UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

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EFEITOS DA POLARIZAÇÃO DE MACRÓFAGOS M1 E M2 NA MIGRAÇÃO CELULAR E SEU PAPEL NO PROGNÓSTICO DE PACIENTES COM CARCINOMA ESPINOCELULAR ORAL

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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgião-Dentista.

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RESUMO

O carcinoma espinocelular oral (CEC oral) é o tipo de câncer mais frequente na cavidade oral, língua e lábios. As células tumorais secretam inúmeros fatores que atuam sobre células adjacentes levando a reprogramação destas, que passam então a atuar em prol do tumor, favorecendo seu crescimento, progressão, invasão e formação de metástases. Este conjunto recebeu o nome de microambiente tumoral e entre os seus componentes estão: fibroblastos, macrófagos, linfócitos e neutrófilos. Os macrófagos se destacam pela presença em inúmeros tipos de câncer, sendo. Atualmente se sabe que os macrófagos possuem dois perfis denominados: M1, que pelas características de citocinas liberadas possui atividade anti-tumoral; e M2 que é considerado pro tumoral, sendo relacionado a pior prognóstico em diversos cânceres. Os objetivos deste trabalho foram: 1- analisar o papel de macrófagos no prognóstico de pacientes com CEC; 2- observar o padrão inflamatório em tumores de pacientes e a influência dos macrófagos na migração de células de CEC. Para responder ao objetivo 1 realizamos uma revisão sistemática de literatura, com buscas realizadas nas bases PubMed, Scopus e ISI Web of Knowledge em que, de um total de 286 artigos, 14 atendiam aos critérios de inclusão. A maioria dos trabalhados utilizava os marcadores CD68 um pan marcador de macrófagos e/ou CD163 marcador de M2, sendo que altos níveis destes marcadores estão associados a menor sobrevida livre de doença, presença de metástases e pior prognóstico. Para o objetivo 2 realizamos uma série de experimentos: análise proteômica e quantificação de macrófagos M1 e M2 em tumores de pacientes com CEC, e avaliação do papel do meio condicionado (CM) de macrófagos em uma linhagem de carcinoma espinocelular oral (SCC25). A análise proteômica mostrou que há diferença entre proteínas relacionadas a inflamação da zona adjacente do tumor (TAE) em relação ao centro do tumor (CT). Quando verificamos o perfil inflamatório em pacientes com CEC oral, houve uma similaridade entre macrófagos M1 e M2 na TAE e na CT. Os testes com CM de macrófagos polarizados sobre SCC25 mostrou que M2-CM aumenta a velocidade de migração em 18% ($p<0,05$) em relação ao controle e ainda alterou a direcionalidade das células. Concluímos que os macrófagos, principalmente do tipo M2, se relacionam a pior prognóstico em pacientes com CEC oral. Já a presença de proteínas relacionadas a inflamação apresentou um padrão entre as áreas avaliadas, dados similares foram vistos quando avaliamos a quantidade de macrófagos. Por fim, M2-CM altera o perfil de migração de células de SCC25, provavelmente através de citocinas liberadas no meio que atuam sobre a velocidade e direcionalidade de migração.

Palavras-chave: Microambiente tumoral. Macrófagos. Inflamação. Câncer oral. Prognóstico.

ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most frequent type of cancer in the oral cavity, tongue and lips. Tumor cells secrete several factors that act on adjacent cells leading to the reprogramming of these cells, that they start acting on the tumor, favoring its growth, progression, invasion and formation of metastases. This characteristic is called tumor microenvironment and among its components are observed: fibroblasts, macrophages, lymphocytes and neutrophils. Macrophages are distinguished by the presence n innumerable types of cancer, and even more numerous than the tumor cells. It is known that macrophages have two profiles called M1, which, due to the characteristics of released cytokines, have anti-tumor activity and type M2, which is considered pro-tumoral, and is related to a worse prognosis in several cancers. The objectives of this study were: 1- to analyze the role of macrophages in the prognosis of patients with OSCC; 2- to observe the inflammatory pattern in tumors of patients and to analyze the influence of macrophages in the migration of OSCC cells. In order to respond to objective 1, we carried out a systematic review of the literature, with searches performed in the PubMed, Scopus and ISI Web of Knowledge databases which, from a total of 286 articles, 14 met the inclusion criteria. Most of the articles used the CD68 markers as a pan marker of macrophages and / or CD 163 marker of M2. High levels of these markers were associated with reduced disease-free survival, presence of metastases and worse prognosis. For objective 2 we performed a series of experiments: proteomic analysis and quantification of M1 and M2 macrophages in tumors of patients with OSCC, and evaluation of the role of conditioned medium (CM) of polarized macrophages in an oral squamous cell carcinoma cell line (SCC25). Proteomic analysis showed that there is a difference between proteins related to the inflammation of the adjacent area of the tumor (TAE) in relation to the center of the tumor (CT). When we verified the inflammatory profile in patients with oral SCC, there was a similarity between M1 and M2 macrophages in TAE and in CT. The tests with CM on SCC25 cells showed that M2-CM increases the migration rate by 18% ($p < 0.05$) over the control and also altered the directionality of the cells. We conclude that macrophages, mainly the M2-type, are related to a worse prognosis in patients with oral CEC. Also, the presence of inflammation-related proteins and the amount of macrophages were higher in CT. Finally, M2-CM altered the migration profile of SCC25 cells, probably through cytokines released in the medium which act on the speed and directionality of migration.

Keywords: Tumor microenvironment. Macrophage. Inflammation. Oral cancer. Prognosis.

SUMÁRIO

1 INTRODUÇÃO

1.1 NEOPLASIAS E MICROAMBIENTE TUMORAL

O câncer é uma desordem genética que ocorre na maioria dos casos em células somáticas. Vários fatores podem levar a mutações no DNA dessa população celular, sendo as seguintes as mais pesquisadas: a dieta, o consumo de tabaco e álcool, considerados fatores exógenos e os radicais livres que são fatores endógenos. As mutações do DNA, aliadas à perda da capacidade de checagem e correção destas se acumulam ao longo do tempo levando as células a adquirirem um fenótipo cancerígeno. As células tumorais apresentam graus variados de diferenciação, quando comparadas a células que as originaram e ainda perda dos mecanismos de controle da proliferação celular e aquisição da capacidade de se tornarem imortais (1).

O carcinoma espinocelular oral (CEC oral) é o tipo de câncer mais frequente na cavidade oral, língua e lábios. Para o ano de 2012 foram estimados, segundo a International Agency for Research on Cancer, 300.373 novos casos diagnosticados em todo o mundo, ocupando a 15ª posição entre todos os cânceres (2). No Brasil, segundo dados do Instituto Nacional do Câncer, o câncer oral é a 5° neoplasia mais frequente em homens e a 12ª em mulheres, com previsão de 11.200 casos novos em homens e 3.500 em mulheres para cada ano do biênio 2018-2019 (3). A maior ocorrência em homens está relacionada a maior tendência deste grupo em desenvolver hábitos como, alcoolismo e tabagismo (4). Estudos recentes apontam o vírus do papiloma humano (HPV) como um fator de risco independente (5).

As células tumorais em seu estágio inicial secretam inúmeras citocinas, as quais recrutam células mesenquimais dos tecidos normais adjacentes, e também da medula óssea vermelha. Estas células são reprogramadas a produzirem outras citocinas que irão atuar sobre as células neoplásicas, favorecendo seu crescimento, promoção e a invasão de tecidos. Cria-se assim um microambiente tumoral, ao redor das células neoplásicas, que é caracterizado por apresentar um estado de inflamação crônica. Neste novo ambiente estabelecido, há a presença de células imunossupressoras com atividade pró-tumoral: linfócitos T reguladores (células treg),

macrófagos M2, neutrófilos N2 e as células supressoras derivadas de mielóides(MDSC) e fibroblastos (6).

Entre estas células do microambiente, os macrófagos associados a tumores (TAMs) são as mais influentes na progressão do tumor e sua presença está relacionada a um pior prognóstico (7). Estas células têm sido relacionadas à proliferação e sobrevida de células tumorais, angiogênese, invasão de tecidos adjacentes e metástase e a presença de macrófagos, principalmente tipo M2, se relaciona a menor sobrevivência em diversos tumores como os de mama, rim, bexiga e melanoma, glioma, câncer de pâncreas e CEC oral (8-10).

1.2 MACRÓFAGOS E CÂNCER

Os macrófagos são células importantes da defesa imune inata, protegendo o organismo contra invasão de patógenos, liberando fatores inflamatórios, atuando como apresentadores de antígenos e ainda participando da cicatrização de feridas. Os macrófagos são derivados do sistema de fagócitos mononucleares, na medula óssea vermelha. As unidades formadoras de colônias de macrófagos/granulócitos (GM-CFUs) dão origem às unidades formadoras de colônias de macrófagos (M-CFUs), subsequentemente formam-se monoblastos e destes os pró-monócitos que, na circulação, se diferenciam em monócitos. Os monócitos deixam a corrente sanguínea migrando para os tecidos onde se diferenciam em macrófagos (11).

Uma das principais características dos macrófagos é a sua plasticidade, pois, a partir de sinais provenientes do microambiente que os cerca, adquirem fenótipos distintos. Tipicamente os macrófagos são divididos em dois principais grupos: M1 e M2. Esta plasticidade também se observa quando por fatores estimulantes M1 passa a apresentar características de M2 (12).

Macrófagos do tipo M1 desempenham um importante papel contra patógenos e células tumorais, por ativar uma resposta imune do tipo I. A polarização em M1 ocorre pelo estímulo de: citocina interferon gama (INF-γ), fator de necrose tumoral (TNF) e ativação de receptores toll-like (TLRs) via exposição a microrganismos ou seus produtos como lipopolissacarídeos (LPS) (13). Elevados níveis do fator estimulador de colônia de granulócitos-macrófagos (GM-CSF), interleucina 12 (IL-12), sintase induzida por óxido nítrico (iNOS), espécies reativas

de oxigênio (EROs), quimiocina com cisteína no terminal 10 (CXCL-10) presentes no microambiente tumoral, também estão relacionados a ativação de macrófagos M1 (6).

Depois de ativados os macrófagos M1 secretam altos níveis de citocina IL-12 e IL-23 e baixos níveis de IL10. A IL-12 produzida por macrófagos promove a diferenciação de células T helper1 (Th1) que podem melhorar a fagocitose de antígenos. Já a IL-23 está relacionada ao desenvolvimento das células Th17 que secretam IL-17 contribuindo para doenças inflamatórias autoimunes (10). Os M1 secretam ainda uma série de citocinas pró inflamatórias como a IL-1, IL-6, e INF- γ, que são associadas a ativação da Th1, promovendo uma resposta imune antitumoral (11).

M1 também tem a grande capacidade de gerar EROs e ativar o gene iNOS que produz óxido nítrico (NO). Além destas funções, macrófagos M1 produzem quimiocinas com cisteína N-terminal separadas por um aminoácido (CXCL9/Mig e CXCL10/Ip), que atraem linfócitos Th1. E ainda funcionam como apresentadores de antígenos o que é considerado uma potencial função antitumoral (11, 14).

O macrófago do tipo 2 (M2), também chamado de alternativamente ativado, segundo vários estudos é o fenótipo mais encontrado nos TAMs (15). Uma classificação alternativa foi proposta por Mantovani et al, no qual os macrófagos do tipo 2 são subdivididos em três populações de acordo com seu estímulo para ativação: M2a induzido por exposição a IL-4 e IL-13; M2b induzidos por receptor toll-like (TLRs), LPS e complexos imunes; e M2c ativados por IL-10, TGF-β e hormônios glucocorticóides (16). Os grupos M2a e M2b promovem a resposta imune Th2 e o grupo M2c suprimem a inflamação (7). Ainda participam da ativação de M2 os seguintes fatores, M-CSF, quimiocinas com duas cisteínas adjacentes (CCL2, CCL5, CCL22), e o fator induzido por hipóxia alfa (HIF-1α) (6, 17).

Macrófagos M2 se caracterizam por produzirem altos níveis de IL-10 e baixos níveis de IL-12 e ainda produzem quimiocinas com duas cisteínas adjacentes como CCL17/TARC, CCL22/MDC e CCL24/Eotaxin-2, associadas ao recrutamento de células reguladoras T(Treg), T helper2 (Th2), eosinófilos e basófilos e não serem eficientes como apresentadores de antígeno (17).

A grande maioria dos tumores apresentam macrófagos com fenótipo M2. Estes liberam fatores de crescimento como fator de crescimento endotelial vascular

(VEGF), fator de crescimento derivado de plaquetas (PDGF), fator de crescimento transformador (TGF- β) e fator de crescimento de fibroblastos (FGF) que podem promover angiogênese em vários tumores, dentre os quais, gliomas, CECs orais, esôfago, mama, bexiga e próstata (11, 18).

Os TAMs também liberam metaloproteinases da matriz (MMP-1, MMP-2, MMP-3, MMP-9 e MMP-12) e também ativador de plasminogênio tipo-uroquinase (uPA). A MMP-9 é a mais importante protease seguida da MMP-2 sendo que ambas degradam a matriz extracelular e promovem a invasão tumoral. Durante a degradação deste substrato, são liberados vários fatores sequestrados na matriz, como o fator de crescimento do endotélio vascular (VEGF), aumentando a angiogênese (7).

A marcação de TAMs utilizando o marcador CD68+ em tumores ocorre simultaneamente tanto em macrófagos do tipo M1 quanto do tipo M2, em quantidades variáveis. Sendo assim, a presença de CD68+ nem sempre indica um pior prognóstico, pois os níveis de M1 que possui atividade antitumoral podem estar elevados. Marcadores com maior especificidade devem ser utilizados. Em diversos estudos foram utilizados para identificação de M1 os marcadores CD11b, IL-12, iNOS, EROs. E para M2 os marcadores CD163, CD206 (9, 19, 20).

1.3 MACRÓFAGOS E PROGNÓSTICO DO CARCINOMA ESPINO CELULAR ORAL

Uma alta densidade de macrófagos infiltrados no tumor é um indicativo de pior prognóstico em vários tumores sólidos. Destaca-se a presença de macrófagos do tipo M2 relacionada a pior sobrevida em tumores de mama, fígado, pulmão, bexiga, melanoma, ovário (21). Nos tumores da cavidade oral a marcação positiva para CD 163 mostrou pior prognóstico e sobrevida livre de doença (22-24). Pacientes com metástase em linfonodos exibem uma alta infiltração tanto no tumor quanto no linfonodo, desta forma TAMs associam-se a disseminação metastática do tumor (25).

2 HIPÓTESE

Os fatores secretados pelos macrófagos polarizados M2 aumentam a migração de células tumorais, o que se relaciona a um pior prognóstico em pacientes com CEC oral.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o papel de macrófagos polarizados do tipo M1 e M2 sobre o comportamento de carcinoma espinocelular e sobre o prognóstico de pacientes com CEC oral.

3.2 OBJETIVOS ESPECÍFICOS

a) Objetivo específico 1 - Correlacionar o papel dos macrófagos com o prognóstico de pacientes com CEC oral.

b) Objetivo específico 2 – Avaliar o perfil proteômico e da proporção de M1 e M2 no epitélio adjacente ao tumor e na zona de centro de tumor provenientes de biópsias de pacientes com CEC.

c) Objetivo específico 3 - Analisar o papel dos macrófagos polarizados sobre o comportamento migratório de CEC Oral

4 ARTIGO 1

Este artigo contempla os resultados obtidos a partir do desenvolvimento do objetivo específico 1. Para responder como o perfil dos macrófagos influência no prognóstico de pacientes com CEC, neste capítulo apresentaremos a seguinte revisão sistemática da literatura: Macrophages and Prognosis of Oral Squamus Cell Carcinoma: A sistematic review, publicada no periódico: The Journal of Oral Pathology and Medicine Volume 47, Issue 5, May 2018, P 460-467. (ANEXO A).

5 ARTIGO 2

Este contempla o desenvolvimento dos objetivos específicos 2 e 3. Os dados experimentais, realizados em cultura celular e amostras de biopsias de pacientes, referentes a tais objetivos, serão descritos na forma de artigo. O artigo será submetido ao periódico Cancer Letters.

Macrophage and inflammation in oral squamous cell carcinoma.

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Running title: M2 macrophages up regulation in oral cancer

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ABSTRACT

Inflammation is present in the tumor microenvironment and macrophages are of great relevance in this process. There are two types of macrophages: the M1 type, considered anti-tumoral, and the M2, considered pro-tumoral. Our objective was to evaluate the profile of inflammatory proteins, the M1/M2 ratio in patients with oral squamous cell carcinoma (OSCC) and the effect of macrophages on migration of OSCC. It was observed differences among inflammation-related proteins at the center of the tumor (CT) and the tumor adjacent epithelium (TAE) which was accompanied by a ~2 fold increase on M2 macrophages at CT. Also, the conditioned medium of M2 macrophages induced higher migration velocity and directionality of OSCC. These data reveal that macrophages might be considered a new therapeutic target in OSCC due to its contribution to tumor invasion and metastasis.

Keywords: tumor microenvironment, macrophage, inflammation, oral cancer.

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) corresponds to 95% of the malignant tumors of the oral cavity and, due to the late diagnosis and the metastatic behavior of the tumor, the 5 years patient survival rate is only 60% [1]. Like other cancers, the OSCC microenvironment is composed not only of tumor cells, but also of stromal cells - immune cells, fibroblasts, endothelial cells - which support tumor growth and is characterized by intense cellular communication through releasing of inflammatory cytokines, such as IL-6, TNFα and IL-1β [2, 3]

Tumor cells in their early stage secrete innumerable cytokines, which recruit mesenchymal cells from adjacent normal tissues, and also from red bone marrow. These cells are reprogrammed to produce cytokines that will act on neoplastic cells, favoring their growth, promotion and invasion of tissues. A tumor microenvironment is created around the neoplastic cells, which is characterized by a chronic inflammation state, in this environment there are the presence of immunosuppressive cells with pro-tumor activity: regulatory T lymphocytes (Treg cells), M2 macrophages, N2 neutrophils and myeloid-derived suppressor cells (MDSC) [4].

Among these microenvironment cells, tumor-associated macrophages (TAMs) are the most influential in tumor progression and their presence is related to a worse prognosis [5]. One of the main characteristics of macrophages is their plasticity, because, from signals from the surrounding microenvironment, they acquire different phenotypes,typically macrophages are divided into two main groups: M1 and M2. [9]. Macrophage M2 was related to proliferation and survival of tumor cells, angiogenesis, invasion of adjacent tissues and metastasis and the presence of macrophages, and with to the lower survival in several tumors such as breast, kidney, bladder and melanoma, glioma, pancreatic and cancer oral SCC [6-8].

In the present study, it was observed that there are differences in the profile of macrophages in patients with oral SCC and that polarized macrophages alters the migration of oral squamous cell carcinoma cell linage.

MATERIALS AND METHODS

Human Biopsies and OSCC cell culture

Head and neck tissue fragments were removed from patients (n=3) during the removal surgical procedure from regions corresponding to the center of the tumor (CT) and the carcinoma edge tissue, named as tumor adjacent epithelia (TAE) were collected. The diagnosis and the tumor inflammatory level were confirmed histopathologically by a pathologist. SCC25 cells (ATCC® CRL-2095™) were cultivated in DMEM/F12 (Thermo Fisher, 12634010) with 15mM HEPES and 0.5mM sodium pyruvate (Thermo Fisher, 11360070) supplemented with FBS 10% and hydrocortisone (400ng/ml, Sigma Aldrich). Cells were maintained in incubator (37°C, 5% CO2). The experimental design was according to Helsinki resolution and was approved by the Ethical Committee of Federal University of Rio Grande do Sul - Brazil and of Hospital de Clínicas de Porto Alegre - Brazil (CAE#140229). All patients provided written informed consent.

Mass spectrometry

The Pierce BCA Protein Assay was used to determine and adjust the protein amounts of lysates from tumor adjacent epithelium and center of the tumor samples. Chloroform/methanol precipitation was performed with 100 µg of each sample, according to Wessel and Flügge (1984). Pellets were suspended in 100 mM TEAB, pH 8.5, and 8 M urea and proteins digested with a protocol adapted from Klammer and MacCoss (2006). Briefly, disulphide bonds were reduced in 5 mM TCEP for 30 min at 37°C and then cysteines were alkylated in 25 mM iodoacetamide (IAM) for 20 min at room temperature in the dark. Urea was diluted to 2 M with 100 mM TEAB, and the complex protein extract was digested with trypsin in a ratio of 1:100 enzyme/protein along with 2 mM CaCl2 by overnight incubation at 37 °C.

Dimethyl labeling of 25 ug of each digested sample was performed as described by Boersema et al. (2009). Peptides were labeled at free-amines (N-terminus and lysine side chain), by combining isotopic forms of formaldehyde and

sodium cyanoborohydride, resulting in mass shifts of +28.0313 Da (Light: CH2O + NaBH3CN) and+32.0564 Da (Intermediate: CD2O + NaBH3CN) per incorporated label. Tumor adjacent epithelium was labeled as "Light" and center of tumor sample as "Intermediate". The two different labeled samples were mixed and pressure-loaded into a biphasic column for Multidimensional Protein Identification Technology (MudPIT) analysis.

MuDPIT columns were prepared by first creating a Kasil frit at one end of a deactivated 250 µm ID/360 µm OD capillary (Agilent Technologies). Kasil frits were prepared by dipping 20 cm capillary in 300 µL Kasil 1624 (PQ Corporation) and 100 µL formamide solution, curing at 100°C for 3 hours and cutting the frit to a length of ~2 mm. A biphasic MuDPIT column was produced in the fritted column by packing in-house 2.5 cm of strong cation exchange (SCX) particles (5 µm Partisphere, Phenomenex) followed by 2.5 cm of reversed phase particles (5 µm ODS-AQ C18, YMC) from particle slurries in methanol. Analytical reversed phase columns were fabricated by pulling a 100 µm ID/360 µm OD (Polymicro Technologies) to a 5 µm ID tip. Reversed phase particles (5 µm ODS-AQ C18, YMC) were packed directly into the pulled column until 18 cm in length. MuDPIT columns and analytical columns were connected using a zero-dead volume union (Upchurch Scientific).

Peptide mixtures were analyzed by LC-MS using quaternary HP 1100 series HPLC pump (Agilent technology) connected to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). Electrospray was performed directly from the tip of the analytical column. Solution A was 5 % acetonitrile and 0.1 % formic acid, solution B was 80 % acetonitrile and 0.1% formic acid, solution C was 500 mM ammonium acetate, 5% acetonitrile and 0.1% formic acid. Flow rate was approximately 300 nL/min. MuDPIT experiments were performed using a 30-minutes transfer step followed by solution C injection and five 60-minute, one 120-minute and one 180-minute separation steps. The LTQ Orbitrap Velos was also operated in a data dependent mode, ESI voltage of 3.5 kV and inlet capillary temperature of 275 °C. Full MS1 scans were collected in the Orbitrap, with mass range of 300 to 1200 m/z at 60.000 resolution and an AGC target of 1x106. The 20 most abundant ions per MS1 scan were selected for CID MS2 in the ion trap with an AGC target of 1x104 and threshold intensity of 500. Maximum fill times were 250 and 100 ms for MS1 and MS2 scans, respectively, and dynamic exclusion was enabled.

Macrophage-conditioned medium

Peripheral blood mononuclear cells (PBMCs) from healthy donors were separated from blood (20 ml) using Histopaque 1077 (Sigma Aldrich 10771) according manufacturer information. Isolated cells were plated on 100 mm plate and maintained with RPMI medium (Thermo Fisher 12633012), 5% of human serum (Sigma Aldrich H4522) and antibiotics. After 24 hours cell were washed with PBS to remove no adherent cells. The monocytes (adherent cells) were cultivated for the next seven days in RMPI medium, supplemented with 10% de FBS and M-CSF (Prepo Tech 300-25) 20ng/ml (added to the medium for differentiation of monocytes in macrophages). Differentiated macrophages were exposure to 20ng/ml of IFNy (Prepo Tech, 300-02) or IL- 4 (Prepo Tech, 200-04) during 48 hours to induce M1 or M2 differentiation respectively. Then, M1 and M2 polarized macrophages were washed and cultivated with FBS free RPMI medium for 24 hours and the conditioned media was collected. Confirmation of macrophage differentiation was performed by flow cytometry.

Flow Cytometry Strategy

In order to analyze the macrophage profile in patient OSCCs, tumor tissues collected from CT and TAE (n=3) were digested in a 2% type I collagenase solution buffer and the single cells obtained were incubated with fluorescent conjugated primary antibodies (Bd Biosciences). Flow cytometry gate strategy was performed to first identify the cell population 7AAD-CD45+CD68+, and next CD11b+ (M1) or CD163+ (M2) cells. Similar assay was used to confirm macrophage polarization from blood cells. Analyses were performed using a FACSAriaIII (BD Biosciences). Average of percentage of inflammatory cells and standard deviation was performed using the GraphPad Prism (GraphPad Software, Inc., CA, USA).

Time-lapse videos

Imaging acquisition and analysis for migration assays were performed as previously described(29). Cells were trypsinized (0.05% Trypsin), centrifuged (1000 rpm, 5 min) and suspended in CCM1 serum-free medium (HyClone, Thermo Fisher Scientific Inc.). Then, OSCC cells were plated in migration promoting conditions according to Ramos et al. (2016): SCC25 were plated on fibronectin (2µg/ml) and Cal27 in poly-l-lysine (1mg/ml) + laminin (2µg/ml). After 1 hour of plating, cells were incubated with rhIL-6 (10 and 100ng/ml), rhTNF-α (25ng/ml) or rhIL-1β (1 and 10 ng/ml) in the presence/absence of Stattic® (Abcam, Cambridge, MA, a STAT3 inhibitor. Alternatively, SCC25 single cells were plated on a 35mm plate coated with fibronectin (2 µg/ml) limited by a silicone ring and were exposure to DMEM F12 (control group) or macrophage-conditioned medium from M1 or M2. In all experimental conditions, cells were transferred to a Nikon TE300 microscope (10x 0.25 NA CFI Achro DL106 Nikon objective), equipped with a temperature controller (37ºC) and a charge coupled device camera (Orca II, Hamamatsu Photonics) and operated with Metamorph software (Molecular Devices). Images were obtained at 10 min intervals for 20 hours under low light conditions. For analysis of speed migration and directionality, the nucleus of each migratory cell was marked on “manual tracking” ImageJ software plugin (http://rsbweb.nih.gov/ij/). Migration speed was calculated by the ratio between the total distance traveled (distance) and the number of images (time) that cell migrated. For directionality, it was obtained the X and Y coordinates of the migratory cell in each image and normalized to start at a virtual X=0 and Y=0 position. A polar plot graph was made to demonstrate the directionality of the cells in each group[10] (29).

Statistical analysis

Statistical analysis was realized using Statistical Package for the Social Sciences 21 (SPSS Inc, Chicago, IL, EUA). Tests performed were Student-t test or One-way analysis of variance (ANOVA) followed by Tukey’s post-test. Statistically significant differences were considered only when $p < 0.05$

RESULTS

Inflammatory-related proteins are different in CT and TAE

Tumor microenvironment contains several proteins that may influence tumor and non-tumor cell behavior. To address the inflammatory protein profile in the CT in relation to TAE region, we evaluated OSSCs (n=3) biopsies with different levels of inflammatory severity (lower (+), intermediate (++) and higher (+++) inflammatory infiltrate) using mass spectrometry (Figure 1A-C). In tongue OSSCs, 134 of a total of more than 3,000 proteins reported were differentially expressed in all tumors, with 159, 388, and 199 of them exclusively present in the samples with lower, intermediate, and higher inflammatory infiltrate respectively. Comparing all three tumors, 11 from 134 proteins were related to inflammatory role. Considering only the 2 tumors with higher inflammatory levels (++ and +++), more 148 proteins were altered in CT in relation to TAE, from which 9 could be involved in inflammatory response. This approach showed similarity to the others 2 tumors analyzed (Supplementary Material 1). Neutrophil defensing protein (HPN-1) was the more pronounced in all tumor fragments in relation to control. Other proteins found were: leukocyte elastase inhibitor (LEI), histidine-rich glycoprotein (HPRG) and alpha-1-antichymotrypsin (ACT). (Fig. 1).

Increased Presence of M2/M1 Cells in most of patient tumor Tissue

Inflammatory cell profile from human tongue OSCCs biopsies (n=4) was verified in order to investigate the proportion of polarized CD163+/CD11b+ macrophages (M2/M1) in TAE and CT. Immune infiltrates were detected in both TAE and CT tissue (Figure 2). Expression of M1 and M2 follows a similar pattern in both regions in the same tumor, being the proportion M2/M1 higher in 3 tumors. This data indicates that the macrophage pattern is similar between CT and TAE regions.

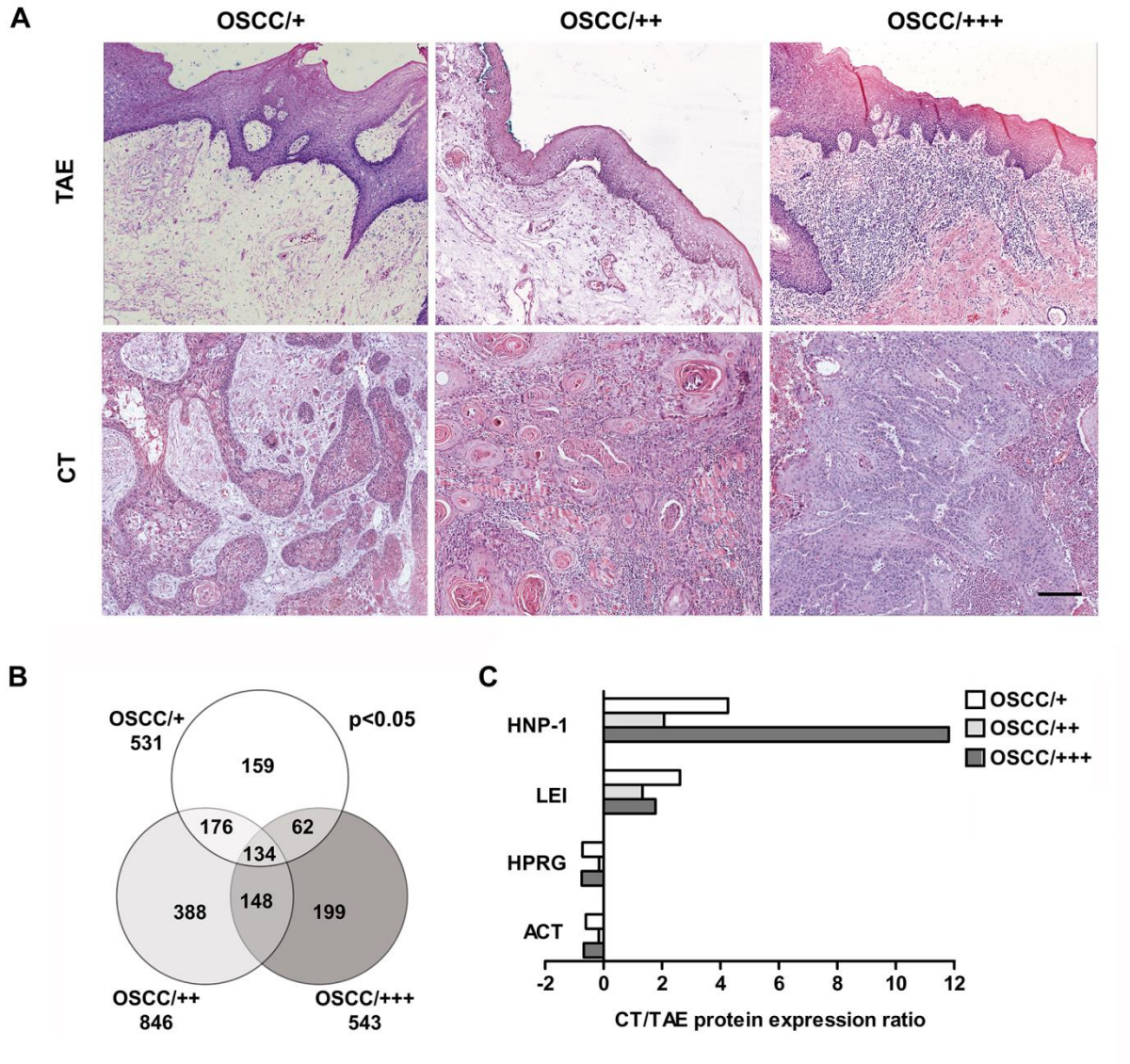

Figure 1. Center of Tumor (CT) and tumor adjacent epithelium (TAE) of oral squamous cell carcinoma (OSSC) show altered expression of inflammatory-related proteins. (A) Hematoxylin and eosin staining represent the OSSCs biopsies (n=3) with different levels of inflammatory severity (OSCC/+, OSCC/++, and OSCC/+++) that were processed to mass spectrometry assay. (B) Veen diagram shows the distribution of proteins that were differentially expressed (p≤0.05) in CT/TAE in OSCC/+, OSCC/++ or OSCC/+++. (C) From 134 proteins differentially expressed in all tumors, the inflammatory proteins HNP-1, LEI, HPRG and ACT were up or downregulated in CT in relation to TAE. Scale bar: 100µm. Abbreviation: HPN-1, neutrophil defensing protein; LEI, leukocyte elastase inhibitor; HPRG, histidine-rich glycoprotein; ACT, alpha-1-antichymotrypsin.

M2 Conditioned Media increases the migration profile and directionality in OSCC cells

The influence of macrophages on tumor cells linage (SCC25) was evaluated by time lapse assays when cell migration and directionality was quantified. For this, conditioned medium of M1 and M2 type macrophages was used on the cells. It was observed that M1-CM increased migration velocity in 5%, while M2- CM increased speed at 18% ($p<0.05$), when compared to the respective control (RPMI) (Fig. 2B). The analysis of cell directionality showed that M1 and M2 CM makes cells migrate further than control cells with a more pronounced effect for M2-CM (Fig. 2C). Taken together this data shows that the conditioned medium of M2 macrophages increases the rate of migration of SCC 25 and also the directionality which may be related to a greater capacity for tumor invasion and formation of metastases.

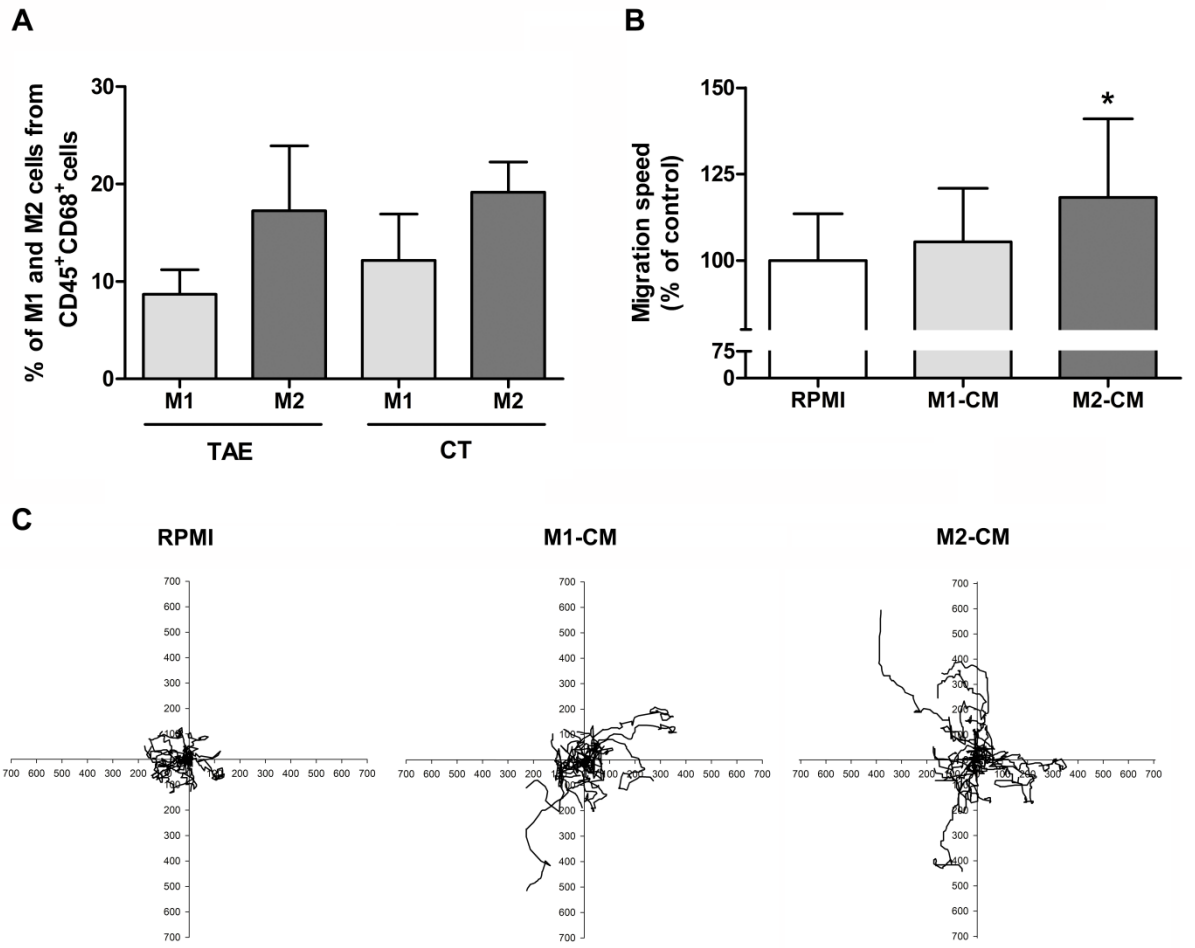

Figure 2. Macrophages role in OSCC (A) Flow cytometry levels of M2 macrophage were higher in relation to M1 macrophages in TAE and CT regions from human OSCC biopsies (n=3) (B) M2 –CM increased 18% the rate of individual migration of SCC25 in relation to control RPMI (n=3, $p<0.05$). (C) The exposure to macrophage conditioned medium increases the directionality of SCC25 cells.

DISCUSSION

The tumor microenvironment plays a key role in tumor growth and progression [11]. Among the cells that are part of the microenvironment we find fibroblasts, endothelial cells, immune cells such as lymphocytes, neutrophils and mainly macrophages [12, 13]. Those cells are able to secrete inflammatory cytokines that are important for different stages of tumor development, such as proliferation, escape from immune system, extracellular matrix degradation and preparation of metastatic niche [29, 30].

In order to investigate the tumor inflammatory protein profile, we identified the proteins differentially expressed in TAE and CT regions of tongue OSCCs. From 134 proteins altered in all tumors, 11 were more directly related to inflammation role, from which 4 presented similar patterns: HPN-1 and LEI were upregulated, whereas HPRG and ACT were downregulated comparing tumor to neighboring adjacent tissue. [14, 15].

In hepatocellular carcinoma cells, the nuclear presence of ACT in hepatic tumor cells was related to control chromatin condensation, reduce cell proliferation, increase cell apoptosis by activating PI3K/AKT/mTOR-mediated apoptosis and its overexpression generate smaller tumor in comparison to mice with absence of ACT [14, 16]. Whereas, in melanomas higher levels of cytoplasmatic ACT were correlated with melanoma progression and high mortality, showing poor survival for patients with metastatic lesions and was also higher in prostate cancer than in benign prostatic hyperplasia [17, 18].

We found that HNP-1 was the most prevalent protein relating CT/TAE region. HNP-1 has been reported upregulated in oral gastric, colorectal tumors, malignant pancreatic ductal epithelia and in serum of patients with bladder cancer with metastatic disease [19-22]. HNP-1 is related on regulating of the production of IL-6 and IL-8 in rheumatoid fibroblast-like synoviocytes and has been considered to be a potent chemotaxin for macrophages [15, 22]. Several studies have shown that

macrophages called TAMs are the predominant inflammatory component of the microenvironment in breast, intestine, and oral squamous cell carcinoma tumors [23].

Under the influence of tumor cells, the component cells of the tumor microenvironment are reprogrammed to produce cytokines and factors that act in favor of the tumor[4]. In addition there is a remodeling of the extracellular matrix, which becomes denser, this process is called desmoplasia, this change leads to a compression of intratumor blood vessels, which affects the flow of chemotherapeutic drugs and cells of the immune system [24].

One of the main characteristics of macrophages is their plasticity, because, from signals from the surrounding microenvironment, they acquire distinct phenotypes called macrophages M1 or macrophages M2 [25, 26]. M1 macrophages are polarized by interferon-gamma IFNγ and lipopolysaccharides, whereas M2-type macrophages are induced by interleukins IL-4, IL-10 e IL 13 [27]. For M1 macrophages, the markers are CD11c, CD80, and HLA-DR, while for M2 detection, it is used CD163, CD11b, CD206, and MRC1 [28-30]. M2 macrophages are involved in tumor growth, invasion, angiogenesis and metastasis formation, by secreting chemokines, growth factors, cytokines, and matrix metalloproteases, such as TGF-β1, IL-10, IL-6, and IL-8. [31, 32].

Besides macrophage M1 and M2 immunoexpression had not altered significantly between TAE and CT regions in our results, it was noticed a tendency to higher levels of M2 in relation to M1 in both sites.. Higher HRPG expression has been described to induce M1 macrophage polarization and by this mechanism acts normalizing vessel formation in fibrosarcoma cells [33, 34]. In opposite, inhibition of HRGB is related to induce M2 macrophage profile, which is implicated to impairment of drug delivery [33, 34].

In gastric, breast, pancreatic tumors, the presence of macrophages is associated with worse prognosis [35, 36]. In oral squamous cell carcinoma the presence of M2 (CD 163+) macrophages is well related to worse prognosis and overall survival, disease-free survival and the presence of metastases [37-40]. These data indicate that the TAMs are important for the prognosis in patients with OSCC.

In our study we evaluated the effect of conditioned medium from polarized macrophages to the profile M1 and M2 on the migration speed and directionality of the OSCC lineage (SCC25). Our data indicate that the conditioned medium of M2 increases the rate of migration, and the directionality of the cells in relation to the control. The mechanisms by which M2 macrophages influence the migration processes are not well understood. Yao et al. showed that hepatic carcinoma cells (HCC) when submitted to M2-CM presented higher levels of mesenchymal epithelium (EMT), vimentin and N-cadherin transition markers and decreased E-cadherin, which favors migration. And even though M2-CM over regulation of TLR4 in HCC activates the signaling pathwayTLR4/STAT3[41].

Our data showed that in the sample of tumors evaluated, there were the expression of inflammation-related proteins some of these already described in other types of tumors as associated with M2-type macrophages polarization and worse prognosis in patients. And the greater presence of M2 in the tumors, may be related to the profile of proteins, that act in the polarization of M2. The increase in the migration velocity and directionality of SCC25 cells when in the prsence of M2-CM reveals that factors released in the medium by macrophages act on the cells, changing the migration profile of these, the way in which this happens is still unknown. Taken together, our results indicates macrophages are an important component in OSCC and the remodeling of the M2 profile to M1 may be a therapeutic alternative.

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6 CONSIDERAÇÕES FINAIS

O macrófago é um componente importante do microambiente tumoral, e observamos que vários estudos associaram macrófagos principalmente do tipo M2 com pior prognóstico de pacientes com CEC oral. Entretanto, estes estudos não levam em conta zonas do tumor e relação M1/M2, além de utilizarem um pan marcador CD68 ao invés de marcadores específicos para M2.

A presença de proteínas inflamatórias diferentes em cada zona tumoral pode ser relacionada a intensidade de inflamação apresentada em cada zona, o que ficou evidenciada pela diferença de macrófagos em tumores de pacientes. Além disso algumas das proteínas analisadas estão envolvidas com a polarização de macrófagos e já foram relatadas em outros tumores como associadas a pior prognóstico. Por fim estudos *in vitro* mostraram que meio condicionado macrófagos do tipo M2 alteram a migração celular por aumentar a velocidade de migração e a direcionalidade em SCC25, o que pode fazer com que células tumorais consigam invadir e disseminar com mais eficiência.

Com este trabalho, propomos que a avaliação do tipo de macrófagos presente nos tumores pode auxiliar no prognóstico do paciente e que estas células de defesa podem ser um alvo para o desenvolvimento de terapias, que busquem uma remodelação de perfil de M2 para M1.

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APÊNDICE

REVIEW ARTICLE

Macrophages and prognosis of oral squamous cell carcinoma: A systematic review

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Oral Squamous Cell Carcinoma (OSCC) presents a tumor microenvironment rich in inflammatory cells. Depending on the stimulus, macrophages can polarize in M1 or M2 profile, where M1 acts as proinflammatory and antitumor, and M2 is anti-inflammatory and shows protumor activity. Several studies have shown that macrophages are important to the prognosis of patients with different types of cancer. Our aim was to conduct a systematic review to evaluate the role of macrophages in the prognosis of OSCC patients. A search in the Pubmed, Scopus, and ISI Web of Knowledge database was performed, and it was included only studies that evaluated the importance of macrophages in the prognosis of OSCC patients. From initial 286 articles, 14 fully attended the inclusion criteria. In the majority of the articles, it was evaluated only CD68, a panmacrophage marker, or CD163, a M2 marker. Only one article evaluated the M1 marker, CD11c. Besides, 5 articles analyzed the presence of macrophages in different areas of the tumor. Higher concentrations of CD68 and CD163 were associated with worse survival. In conclusion, macrophages are important to OSCC patients' prognosis; however, it is necessary to address in which tumor region the presence of polarized macrophage is more important to the outcome.

KEY WORDS
disease-free survival, head and neck cancer, M1, M2, outcome, overall survival

1 | INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is the most common cancer in the oral cavity and represents more than 90% of all head and neck cancers.[1] Despite advances in the understanding of the biology of OSCC, the patient survival rate after 5 years is around 50%, mainly due to the presence of regional lymph node metastasis.[2] Like other cancer types, OSCC has a complex tumor microenvironment (TME) with the presence of several stromal cells[3,4] that might be associated with tumor aggressiveness and the resistance and failure to antineoplastic treatment.[5,6]

Tumor microenvironment is an emerging field in cancer therapy, and it is defined as a complex environment that supports cancer progression, proliferation of neoplastic cells, and invasion of adjacent tissues. It is composed by cancer cells and stromal cells, such as fibroblasts, endothelial cells, pericytes, and immune cells.[7,8] These cells produce a multitude of molecules with the potential to influence the tumorigenesis process. For instance, cytokines from tumor microenvironment recruit cells from normal adjacent tissues, which are reprogramed to produce various growth factors and other cytokines, and then contribute to tumor cell growth, tissue invasion, metastasis, and, consequently, poor prognosis.[9] Since these cells are linked to the majority of the hallmarks of cancer,[10] researchers have focused on new therapeutic modalities targeted to modulate the behavior of the cellular components of TME. Furthermore, stromal cells do not have mutations like tumor cells, and the changes in cell behavior are modulated by several cytokines expressed in the TME.[7,10]

Among immune cells, tumor-associated macrophages (TAMs) are the most abundant and important stromal cells in the TME. TAMs are considered important players in tumor progression and are related to proliferation and survival of tumor cells, angiogenesis,

invasion of surrounding tissues, and metastasis.[11,12] Furthermore, TAMs' density is related to worse prognosis in several types of cancer.[13] According to stimuli in TME, TAMs can be polarized in two main groups: M1, which is considered proinflammatory and antitumor, and M2, which is considered immunosuppressive and protumor.[11,12,14]

M1 macrophage polarization occurs mainly in the presence of interferon gamma (IFN-c) or via exposure to microorganisms or their products such as lipopolysaccharide (LPS).[11,14,15] Then, M1 secretes several proinflammatory cytokines such as IL-1, IL-6, INF-c, which are associated with activation of Th1 response and Th1 lymphocytes attraction.[16] Moreover, M1 functions as an antigen-presenting cell with the potential to play an antitumor role.[11,16]

M2 macrophage polarization is stimulated in the presence of IL-4, IL-10, IL-13, or TGFb.[12,14,15,17] Some authors suggest that this type of polarization may be divided into four groups: M2a, M2b, M2c, and M2d.[18] This classification is related to the immunosuppressive response and the attraction of regulatory T cells (Treg) and Th2 lymphocytes. M2 releases growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF-b), and fibroblast growth factor (FGF) that can promote angiogenesis in various tumors[16,19] and release matrix metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-9 and MMP-12) and plasminogen activator urokinase-type (uPA).[20]

Herein, we conducted a systematic review to evaluate the influence of macrophages on the prognosis of patients with OSCC. We observed that higher concentrations of TAMs, mainly CD68+ and CD163+, are related to poor prognosis.

2 | METHODS

2.1 | Search strategy

The search was performed on PUBMED, SCOPUS, and ISI Web of Knowledge database with the following terms: "head and neck neoplasms" OR "mouth neoplasms" OR "oral squamous cell carcinoma" OR "head and neck squamous cell carcinoma" OR "head and neck cancer" OR "oral cancer" AND "survival" OR "mortality" OR "prognosis" OR "disease-free survival" OR "survival analysis" AND "macrophage" (the last access was realized on August 2017). Furthermore, searches were done in the references of the selected papers. Two independent authors (AMA e LFD) reviewed the articles, and the studies that generated disagreement between reviewers were reevaluated to be reached on a consensus. PROSPERO registration: 72257.

2.2 | Inclusion and exclusion criteria

In this paper, it was included exclusively studies that evaluated the importance of macrophages to the prognosis of the patients with OSCC. Patients in treatment, in vitro and animal models studies, and review articles were excluded.

2.3 | Data extraction

Using a standardized instrument, two authors independently extracted the following data: first author, year, sample size, markers, method, median follow-up, evaluated tumor zone, and main results.

3 | RESULTS

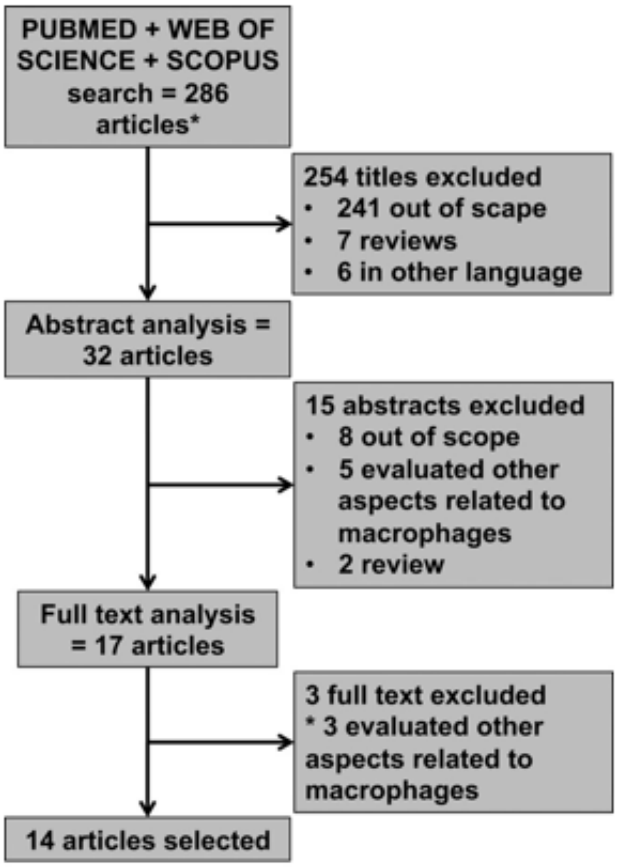
The search in the PUBMED, Web of Science, and Scopus database resulted in 286 articles. Of these, 254 were removed in the title analysis phase: 241 were out of research scope, six were written in a language different than English, and another seven were reviews. Another 15 studies were declassified by the abstract evaluation: Eight were out of research scope, five analyzed other aspects of macrophages related to OSCC, and two were reviews. After these two steps, it remained 17 works in which the full text was analyzed. At this moment, we discarded three papers due to the analysis of other aspects of macrophages related to OSCC. At the end, 14 studies were included in this review (Figure 1). Table 1 summarizes the demographic and pathological characteristics of the samples from the 14 selected studies, while Table 2 shows the data related to follow-up time, markers, methodology, tumor areas evaluated, and main results.

The publication date of the articles ranged from 2004 to 2017. The median sample size was 92 patients (range from 17 to 240), and mean age ranged from 51 to 68.9 years. In all studies, tongue was the most common site followed by buccal mucosa, gingiva, and floor of the mouth. And, in relation to smoking and drinking, few studies had this information (Table 1).

Regarding tumor characteristics, in half of the studies, most of the samples were well differentiated, and the other half were classified as moderate to poor differentiated. Moreover, in most of the studies, tumors were classified as T1/2, N0, and clinical stage 3/4 (Table 1).

The median for patient follow-up was 40 months, varying from 18 to 61.5 months. All studies applied immunohistochemistry staining for macrophage markers, and the most used markers in the selected studies were CD68 (panmacrophage antigen, n = 11) and CD163 (M2 macrophage antigen, n = 8). Other three different cell markers were also used in the studies: MRC1 (M2 macrophage antigen, n = 1), CD11b (M2 macrophage antigen, n = 1), and CD11c (M1 macrophage antigen, n = 1) (Table 2).

From the 14 selected articles, only six reported that evaluated different areas of the tumor—the main areas assessed were tumor stroma and tumor epithelium. Two articles related that the higher density of CD68+ or CD163+ or MRC1 macrophages in the epithelial fraction/tumor nest was related to poor outcome. Other two articles related that higher density of CD68+ or CD163+ macrophages in the tumor stroma was related to poor prognosis, and one study showed that the higher density of CD163 in the invasive front of the tumor was associated with worse prognosis. Even in relation to prognosis, all articles evaluated overall survival (OS) followed by disease-free survival (DFS), and one article also evaluated local failure-



FIGU RE 1 Flowchart showing the different steps for the selection of articles included in the systematic review

free survival (LFFS), distant metastasis-free survival (DMFS), and progression-free survival (PFS). Together, the results of these studies revealed that the increased concentration of TAMs CD68+/CD163+/MRC1+, depending on the tumor region, was related to worse prognosis. Two papers showed no association between TAMs CD68+ and tumor outcome (Table 2).

4 | DISCUSSION

Tumor microenvironment is a complex system where tumor cells reprogram stromal cells for their own benefit. From the ten hallmarks of cancer described by Hanahan and Weinberg[10], these reprogramed cells contribute to at least seven hallmarks: sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, deregulating cellular energetics, resisting cell death, inducing angiogenesis, and activating invasion and metastasis.[7] For instance, during oral squamous cell carcinoma development, the reprograming of stromal cells already starts in potentially malignant disorders, where altered non-neoplastic cells induce angiogenesis and modifications in immune cells in the adjacent connective tissue.[3,35] When these altered non-neoplastic cells become malignant cells and invade adjacent connective tissue shortly after basal membrane rupture, there is a switch to an immunosuppressive TME, which allows tumor development.[3,5] This review included only studies that evaluated the role of macrophages, the main player on TME, in the prognosis of OSCC. The results of the selected studies showed that a high concentration of macrophages was related to a worse prognosis. Thus, the development of new therapeutic modalities target to TME-related cells might improve the success rate for the treatment of the cancer.[36]

Tumor-associated macrophages may be polarized into two different phenotypes: classic M1, which exerts antitumor role; and alternative M2—which exerts protumor role.[11] For M1 macrophages, the most used markers are CD11c, CD80, and HLA-DR, while for M2 detection, it is used CD163, CD11b, CD206, and MRC1. Moreover, it can be used CD68, which is a panmacrophage marker.[11,13,14,24,31] However, some studies related that CD68 is not specific for macrophages, since fibroblasts and tumor cells might be detected by this marker.[22,37] From the articles selected for this study, only one used an appropriated marker for M1,[21] and some articles used only the panmacrophage marker CD68. Due to the variability of the data and the methodology of the studies, we did not perform a meta-analysis. The failure to use specific markers may lead to unspecific findings and does not identify the true role of macrophages in tumor progression and prognosis.

A systematic review conducted by Zhang et al[13] has shown that TAMs are important for the prognosis of patients in different types of solid tumors. In human gastric cancer, it was observed that the higher density of CD68+ macrophage was associated with worse overall survival.[38] In addition, CD68 expression was related to a higher expression of vimentin and low expression of E-cadherin, important markers of epithelial to mesenchyme transition (EMT), suggesting that macrophages can induce EMT.[38] Other study conducted in patients with pancreatic cancer, the presence of M2 macrophages was associated with worse prognosis.[39] The articles selected in this review, except for one,[34] showed association among macrophages, CD68+, CD163+, or MRC1+, and worse prognosis. These findings indicate that the TAMs are important for the prognosis of patients with OSCC.

Although it may be concluded that macrophages - CD68+, CD163+, or MRC1+ - are important for the outcome of OSCC patients, it has not been possible to conclude in which region of the tumor, whether epithelial part, stroma, or invasive front of the tumor, TAMs have more influence on the prognosis. The organization of TME might vary according to the tumor region due to anatomical and biological challenges to the recruitment of stromal cells.[6,17,40] In lung cancer, a meta-analysis showed that the infiltration of M2 into the tumor stroma was associated with poor prognosis, while the presence of M1 TAMs into the tumor islands was related to good prognosis.[41] In breast cancer samples, the infiltration of CD68+ macrophages in intratumor compartment[42] or the presence of CD163+ macrophages in the tumor stroma[43] was related with poor prognosis. In the present review, the high density of macrophages CD68+, CD163 or MRC1+ in the epithelial part[22,23] or the high density of macrophages CD68+ or CD163+ in tumor

TABL E 1 Demographic and pathological characteristics of the selected studies

Authors/Year	Sample	Age mean (range)	Gender	Tumor location	Smoking	Drinking	Differentiation	T stage	N stage	M stage	Clinical stage
Fang et al (2017)[21]	78	60 (24-82)	57 (M)	NR	47 (yes)	42 (yes)	57 (well)	58 (T1 and T2)	48 (N0)	NR	36 (I and II)
			21 (F)		31 (no)	36 (no)	21 (moderate/poor)	20 (T3 and T4)	30 (N1-N3)		42 (III and IV)
Hu et al (2016)[22]	127	61 (34-88)	74 (M)	52 (tongue)	NR	NR	76 (well)	89 (T1 and T2)	72 (N0)	125 (M0)	54 (I and II)
			53 (F)	21 (gingiva) 31 (buccal mucosa) 4 (lip) 2 (floor of the mouth) 12 (others)			51 (moderate/poor)	38 (T3 and T4)	55 (N1-N3)	2 (M+)	73 (III and IV)
Weber et al (2016)[23]	17	63	12 (M)	2 (tongue)	NR	NR	17 (well)	17 (T1 and T2)	17 (N0)	17 (M0)	17 (I and II)
			5 (F)	2 (buccal mucosa) 2 (mandibular mucosa) 7 (floor of the mouth) 3 (soft palate)			0 (moderate/poor)	0 (T3 and T4)	0 (N1-N3)	0 (M+)	0 (III and IV)
Ni et al (2015)[24]	91	55 (20-78)	38 (M)	NR	20 (yes)	NR	35 (well)	66 (T1 and T2)	31 (N0)	NR	41 (I and II)
			53 (F)		71 (no)		56 (moderate/poor)	25 (T3 and T4)	60 (N1-N3)		50 (III and IV)
Matsuoka et al (2014)[25]	60	68.9 (33-87)	36 (M)	19 (tongue)	NR	NR	46 (well)	24 (T1 and T2)	34 (N0)	NR	6 (I and II)
			24 (F)	10 (mandible) 12 (maxilla) 9 (floor of the mouth) 10 (buccal mucosa)			14 (moderate/poor)	36 (T3 and T4)	26 (N1-N3)		54 (III and IV)
Fujita et al (2014)[26]	50	68.6 (48-93)	32 (M)	17 (tongue)	NR	NR	40 (well)	35 (T1 and T2)	32 (N0)	50 (M0)	27 (I and II)
			18 (F)	7 (upper gingiva) 16 (lower gingiva) 6 (buccal mucosa) 3 (floor of the mouth) 1 (mandibular bone)			10 (moderate/poor)	15 (T3 and T4)	18 (N1-N3)	0 (M+)	23 (III and IV)
Balermpas et al (2014)[27]	106	60.6	70 (M) 36 (F)	NR	59 (yes) 47 (no)	NR	5 (well) 101 (moderate/poor)	10 (T1 and T2) 96 (T3 and T4)	15 (N0) 91 (N1-N3)	NR	NR
He et al (2014)[28]	43	NR	NR	24 (tongue) 7 (gingiva) 8 (buccal mucosa) 3 (floor of the mouth) 1 (others)	NR	NR	12 (well) 31 (moderate/poor)	32 (T1 and T2) 11 (T3 and T4)	23 (N0) 20 (N1-N3)	43 (M0) 0 (M+)	NR
Wang et al (2014)[29]	240	53 (21-78)	152 (M) 88 (F)	83 (tongue) 21 (lower gingiva) 44 (buccal mucosa) 49 (floor of the mouth) 43 (others)	NR	NR	96 (well) 144 (moderate/poor)	NR	86 (N0) 154 (N1-N3)	NR	120 (I and II) 120 (III and IV)
Costa et al (2013)[30]	45	61.7 (42-90)	32 (M) 13 (F)	18 (tongue) 13 (floor of the mouth) 14 (others)	40 (yes) 5 (no)	30 (yes) 15 (no)	18 (well) 27 (moderate/poor)	11 (T1 and T2) 34 (T3 and T4)	20 (N0) 25 (N1-N3)	NR	NR
Fuji et al (2012)[31]	108	66.4 (23-93)	67 (M)	41 (tongue)	NR	NR	82 (well/moderate)	61 (T1 and T2)	71 (N0)	NR	46 (I and II)
			41 (F)	15 (upper gingiva)			26 (poor)	47 (T3 and T4)	37 (N1-N3)		62 (III and IV)
				33 (lower gingiva)							
				6 (buccal mucosa)							
				10 (floor of the mouth)							
				3 (palate)							
Lu et al (2010)[32]	92	51 (21-76)	75 (M)	34 (tongue)	72 (yes)	63 (yes)	82 (well)	49 (T1 and T2)	44 (N0)	NR	33 (I and II)
			17 (F)	45 (buccal mucosa)	20 (no)	29 (no)	10 (moderate/poor)	43 (T3 and T4)	48 (N1-N3)		59 (III and IV)
				13 (others)							
Liu et al (2008)	112	NR	93 (M)	55 (tongue)	NR	NR	41 (well)	66 (T1 and T2)	64 (N0)	NR	50 (I and II)
			19 (F)	10 (gingiva)			71 (moderate/poor)	46 (T3 and T4)	48 (N1-N3)		62 (III and IV)
				30 (buccal mucosa)							
				4 (floor of the mouth)							
				7 (palate)							
				6 (lips)							
Marcus et al (2004)[34]	102	56 (33-74)	76 (M)	NR	NR	NR	14 (well)	10 (T1 and T2)	33 (N0)	NR	22 (I and II)
			26 (F)				88 (moderate/poor)	92 (T3 and T4)	69 (N1-N3)		80 (III and IV)

M, male; F, female; NR, not reported.

TABL E 2 Characteristics of the selected studies in the systematic review

Authors/Year	Median follow-up	Markers	Methods	Evaluated tumor zone	*P*-value	Confidence interval	Main results
Fang et al (2017)[21]	48 mo	CD68	IHC	Tumor epithelium, tumor stroma, and advancing tumor margin	.293	0.411-1.308	Association was not found
Hu et al (2016)[22]	39 mo	CD68, CD163	IHC	Tumor epithelium and tumor stroma	a) .01 b) .02	a) 2.236-3.914 b) 1.991-3.669	Higher expression of CD68(a) and CD163(b) in tumor epithelium were associated with worse OS
Weber et al (2016)[23]	Minimum of 36 mo	CD68, CD11c, CD163 e MRC1	IHC	Tumor epithelium, tumor stroma, and tumor epithelium + tumor stroma	.001	-	Higher expression of MRC1 in tumor epithelium + tumor stroma was associated with poor outcome[a]
Ni et al (2015)[24]	NR	CD68	IHC	Normal tissue, tumor epithelium and tumor stroma	.033	1.512-10.379	Higher expression of CD68 in tumor stroma was associated with worse OS
Matsuoka et al (2014)[25]	NR	CD163	IHC	NR	a) .003 b) .007	a) - b) -	Higher expression of CD163 was associated with worse OS(a) and worse DFS(b)
Fujita et al (2014)[26]	NR	CD163	IHC	Tumor epithelium and advancing tumor margin	a) .006 b) .002	a) - b) -	Higher expression of CD163 in the advancing tumor margin was associated with worse OS(a) and worse DFS(b)
Balermpas et al (2014)[27]	40 mo	CD68, CD163, CD11b	IHC	Tumor epithelium, tumor stroma, and advancing tumor margin	a) .017-.007 b) .010-.006 c) .006-.007 d) .011-.010	a) - b) - c) - d) -	Higher expression of CD68 and CD163 in tumor stroma were associated with worse OS(a), worse LFFS(b), worse DMFS(c) and worse PFS(d)
He et al (2014)[28]	24 mo	CD68, CD163	IHC	NR	.0319	-	Higher expression of CD163 was associated with worse OS
Wang et al (2014)[29]	61.5 mo	CD163	IHC	NR	≤.001	2.578-7.547	Higher expression of CD163 was associated with worse OS
Costa et al (2013)[30]	Minimum of 18 mo	CD68	IHC	NR	.08	-	Association was not found
Fuji et al (2012)[31]	NR	CD68, CD163	IHC	NR	.007	-	Higher expression of CD163 was associated with worse OS
Lu et al (2010)[32]	NR	CD68	IHC	NR	a) ≤.001 b) .001	-	Higher expression of CD68 was associated with worse OS(a) and worse DFS(b)
Liu et al (2008)	NR	CD68	IHC	NR	.001	-	Higher expression of CD68 was associated with worse OS
Marcus et al (2004)[34]	41.1 mo	CD68	IHC	NR	-	-	Association was not found

OS, overall survival; DFS, disease-free survival; LFFS, local failure-free survival; DMFS, distant metastasis-free survival; PFS, progression-free survival; IHC, immunohistochemistry; NR, not reported; CD68, panmacrophage marker; CD163, CD11b, and MRC1, M2 macrophage; CD11c, M1 macrophage.
P-value and confidence interval are related to univariate analysis.
[a]Poor outcome = occurrence of local recurrence, a second oral tumor, lymph node metastasis or distant metastasis during the follow-up period.

stroma[24,27] was related with worse prognosis. In addition, one study[26] showed that high density of macrophages CD163+ in invasive front of the OSCC was related with worse prognosis. These data show the relevance of assessing not only the polarization of the macrophages, but also the zone of the tumor where it is present.

Since macrophages show dynamic plasticity within TME that could result in pro- or antitumor activity, we suggest studies with the use of specific markers for the detection of M1 (CD11c, CD80 and HLA-DR) or M2 (CD163, CD11b, CD206 and MRC1) TAMs at different regions of patient biopsies. With this approach, the association of M1 or M2 with prognosis will be more precise, corroborating for the development of therapeutic strategies that targets the polarization reprogramming of these cells. Also, analysis of TAMs at the epithelia adjacent to tumor will contribute to a better understanding of the role of TAM within TME and its potential role in tumor recurrence.

One of the main causes of decreased survival in cancer patients is related to the development of metastasis. Besides the modulation of the tumor cell migration performance,[44] components of TME might contribute to the preparation of the pre-metastatic niche.[8,45] It was demonstrated in cancer that TAMs contribute to the formation of the pre-metastatic niche by releasing molecules that promote angiogenesis as well as the chemoattraction of tumor cells and naive macrophages to the local of metastasis.[45] In an in vitro study with triple negative breast cancer, it was demonstrated that the metastatic suppressors reduce the macrophage infiltration in the tumor and consequently decreased metastasis.[46] For OSCC, patients with metastasis in lymph nodes exhibited high infiltration of M2 macrophages both in the tumor and in the lymph node.[47] Based on these results, it is possible that TAMs not only participate in the invasive process of tumor cells, but also modify the pre-metastatic niche to receive the metastatic cells.

The present review showed that macrophages are important components of OSCC-TME, and M2 TAMs are related with worse overall survival and disease-free survival. Nevertheless, it was not possible to identify in which tumor zone the presence of these cells are more relevant to prognosis, being necessary other studies that evaluate the characteristics of TAMs, whether M1 or M2, and in which region of the TME is more relevant to the outcome.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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ANEXO A

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO INDIVÍDUOS SAUDÁVEIS - COLETA DE SANGUE PERIFÉRICO

Você é um indivíduo saudável e gostaríamos de convidá-lo a ser um doador voluntário de sangue periférico para um estudo o qual envolve o isolamento de um tipo específico de célula que está presente no sangue, o qual se chama monócito. Esta pesquisa, que tem o título de “Análise do papel da inflamação sobre o processo invasivo do carcinoma espinocelular oral” tem o objetivo analisar o papel de substâncias envolvidas na evolução do câncer de boca. Para isso, serão realizados testes no laboratório com a ajuda de microscópicos utilizando as suas células em lesões de pacientes com câncer de boca. Se você concordar, sua amostra será utilizada para este estudo e após a utilização, o material restante, se houver, será descartado. Caso decida autorizar a utilização do seu tecido, você poderá mudar de ideia a qualquer momento. Para isso você deve entrar em contato com o pesquisador e pedir a retirada do seu tecido da pesquisa. Os riscos que podem existir são os devido à uma extração de sangue convencional. Nesse estudo não está previsto nenhum tipo de pagamento pela participação no estudo e o Sr. (a) não terá nenhum custo com respeito aos procedimentos envolvidos. O pesquisador responsável compromete-se a manter em segredo as informações que possam identificá-lo, como nome, endereço e número de telefone. No futuro, pesquisadores poderão precisar de informações sobre sua saúde. Estes dados serão fornecidos pelo pesquisador responsável, porém sem conter seu nome, endereço, número de telefone ou qualquer outra informação que permita a sua identificação. Qualquer dúvida referente a este termo de consentimento pode ser resolvida diretamente com o pesquisador responsável Dr. Marcelo Lazzaron Lamers, no Serviço de Patologia Bucal, sala 503 na Faculdade de Odontologia da UFRGS, telefone (51- 3308 5023) ou ao Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre (51-3359-7640) das 8h às 17h de segunda a sexta. Esse documento será elaborado em duas vias, onde uma via ficará com os pesquisadores e a outra via ficara com o Sr. (a) para que possa ter nosso contato caso seja necessário.

Nome do participante:_____

Assinatura:_____
Data_____/_____/____

Nome do pesquisador:_____
Assinatura:_____
Data_____/_____/_____

ANEXO B

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO CIENTÍFICA

A Comissão Científica do Hospital de Clínicas de Porto Alegre analisou o projeto:

Projeto: 140229
Data da Versão do Projeto:

Pesquisadores:
MANOEL SANTANA FILHO
MARCELO LAZZARON LAMERS
PANTELIS VARVAKI RADOS
ALESSANDRA SELINGER MAGNUSSON
GRASIELI DE OLIVEIRA RAMOS
ALESSANDRO MENNA ALVES
LISIANE BERNARDI

Título: Análise do papel da inflamação sobre o processo invasivo do carcinoma espinocelular oral

Este projeto foi APROVADO em seus aspectos éticos, metodológicos, logísticos e financeiros para ser realizado no Hospital de Clínicas de Porto Alegre.
Esta aprovação está baseada nos pareceres dos respectivos Comitês de Ética e do Serviço de Gestão em Pesquisa.

- Os pesquisadores vinculados ao projeto não participaram de qualquer etapa do processo de avalição de seus projetos.

- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao Grupo de Pesquisa e Pós-Graduação (GPPG)

Porto Alegre, 22 de maio de 2014.

Prof. Eduardo Pandolfi Passos
Coordenador GPPG/HCPA