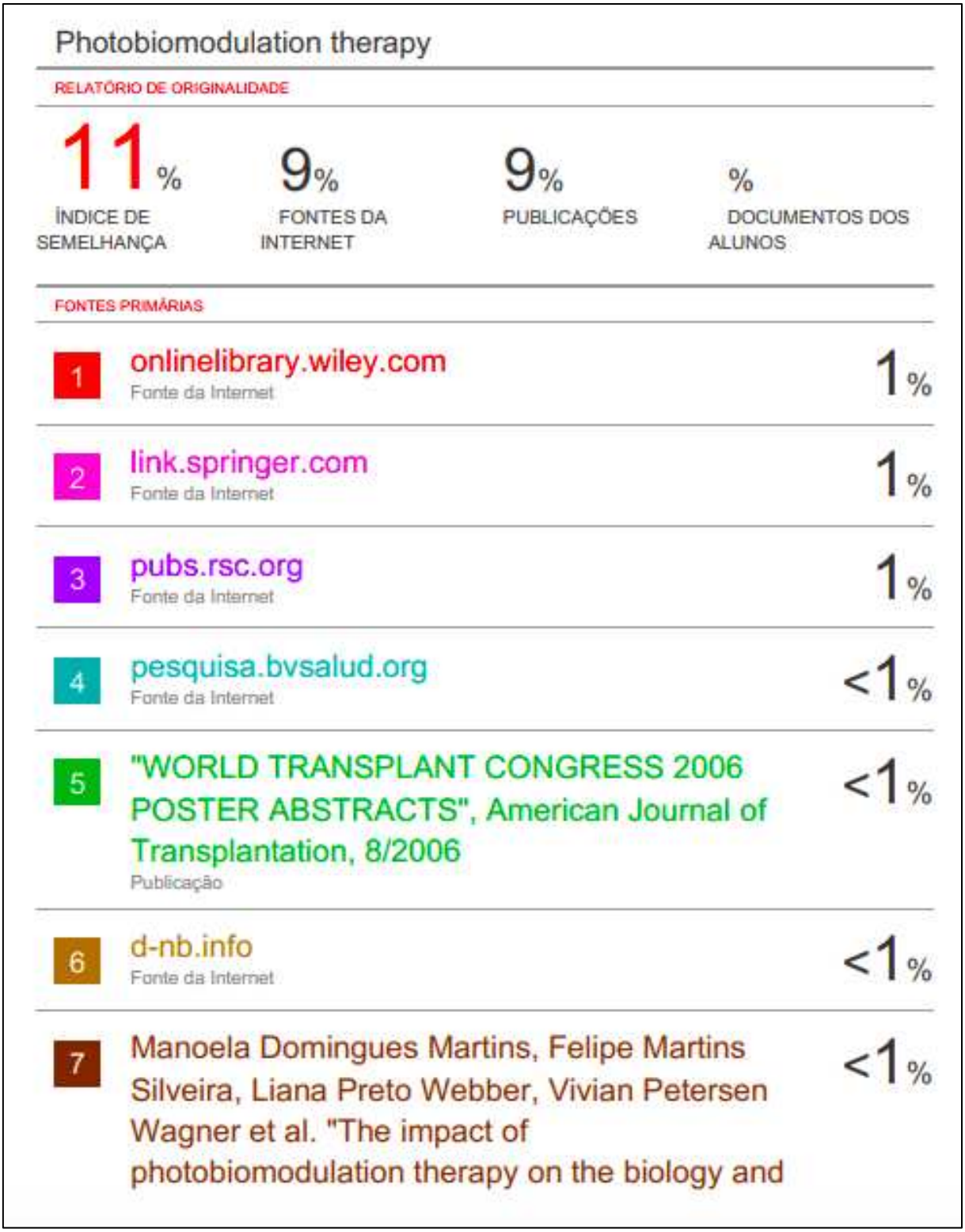
Espécie/Linhagem	Sexo/Idade	Quantidade
CAMUNDONGO MUTANTE	null/7 Semana(s)	40

- Os membros da CEUA/HCPA não participaram do processo de avaliação onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

Profª Patrícia Ashton Prolla
Coordenadora GPPG/HCPA

Impresso do sistema AGHU-Pesquisa por RAFAEL LEAL ZIMMER em 17/07/2018 14:39:34

Anexo 3 - Relatório de verificação de originalidade e prevenção de plágio



Photobiomodulation therapy

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