UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE ODONTOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

NÍVEL DOUTORADO

ÁREA DE CONCENTRAÇÃO PATOLOGIA BUCAL

A RELAÇÃO DAS VESÍCULAS EXTRACELULARES COM O CARCINOMA ESPINOCELULAR DE CABEÇA E PESCOÇO

KELLY BIENK DIAS

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por

KELLY BIENK DIAS

Orientador: Prof. Dr. Pantelis Varvaki Rados

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Tese apresentada ao programa de Pós-Graduação em Odontologia, nível Doutorado, da Universidade Federal do Rio Grande do Sul, como pré-requisito final para a obtenção do título de Doutor em Odontologia – Área de concentração em Patologia Bucal.

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“Se cheguei até aqui foi porque me apoiei no ombro de gigantes.” - Isaac Newton

RESUMO

DIAS, Kelly Bienk. **A relação das vesículas extracelulares com carcinoma espinocelular de cabeça e pescoço.** Tese (Pós Graduação em Odontologia com ênfase em Patologia Bucal) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2020.

O carcinoma espinocelular de cabeça e pescoço (CECCP) é uma patologia composta por células agressivas e altamente tumorigênicas responsáveis por um curso clínico de alta recidiva e baixa sobrevida. O diagnóstico precoce é a melhor chance de redução de morbidade e mortalidade desta patologia uma vez que a contenção da disseminação da doença tem sido um desafio terapêutico importante, onde pacientes com recorrência e/ou metástase sobrevivem por curtos períodos de tempo. Dentro deste contexto**,** o uso de marcadores prognósticos e preditivos pode contribuir no monitoramento dos pacientes acometidos pela doença. As vesículas extracelulares (VEs) são nanoestruturas secretadas por diferentes tipos de célula que oferecem, através do seu isolamento, a possibilidade de detecção de conteúdos que representem a origem celular de doenças como o câncer. Estão relacionadas à progressão tumoral, resistência à quimioterapia e evasão imunológica através da transferência de moléculas, participando das etapas necessárias para a metástase com possibilidade de utilização como biomarcadores para o CECCP. Inicialmente, objetivamos analisar a sobrevida por 10 anos de uma coorte de 50 pacientes com CECCP, correlacionando os parâmetros clínicos e histopatológicos do diagnóstico inicial com a evolução do estado de saúde dos pacientes. Observamos que 64% dos pacientes apresentou tumores menores de 4 cm sem metástase regional e não houve nenhum paciente com metástase à distância no momento do diagnóstico, no entanto, a maior causa de morte dos pacientes foram eventos relacionados à metástase. Diante dessas evidências, consideramos que o estudo de fatores capazes de refletir o perfil molecular do CECCP bem como a sua mudança comportamental ao longo do tempo se torna essencial para a melhora da sobrevida dos pacientes. Procuramos estabelecer a relação das VEs com o CECCP através de revisão sistemática de literatura. Nesta pesquisa, observamos que ainda existem limitações técnicas no estudo dessas estruturas, no entanto, a sua influência nas diferentes vias envolvidas na carcinogênese as torna um campo de interesse sobre diagnóstico, alvos terapêuticos e marcadores prognósticos do

CECCP. Por fim, foi realizado um estudo *in vitro* analisando o efeito de VEs de carcinoma espinocelular bucal (CECB) sobre a migração celular, onde as VEs de CECB aumentaram a velocidade e direcionalidade de migração de queratinócitos e fibroblastos, sugerindo uma possível transferência de conteúdos funcionais que influenciem comportamentos pró-metastáticos. Através dos estudos realizados, podemos concluir que sinalizações bioquímicas podem influir no processo de disseminação e metástase tumoral do CECCP e as VEs presentes nos fluidos corporais poderiam ser biomarcadores específicos desse processo.

Palavras chave: câncer de cabeça e pescoço, biomarcadores tumorais

ABSTRACT

DIAS, Kelly Bienk. **The relationship of extracellular vesicles with squamous cell carcinoma of the head and neck.** Tese (Pós Graduação em Odontologia com ênfase em Patologia Bucal) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2020.

Head and neck squamous cell carcinoma (HNSCC), pathology composed by aggressive and highly tumorigenic cells, is responsible for a clinical course of high recurrence and low survival. Early diagnosis is the best way of reducing the morbidity and mortality of this pathology, since containing the spread of the disease has been an important therapeutic challenge, where patients with recurrence and / or metastasis survive have short periods of time. In this context, prognostic and predictive markers can contribute to patients monitoring affected by the disease. Extracellular vesicles (EVs) are nanostructures secreted by different cell types that offer, through their isolation, the possibility of detecting contents that represent the cellular origin of diseases such as cancer. They are related to tumor progression, chemotherapy resistent and immune evasion through the transfer of molecules, participating in the necessary steps for metastasis with the possibility of being used as biomarkers for HNSCC. Initially, we aimed to analyze the 10-year survival of a cohort of 50 patients with HNSCC, correlating the clinical and histopathological parameters of the initial diagnosis with the patients' evolution health status. We observed that 64% of the patients had tumors smaller than 4 cm without regional metastasis and there were no patients with distant metastasis at the time of diagnosis, however, the major cause of death of the patients were events related to metastasis. In view of this evidence, we consider that the study of factors capable of reflecting the molecular profile of the HNSCC as well as its behavioral change over time becomes essential for improving patient survival. We aimed to establish the relationship between EVs and HNSCC through a systematic literature review. In this research, we observed that there are still technical limitations in the study of these structures, however, their influence on the different pathways involved in carcinogenesis makes them a field of interest in HNSCC diagnosis, therapeutic targets and prognostic markers. Finally, an in vitro study was carried out analyzing the effect of EVs of oral squamous cell carcinoma (OSCC) on cell migration, where EVS of OSCC increased the speed and directionality of migration of keratinocytes and fibroblasts, suggesting a possible transfer of functional contents that influence pro-metastatic behaviors. Through the studies carried out, we can conclude that biochemical signals could influence the

dissemination and tumor metastasis process of the HNSCC and the EVs present in the body fluids could be vehicles of specific biomarkers of this process.

Keywords: head and neck cancer, tumor biomarkers.

SUMÁRIO

ANTECEDENTES E JUSTIFICATIVA

1. Carcinoma espinocelular de cabeça e pescoço

O carcinoma espinocelular representa aproximadamente 95% de todas as neoplasias malignas de cabeça e pescoço (CECCP) (Dedivitis Ra, 2004) e é um dos tipos de câncer mais relatados no mundo, com altas taxas de prevalência em localidades como a Ásia, Europa e Brasil (Kaidar-Person, Gil *et al.*, 2018). Considerado como uma doença de curso agressivo onde mais de meio milhão de novos diagnósticos é realizado por ano, aproximadamente 50% dos pacientes acometidos sobrevive durante períodos em média menores que 05 anos (Gazdzicka, Golabek *et al.*, 2019).

Composto por um grupo anatômico heterogêneo, que abrange a boca (CECB), orofaringe (CECOF), hipofaringe (CECHF), laringe (CECL) e nasofaringe (CECN), com fatores etiológicos em comum (tabaco e álcool) e distintos, que podem influenciar diretamente no curso da doença, como o Papiloma Vírus Humano (HPV) (Economopoulou, Kotsantis *et al.*, 2017) é uma neoplasia heterogênea e há a presença de células agressivas e altamente tumorigênicas, responsáveis pela formação, invasão de tecidos adjacentes e ocorrência de metástases regionais e à distância (Bryne, Koppang *et al.*, 1989; Liang, 2011; Yanamoto, Kawasaki *et al.*, 2011) que propiciam altas taxas de recidiva e, baixas taxas de sobrevida (Jimenez, Jayakar *et al.*, 2015).

As células neoplásicas que apresentam estas características representam uma pequena subpopulação dentro do total de células do tumor que, para invadir o tecido conjuntivo adjacente, necessitam alterar o seu perfil molecular. Os mecanismos biológicos que regulam este fenômeno permanecem não completamente compreendidos (Yanamoto, Kawasaki *et al.*, 2011), embora já existam evidências sugerindo que as mesmas vias críticas para o desenvolvimento fisiológico podem desempenhar um papel importante na tumorigênese, em seus estágios iniciais (Zhang, Filho *et al.*, 2012).

2. Diagnóstico e monitoramento do CECCP

O diagnóstico precoce é a melhor chance de redução de morbidade e mortalidade frente às neoplasias malignas uma vez que não existem agentes terapêuticos de alta potência com mínimo efeito colateral para tais enfermidades (Vogelstein, Papadopoulos et al., 2013; Lauritano, Lucchese et al., 2016). O exame anatomopatológico tradicional é eficaz no diagnóstico inicial, no entanto, impotente frente à necessidade de detecção de alterações subclínicas bem como na predicção da evolução desses pacientes. Recidiva e, sobrevida inversamente a mortalidade, dependem de diversos e diferentes fatores histológicos, macroscópicos e microscópicos que são investigados a fim de definir as causas, para auxílio no diagnóstico e refinamento das modalidades terapêuticas (Lauritano, Lucchese et al., 2016).

Fatores prognósticos tradicionais de câncer que incluem idade do paciente, tamanho do tumor, graduação, presença de linfonodos locais e/ou metastáticos e

estadiamento foram critérios utilizados durante décadas na determinação de prognóstico e plano de tratamento para diferentes tipos de neoplasias, no entanto, não eram suficientemente precisos na previsão do comportamento clínico do CECCP bem como de outras malignidades (Duffy e Crown, 2014). Como exemplo, a literatura relata que, considerando-se a presença de linfonodos positivos como fator prognóstico mais importante, observa-se que a precisão de identificação de metástase cervical não ultrapassa 80% mesmo com a melhora tecnológica das análises (Fan, Zeng et al., 2014) e a incidência de ocorrências ocultas varia entre 15% e 60%, dependendo do recurso utilizado.

Dentro desta perspectiva, muitos estudos ainda recomendam o esvaziamento cervical eletivo com finalidade de reduzir o descontrole da doença e assim garantir margem de sobrevida maior (Abu-Ghanem et al., 2016), ainda que anteriormente já tenha sido relatado que, uma porcentagem comprovada de pacientes submetidos ao esvaziamento cervical foram sobretratados, tendo como consequência a diminuição significativa da qualidade de vida (Nibu, Ebihara et al., 2010).

Em pacientes com estadiamento clínico avançado, além do tratamento cirúrgico é rotina a utilização de tratamentos adjuvantes como radioterapia combinados ou não com quimioterapia, de acordo com a presença positiva ou negativa de fatores prognósticos. Nestes casos, além da presença de linfonodos positivos, passou a ser considerada como fator prognóstico a presença de margens negativas de tumor e a extensão extra-capsular, sem consenso sobre

qual fator ou combinação desses fatores determinariam a indicação dessas terapias (Chen, Lai et al., 2016).

A implicação do estadiamento clínico dos pacientes é tão importante no emprego de terapias adjuvantes que o sistema TNM incorporou na edição atualizada, dentre outros fatores, a presença de HPV, profundidade de invasão e classificação da extensão extra-capsular. Esses novos critérios foram adicionados no intuito de otimizar a indicação terapêutica e melhorar a taxa de sobrevivência dos pacientes supondo-se que fatores prognósticos bem consolidados poderiam predizer o comportamento tumoral (Lee, Eskander et al., 2019).

Com o objetivo de validar a nova edição do sistema TNM em CECB foi observado que, na comparação da edição passada com a atual, houve um aumento da categoria dos pacientes e melhora geral da capacidade de predicção da doença (Singhavi, Chakrabarti et al., 2019). Resultados de certa forma contraditórios foram observados quando, em estudo retrospectivo os pacientes que foram reclassificados pioraram o seu estadiamento, porém, permaneceram com índices de sobrevida iguais ou melhores do que os pacientes classificados diretamente pela edição atualizada (Lee, Eskander et al., 2019), corroborando com outro estudo que conclue que, mesmo com a tentativa de melhora dos fatores prognósticos através da adição de novas subcategorias no TNM, há a necessidade de criação de estratégias que cumpram a finalidade com maior precisão (Singhavi, Chakrabarti et al., 2019).

Paralelo ao novo sistema TNM, tem-se sugerido ainda para CECB uma subdivisão da área anatômica pela diferença de comportamento dos CECs de língua. Em CECOF observou-se que a subárea também tem impacto na sobrevivência dos pacientes, devendo ser considerada como fator prognóstico independente (Tham, Ahn et al., 2019). As necessidades de criação de subitens para fatores prognósticos surgem a partir do momento em que tumores sólidos como o CECCP possuem uma heterogeneidade molecular que permite um comportamento complexo e imprevisível. Os materiais obtidos por biópsia única que são utilizados para graduação histológica bem como a condição clínica que une os critérios para o estadiamento do paciente não podem ser representativos por fornecerem uma visão estática em relação à expressão de malignidade, que faz parte de uma dinâmica multifatorial (Duffy e Crown, 2014; Wikner, Grobe et al., 2014; An, Qin et al., 2015).

Muito além da terapêutica, dificuldades em definir prognóstico também geram falta de padronização de condutas em termos de acompanhamento dos pacientes após o tratamento. A maioria dos *guidelines* disponíveis trata o CECCP de maneira única, entretanto, a literatura relata tempos e riscos diferentes de recorrência e desenvolvimento de segundos tumores primários, de acordo com a área anatômica (Brands, Brennan *et al.*, 2018).

3. Microambiente Tumoral

O câncer não é apenas um acúmulo de células neoplásicas malignas, mas sim um arranjo destas num meio composto por células imunes, endoteliais,

mesenquimais, fibroblastos e matriz extracelular, chamado de microambiente tumoral (MT), que forma uma estrutura capaz de interagir com o organismo, em nível sistêmico. Tornou-se cada vez mais evidente que essa interação se assemelha aos processos que ocorrem frequentemente na inflamação, na cicatrização de feridas e nos órgãos em desenvolvimento, com tumores simulando a homeostase de tecidos normais, permitindo que o organismo aceite o seu desenvolvimento e progressão. O perfil molecular do MT é crucial no avanço da doença uma vez que o mesmo se auto-organiza de maneira que ocorram mudanças metabólicas, modificações nas características celulares, estimulação de neoformação vascular e reprogramação de células do sistema imune, adquiridas através da troca contínua de informação entre células neoplásicas e não neoplásicas permitindo além do desenvolvimento tumoral, a formação de metástases (Kim e Zhang, 2016; Wendler, Favicchio et al., 2016).

A resposta do hospedeiro em relação à neoplasia é a produção de um tecido tumoral que contenha elementos normais (Kalluri, 2016). Embora de proporção variável, mesmo dentro do mesmo tipo de tumor, os fibroblastos são, na maioria dos casos, o principal contribuinte para o estroma tumoral, fazendo parte de todas as etapas de progressão da doença, inclusive metástase. O fator de transformação do crescimento β (TGFβ), o fator de crescimento derivado de plaquetas, os ligantes de sinalização e o fator de crescimento de fibroblastos em conjunto com outras moléculas incluindo microRNAs (miRNAs) podem induzir o fenótipo de fibroblastos associados ao câncer (FACs) causando aumento das taxas de proliferação e do perfil migratório, além de maior deposição de matriz

extracelular. Originários de fibroblastos residentes, os FACs podem induzir a transição epitélio-mesenquimal (TEM), através do recrutamento e reprogramação de células-tronco mesenquimais (Kalluri e Zeisberg, 2006; Kalluri, 2016).

A interação entre as células neoplásicas, o MT e o organismo ocorre pela secreção de moléculas através de gaps entre as junções celulares, nano túbulos ou vesículas extracelulares (VEs).

4. Vesículas extracelulares

Descobertas em 1967 e chamadas de “pó de plaqueta”, rapidamente as VEs deixaram de ser “lixo celular” e foram relacionadas a processos importantes como o de neoformação óssea na superfície da cartilagem epifisária. Em 2007 um grupo de pesquisa identificou uma infinidade de miRNAs e RNAs não codificados em seu interior, indicando a função biológica dessas vesículas em condições fisiológicas. Estudos posteriores identificaram essas estruturas como fundamentais na comunicação entre as células pelo transporte de proteínas, lipídios e ácidos nucleicos, despertando ainda mais interesse científico (Roy, Hochberg *et al.*, 2018; Zheng, Bahr *et al.*, 2019).

Atualmente, diversos tipos de VEs têm sido estudados. São caracterizadas por serem fragmentos circulares de membrana, formadas por bicamada lipídica, tendo como mais frequentes as liberadas por compartimentos endossômicos como os exossomos ou por compartimentos mais superficiais como as microvesículas (Zhang, Valencia *et al.*, 2015; Kowal, Arras *et al.*, 2016) (Figura 1). Como a caracterização dessas estruturas ainda é significantemente discrepante entre os

grupos de pesquisa mundiais, a Sociedade Internacional das Vesículas Extracelulares recomenda o uso genérico de “VEs” (Roy, Hochberg *et al.*, 2018; Zheng, Bahr *et al.*, 2019) ainda que a literatura em sua maioria foque para o termo exossomos, e use estes termos como sinônimo (Taylor e Shah, 2015).

4.1 Exossomos

Os exossomos são pequenas VEs, de 30 a 150nm de diâmetro, originários de corpos multivesiculares, secretados por diferentes tipos de células, encontrados em fluidos corporais como sangue, urina, saliva, leite materno e sêmem, e no Sistema Nervoso Central (SNC), que contém proteínas, lipídios, metabólitos, DNA, miRNA, e diversas espécies de RNA não codificado, capazes de modular funções importantes na fisiopatologia, particularmente na transdução de sinais (Kanninen e Malm, 2016; Kanninen, Bister *et al.*, 2016). A biogênese dos exossomos inicia com endossomos jovens se tornando corpos multivesiculares, que se fusionam com a membrana plasmática da célula para serem liberadas para o meio extracelular. (Zheng, Bahr *et al.*, 2019).

Os elementos centrais dos exossomos são representados pelos miRNAs, uma vez que são observados compondo mais de 76% do mapeamento da análise sequencial (Zhang, Valencia et al., 2015) e seriam potenciais biomarcadores de doenças complexas, como doenças cardiovasculares (Kondkar e Abu-Amero, 2015), Alzheimer (Femminella, Ferrara et al., 2015), doenças hepáticas (Enache, Enache et al., 2014) e alguns tipos de câncer (Kinose, Sawada et al., 2014; Yuan, Yang et al., 2014).

4.2 Microvesículas

As microvesículas são VEs maiores em diâmetro (100-1000nm) originárias por brotamentos que ocorrem a partir da membrana plasmática (Gyorgy, Szabo *et al.*, 2011; Zheng, Bahr *et al.*, 2019). Alguns estudos relatam sua origem como das células endoteliais e/ou de eritrócitos que circulam no sangue periférico, por isso são encontradas nos seus derivados (Gyorgy, Szabo *et al.*, 2011) e outros sugerem que as mesmas também se originem de plaquetas, células dendríticas e neutrófilos (Thery, Ostrowski *et al.*, 2009). Sua biogênese envolve eventos como o aumento de cálcio intracelular ou a apoptose da célula, que induz os receptores de superfície de membrana à sua formação e liberação. Embora as microvesículas sejam estruturalmente similares aos exossomos, funcionalmente essas estruturas são diferentes uma vez que somente as microvesículas conseguem transportar plasmídeos (moléculas de DNA que se reproduzem independentemente do DNA cromossômico) (Zheng, Bahr *et al.*, 2019).

A literatura atual sugere que este tipo de VE esteja envolvido com a ativação da atividade coagulatória, como forma de secreção de interleucinas, patogênese da artrite reumatóide, indução de transformação oncogênica bem como contribuição para características invasivas de tumores e comunicação feto-maternal (Gyorgy, Szabo *et al.*, 2011), além disto, ainda existem evidências de sua relação com lúpus eritematoso e a angiogênese tumoral (Melki, Tessandier *et al.*, 2017).

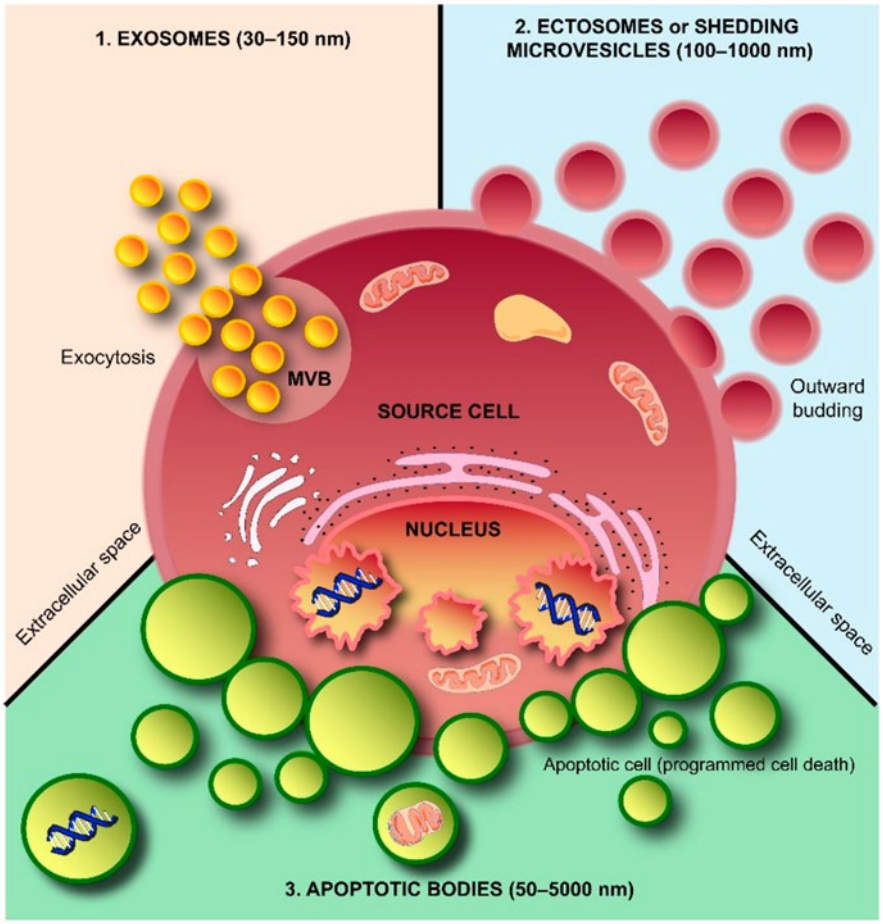


Figura 1: imagem representativa das VEs. Fonte: microvesicles.org.

5. Vesículas extracelulares e microambiente tumoral

As VEs participam da conformação do MT e são estudadas de maneira ampla por transportar e liberar ácido desoxirribonucleico (DNA), ácido ribonucleico mensageiro (mRNA), microRNAs (miRNAs) e proteínas que atingem locais ou células associadas ao tumor (Zhang, Valencia *et al.*, 2015). Podem transferir moléculas entre células ou por via sistêmica para locais anatômicos distantes,

podendo induzir vias de sinalização ou alterar diretamente o fenótipo das células receptoras, tornando a caracterização do perfil molecular do MT importante na aquisição de informações prognósticas (An, Qin et al., 2015; Kim e Zhang, 2016; Lin, Du et al., 2016; Wendler, Favicchio et al., 2016).

É provável que a composição das VEs reflita a fisiologia das células-mãe onde, podendo transportar informações para células receptoras e criando uma conexão, interagem e reprogramam o metabolismo das mesmas, possibilitando a análise do efeito causal da transformação oncogênica (Wendler, Favicchio et al., 2016). As células tumorais podem exercer diversos efeitos nas células circundantes (endoteliais, imunes e FACs), a fim de estabelecer um microambiente propício. Evidências demonstram que VEs derivadas de tumor, como exossomos, contribuem para a inibição imunológica, crescimento tumoral e metástase (Zheng, Bahr *et al.*, 2019) e as microvesículas, através de sua capacidade de reter quimiocinas, facilitam a troca de informações entre as células tumorais e as do MT, causando a ativação de células imunes como neutrófilos e macrófagos associados ao tumor (MAT), promovendo infiltração tumoral (Bian, Xiao *et al.*, 2019).

Outros estudos sugerem que o reparo e a regeneração normal tecidual são processos adquiridos pelo MT. Essa capacidade do tumor, chamada “nicho onco-regenerativo” tem função de garantia de crescimento e progressão, e é composto além das células neoplásicas, por uma rede de células normais e fatores ativados. Nos linfomas tipo B agressivos, sugeriu-se que as VEs das células tumorais apoptóticas seriam veículos críticos de comunicação intercelular, garantindo a

ativação de MAT e da via endotelial, contribuindo para o estudo das terapias antineoplásicas indutoras de apoptose (Gregory e Paterson, 2018) e que as VEs derivadas de câncer colorretal secretam miR-145, que é absorvido pelos macrófagos e, após induzi-los ao fenótipo tipo 2 (M2), tem ação pró-tumoral através do estímulo da angiogênese e inibição seletiva da resposta imune, contribuindo para o seu crescimento (Shinohara, Kuranaga *et al.*, 2017).

6. VEs como biomarcadores e biópsia liquida

Biomarcador é definido como uma molécula biológica encontrada no sangue ou outros fluidos corporais e em tecidos, que sinaliza processos fisiológicos, patológicos, condições e/ou doenças e também pode ser utilizado para avaliar a resposta terapêutica do organismo frente a algum fármaco (Hsieh, Wang *et al.*, 2019).

Tendo como objetivo a identificação de biomarcadores, a biósia líquida, baseada no material contido em fluidos corporais, pode refletir a ampla gama de propriedades da malignidade, incluindo possíveis metástases e alterações bioquímicas adquiridas durante a disseminação, que não estão presentes no tumor primário. Neste caso, contrariando a biópsia única, a heterogeneidade das características do tumor em um mesmo paciente pode ser refletida com uma única amostra, logo, alterações moleculares ou genômicas adquiridas de sítios secundários podem ser identificadas e combatidas mesmo antes de a metástase ser evidenciada. Devido à pequena invasividade, amostras repetidas podem ser coletadas sempre que necessário para monitorar mudanças dinâmicas nas características moleculares da doença, particularmente influenciada pela pressão

seletiva do tratamento medicamentoso (Mader e Pantel, 2017; Hsieh, Wang *et al.*, 2019).

Os padrões de incidência do câncer são refletidos pelo entendimento do comportamento biológico da doença e pelas mudanças na prática médica, tendo como exemplo os testes de rastreamento realizados com biomarcadores como o antígeno prostático específico (PSA). O aumento expressivo na incidência do câncer de próstata bem como melhora dos índices de sobrevivência para homens acometidos é resultado do aumento da detecção da doença ainda assintomática, que permite diagnóstico precoce e melhor capacidade de tratamento (Siegel, Miller *et al.*, 2019).

Alguns biomarcadores podem ser isolados nos flúidos corporais para o estudo do CECCP. No entanto, existe uma série de fatores que podem impactar na confiabilidade e dinâmica deste tipo de material, como momento da coleta, viscosidade do fluido, fatores nutricionais, condições inflamatórias entre outros, que limitam a sua utilização clínica. Marcadores que têm sido estudados tradicionalmente para esta condição como CDK4, indutor de apoptose do tipo fator de necrose tumoral (TNF), fator de crescimento endotelial vascular (VEGF) e outros, nem sempre são específicos para o câncer, podendo não ter capacidade de distinção entre doenças malignas e inflamatórias (Hsieh, Wang *et al.*, 2019).

Atualmente, a possibilidade de utilização de VEs como biomarcadores em várias doenças ou como veículos de imunoterapia têm sido amplamente explorada. Essas estruturas possuem receptores de superfície que permitem a

sua segmentação e captura pelas células receptoras, incorporando mensagens e modificando seu estado fisiológico (Kowal, Arras *et al.*, 2016) uma vez que a sua carga reflete com precisão o estado da célula (sobre stress, ativada ou doente). Por serem liberadas na corrente sanguínea, as VEs são passíveis de ser isoladas e utilizadas para detecção de conteúdos específicos que podem representar a origem celular de diversas doenças, tornando o seu uso interessante para fins diagnósticos, decisão terapêutica e monitoramento (Kanninen, Bister *et al.*, 2016; Lin, Du *et al.*, 2016).

7. Vesículas extracelulares e câncer

Estudos têm demonstrado que VEs liberadas pelos tumores, pelos FACs e pela medula óssea estão relacionadas à progressão tumoral, resistência à quimioterapia e evasão imunológica através da transferência horizontal de moléculas bioativas. Estas estruturas são capazes de modificar o próprio tumor e o MT, participando das etapas necessárias para a metástase fazendo com que o seu conteúdo seja útil como fator diagnóstico e prognóstico (An, Qin *et al.*, 2015). Dentre as hipóteses sobre mecanismos capazes de explicar a transferência de conteúdo das VEs entre as células, a mais aceita propõem que estas estruturas são capazes de se incorporar às proteínas transmembrana da célula receptora e liberar seu conteúdo luminal no citoplasma da mesma (Thery, Ostrowski *et al.*, 2009; Purdie, Pourreyron *et al.*, 2011).

Diversos estudos têm observado a relação das VEs com diferentes tipos de câncer. No plasma, pacientes com neoplasias malignas apresentam quantidades significativamente maiores do que pacientes hígidos (Silva, Garcia *et al.*, 2012;

Matsumoto, Kano *et al.*, 2016). Pacientes com câncer colorretal possuem níveis maiores de VES e sua presença está associada a parâmetros de mal prognóstico e sobrevivência global curta (Silva, Garcia *et al.*, 2012). No carcinoma hepatocelular, a transferência de miRNAs mediada por exossomos é uma mecanismo de comunicação entre as células tumorais indicando que a comunicação intercelular tem potencial para contribuir na disseminação local, metástases intra-hepáticas ou crescimento multifocal da neoplasia (Kogure, Lin *et al.*, 2011).

Em pacientes com tumores metastáticos incluindo sarcomas, adenocarcinomas de mama, tratos geniturinário, gastrointestinal e respiratório, foi observado um número significativamente maior de microvesículas em relação aos pacientes normais. Além disto, o fator tecidual contido por essas VEs aparece em concentrações maiores nos pacientes doentes e está envolvido com a via extrínseca de coagulação, podendo estar relacionado à trombose venosa associada ao câncer, fazendo destas estruturas promissoras na identificação de risco de trombose em pacientes com tumores agressivos (Ender, Freund *et al.*, 2019).

8. Vesículas extracelulares e CECCP

Diversos estudos *in vitro* e *in vivo* relatam algum tipo de relação das VEs em alguma etapa ou via de progressão do CECCP e diferentes tipos de conteúdo são relatados, sendo miRNAs, receptores de TGFβ, e proteínas de membrana como Caveolin 1 e transmembrana como o *Fas Ligant* os mais frequentes (Kim,

Wieckowski *et al.*, 2005; Vered, Lehtonen *et al.*, 2015; Languino, Singh *et al.*, 2016; Li, Li *et al.*, 2016; Sakha, Muramatsu *et al.*, 2016; Sento, Sasabe *et al.*, 2016; Zlotogorski-Hurvitz, Dayan *et al.*, 2016; Dickman, Lawson *et al.*, 2017).

Sugere-se que os exossomos que contém miRNA derivado de células altamente metastáticas são capazes de promover crescimento celular, migração e invasão em células com baixa capacidade de metástase, podendo aumentar o potencial maligno das mesmas (Sakha, Muramatsu *et al.*, 2016). Estas estruturas são capazes de reter seletivamente os miRNAs e a sua liberação conduz ao aumento do crescimento de células neoplásicas, displásicas e do estroma tumoral através de alterações na expressão de TGFβ1 (Dickman, Lawson *et al.*, 2017).

Exossomos isolados a partir de fibroblastos do estroma tumoral contêm componentes da via de sinalização do TGFβ (TβRII), onde a transferência exossomal do MT gera a reativação da sinalização de TGFβ em células epiteliais malignas de CECB que perderam a resposta a esse tipo de ligante, demonstrando que a comunicação do estroma com as células tumorais pode direcionar a sinalização do tumor (Languino, Singh *et al.*, 2016).

Derivados de cultivo celular e sob hipóxia, os exossomos contém altos níveis de miRNAs aberrantes que são associados ao estágio tumoral e metástase locorregional. Essas VEs quando derivadas de tumor podem ser retidas por células normóxicas levando as mesmas à aquisição de fenótipo pró-metastático sugerindo-se que podem funcionar como mensageiros de entrega de miRNAs

entre células tumorais hipóxicas e normóxicas remodelando o MT(Li, Li *et al.*, 2016).

Embora seja necessária a análise clínica aprofundada do CEC primário e extensão local do mesmo, observou-se que células do CECB são capazes de promover a progressão tumoral através da secreção e absorção de seus próprios exossomos (Sento, Sasabe *et al.*, 2016) e que os derivados de fluidos orais de pacientes com CECB diferem molecular e morfologicamente de indivíduos livres da doença. Os estudos servem de base na busca de biomarcadores obtidos a partir de VEs para detecção precoce de CECB em pacientes de alto risco e sem evidência de sinais clínicos de lesões bucais (Zlotogorski-Hurvitz, Dayan *et al.*, 2016).

Tendo em vista que o CECB é o tipo de câncer mais associado à inflamação crônica entre os CECCP, e que os níveis de IL-6 e TNF-α foram positivamente correlacionados ao aumento de concentração de MVs circulantes e plasmáticas, foi sugerido que a liberação dessas MVs no sangue periférico é relacionada à secreção de fatores inflamatórios do MT. Ainda, observou-se um número significativamente maior de MVs circulantes e derivadas de plasma em pacientes com CECB e os pacientes em estágios III e IV apresentaram maior concentração dessas estruturas quando comparados aos pacientes em estágios I e II (Ren, Man *et al.*, 2016).

Visando identificar mecanismos responsáveis pela apoptose das células T, através da atividade biológica das MVs presentes na circulação periférica de

pacientes com CECB, observou-se que MVs que contém FasL são capazes de induzir a morte celular e estão correlacionadas com a carga tumoral e acometimento de linfonodos nos pacientes (Kim, Wieckowski *et al.*, 2005). Em CECCP, as MVs derivadas de soro, em relação aos pacientes saudáveis, apresentaram quantidades maiores dessa mesma proteína de transmembrana entre outras, com a mesma capacidade (Bergmann, Strauss *et al.*, 2009).

Em estudo sobre CECE observou-se que a quantidade de exossomos do plasma dos pacientes doentes era significativamente maior do que em pacientes livres de doença e ainda, através de modelo animal, foi comprovado que os exossomos liberados pelo tumor circulam no sangue periférico (Matsumoto, Kano *et al.*, 2016). Nos pacientes com CECN o aumento dos exossomos circulantes presentes no plasma foi correlacionado com linfonodos metastáticos e sobrevida pobre, mostrando que a concentração de exossomos pode ter relevância clínica e valor prognóstico (Ye, Li *et al.*, 2014).

Visando a estratificação de pacientes resistentes e não resistentes a quimiorradioterapia, estudo relata que as VEs coletadas do plasma dos pacientes com CECCP apresentaram uma gama de proteínas em comum, sendo 119 exclusivas dos pacientes resistentes ao tratamento e 38 exclusivas dos pacientes sensíveis ao tratamento. Foram identificadas novas proteínas e outras já descritas que, no presente momento não tem aplicação clínica, mas são promissoras na futura padronização de biomarcadores para o CECCP (Rodrigues-Junior, Tan *et al.*, 2019).

9. A aplicabilidade das VEs na medicina de precisão em CECCP

Os tratamentos de precisão ou personalizados, selecionados através de um processo de diagnóstico que represente as características biológicas do câncer têm por finalidade a excelência em definição de tipo e doses terapêuticas eficientes. Na indisponibilidade desta modalidade, pacientes de CECCP são frequentemente submetidos a tratamentos agressivos que têm como objetivo combater a alta invasividade e capacidade de recorrência da doença, características resultantes de várias etapas da carcinogênese através da geração de lesões múltiplas em diferentes estágios que podem se desenvolver simultaneamente em grandes áreas expostas à neoplasia, devido ao campo de cancerização (Bettegowda, Sausen *et al.*, 2014). Ainda que se apliquem tratamentos radicais, taxas de sobrevida baixas demonstradas pela literatura sugerem a baixa efetividade deste tipo de prática que, mesmo quando tem a capacidade de erradicar a doença, causam sequelas irreversíveis que impactam negativamente na qualidade de vida dos pacientes.

Dentro deste contexto, o desenvolvimento de métodos não invasivos para observar as doenças malignas é o atual desafio da oncologia (Bettegowda, Sausen et al., 2014; Schmidt, Kulasinghe et al., 2016), que vem explorando pesquisas de novas gerações de biomarcadores, representativos do processo de iniciação e progressão do câncer (Yates e Campbell, 2012; Vogelstein, Papadopoulos et al., 2013).

O uso de biomarcadores prognósticos e preditivos pode contribuir para determinar o tratamento mais efetivo e menos tóxico para cada paciente, evitando sobretratamento e custos desnecessários. Enquanto os marcadores prognósticos ajudam a diferenciar os pacientes com maior ou menor risco de vida, visando identificar quem deve ou não receber terapias adjuvantes seguidas à remoção do tumor primário os preditivos auxiliam na identificação dos pacientes que são responsivos ou resistentes a certos tipos de terapia, ou seja, ao invés de serem necessários na decisão terapêutica, são utilizados na precisão do tipo de terapia administrada (Duffy e Crown, 2014) sendo importantes principalmente em tumores do mesmo tipo histológico que respondem diferentemente a um antineoplásico específico, justificando porque apenas uma minoria de pacientes com a mesma patologia é beneficiada pelos agentes quimioterápicos (Duffy e Crown, 2014; Wikner, Grobe et al., 2014).

Tendo em vista que as VEs participam da etapa evolutiva das neoplasias incluindo a metástase, e que seu conteúdo é de origem específica, a possibilidade de emprego das VEs como biomarcadores para o CECCP torna-se promissora para diagnóstico, monitoramento e tratamento personalizado. Atualmente, centenas de estudos clínicos sobre as VEs têm sido registrados em nível mundial (Roy, Hochberg *et al.*, 2018; Susa, Limongi *et al.*, 2019), no entanto, VEs derivadas de CECCP bem como outros tipos de tumor representam apenas uma parcela do total de vesículas presentes nos fluidos corporais uma vez que uma

variedade de células ativas de diferentes origens e estágios de diferenciação também liberam este tipo de estrutura, que faz parte dos processos fisiológicos (Bergmann, Strauss *et al.*, 2009).

Técnicas de isolamento de VEs ainda não são capazes de separar estruturas provenientes de processos fisiológicos e patológicos, além disto, métodos que preservem o conteúdo destas estruturas vêm sendo amplamente comparados e necessitam de aprimoramento uma vez que a identificação de biomarcadores confiáveis oriundos de VEs depende diretamente da precisa purificação dos mesmos (Taylor e Shah, 2015). Os métodos utilizados atualmente, não são apenas trabalhosos e demorados, mas também resultam em perda substancial de amostras e geração de artefatos, o que impede a precisa detecção e análise bem como a padronização para uso clínico (Lim, Zhang *et al.*, 2019).

Recentemente um compêndio chamado Vesiclopédia exibindo dados obtidos de 1254 estudos registra 38.146 tipos de RNA, 349.988 tipos de proteína e 639 tipos de lipídeos e ou metabólitos como conteúdos em potencial das VEs (Pathan, Fonseka *et al.*, 2019). O conhecimento básico de como e onde o conteúdo dessas vesículas é descarregado e qual o seu papel na homeostase celular não foi elucidado bem como a consequência das interações intercelulares que ocorre através destas estruturas (Russell, Sneider *et al.*, 2019) o que faz do estudo das VEs um desafio, porém promissor campo de pesquisa para o CECCP uma vez que esta patologia não possui um biomarcador “padrão ouro” com aplicabilidade clínica para o seu diagnóstico e monitoramento.

OBJETIVOS

Objetivo geral

Avaliar a relação das vesículas extracelulares com carcinoma espinocelular de cabeça e pescoço.

Objetivos específicos artigo 1

Contextualizar o CECCP

- Analisar a sobrevida de uma coorte de 50 pacientes com CECCP durante um período de 10 anos.
- Correlacionar os parâmetros clínicos e histopatológicos no momento do diagnóstico inicial com recidiva, metástase e óbito pelo tumor durante o tempo de acompanhamento.

Objetivos específicos artigo 2

Contextualizar as VEs em relação ao CECCP

- Estabelecer a relação das VEs do tipo exossomos com o CECCP através de revisão sistemática de literatura.

Objetivos específicos artigo 3

Estudo sobre a relação das VEs e o CECB

- Caracterizar as vesículas extracelulares de linhagens de CECB.
- Avaliar *in vitro* o efeito das vesículas extracelulares na migração das células epiteliais e de fibroblastos.

ARTIGOS CIENTÍFICOS

Artigo 1

Artigo científico de acordo com as normas do periódico *Journal of oral pathology & medicine* (Qualis Capes 2016 - A2, ISSN 1756-8919, Fator de impacto: 2,495).

Clinical outcomes and prognostic factors of head and neck squamous cell carcinoma: a ten-year follow-up study

Abstract

Background

The guidelines for determining head and neck squamous cell carcinoma (HNSCC) prognosis are used in therapeutic decision-making of patients. However, only 50% of patients live for more than 5 years. The present study aimed to analyze the correlation of prognostic factors with the survival of patients diagnosed with HNSCC.

Methods

A sample of 78 patients diagnosed with HNSCC was followed for 10 years after tumor diagnosis and treatment. Information regarding sex, age, ethnic group, and tobacco and/or alcohol consumption, tumors characteristics and treatment were obtained from an interview or hospital records. Data collection was performed at four time points from the hospital records; cases without information relevant to the study were excluded.

Results

The final study sample comprised 50 patients. The events related to patient survival occurred from the first month up to 9 years and 9 months of follow-up. There was a significant increase in number of deaths after 2.5 and 5 years of

follow-up. The number of patients alive without recurrence increased between 7.5 and 10 years of follow-up. At the end of the study, only 14% of patients were discharged, whereas 44% remained under follow-up due to presence of a potentially malignant disorder, treatment for another tumor, or metastases.

Conclusion

The analysis of the 10-year follow-up showed that traditional prognostic factors are not accurate in detecting subclinical changes or predicting the prognosis of patients. The advance in knowledge of tumor molecular biology support the idea that the disease should be considered on an individual basis.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC), a disease related to risk factors such as smoking and alcohol consumption, is one of the cancers with the highest reported prevalence rates worldwide[1]. Other risk factors include sun exposure, eating habits, and human papillomavirus (HPV) infection, which is particularly related to the etiopathogenesis of oropharyngeal disease[2].

Currently, the guidelines for determining prognosis, which include tumor size, histological grade, local and/or distant lymph node involvement, and tumor staging, are carefully used in therapeutic decision-making and follow-up of patients with the disease[3]. However, there has been a discussion regarding these guidelines because only 50% of patients with HNSCC live for more than 5 years[4], with even lower mean survival rates in cases of metastases and recurrence (rarely exceeding 1 year)[5]. In view of this, other clinical and pathological factors associated with survival, such as age, sex, tumor location and treatment, are increasingly being considered in the analysis of prognosis[6], and the development of biomarkers for precision medicine has been increasingly visible[5]. Thus, the present study aimed to analyze the correlation of prognostic factors with the survival of patients diagnosed with HNSCC in a 10-year follow-up period.

2. Materials and methods

2.1 Study sample

The sample comprised 78 patients aged between 37 and 77 years at the time of diagnosis, both men and women, who were diagnosed with primary HNSCC and treated at the Head and Neck Surgery Outpatient Clinic of the Otorhinolaryngology Department of Hospital de Clínicas de Porto Alegre (HCPA) from October 2009 to October 2010. These patients were followed for 10 years after tumor diagnosis and treatment.

The sample was derived from a previous study conducted by our research group[7]. All individuals provided written informed consent, and the study was approved by the Research Ethics Committee of the HCPA (GPPG no. 09-315).

2.2 Clinical parameters

During the diagnostic process, an interview was conducted with the participants to obtain information regarding sex, age, ethnic group, and tobacco and/or alcohol consumption. Data on the characteristics of the tumors were obtained from the hospital records after the patients' treatment, a procedure that did not affect the adequate therapy for each case.

2.3 Treatment

The patients received the treatment indicated in the hospital's HNSCC care protocol for each case, which was either surgery alone, surgery plus radiotherapy, surgery plus radiotherapy plus chemotherapy alone, or chemotherapy plus radiotherapy.

2.4 Clinical staging and histological grading

The tumors were classified according to the TNM system (size, T; regional metastases, N; and distant metastases, M) of the American Joint Committee on Cancer[8].

Histological grading (HG) was performed by two pathologists blinded to the identity of the specimens according to the criteria proposed by Bryne et al. (1992). Thus, the tumors were considered to be of good, moderate, and poor prognoses according to their degree of malignancy[9].

The HG was executed on the surgical specimens after tumor resection in all patients except one, who was treated with radiotherapy and chemotherapy. In this case, histological grading was performed on the tissue sample collected during incisional biopsy for the initial diagnosis.

2.5 Survival analysis

2.5.1 Follow-up

Data collection during the follow-up period was performed at four time points: F1, 2.5 years; F2, 5 years; F3, 7.5 years; and F4, 10 years. The information was obtained from the hospital records; cases without records relevant to the study were excluded. At the different follow-up time points, the sample was categorized into the following: patients without recurrence, patients with recurrence, patients with metastases (regional or distant), and patients who died due to the tumor. Therefore, this categorization varied depending on the patient's condition at the time of data collection.

2.5.2 Prognosis

Patient prognosis was determined according to the patient's progression during the follow-up period, and the sample was categorized as follows: patients with good prognosis (absence of clinical signs, recurrence or metastases, or progression to death); patients with moderate prognosis (potentially malignant

disorders, recurrence, or metastasis); and patients with poor prognosis (death due to the tumor).

2.5.3 Final clinical status

At the end of the 10-year follow-up period, patients were reclassified according to their final clinical status: discharged patients, patients without recurrence, patients with potentially malignant disorders, patients with recurrence, patients with metastases (regional and/or distant), and patients who died due to the tumor. The cause of death related to the tumor was obtained from the medical records.

2.6 Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences version 17.0 (SPSS Inc., Chicago, IL, USA). Age was presented as mean and standard deviation. Data such as sex, ethnic group, smoking habit and alcohol consumption, type of treatment, evaluation of tumor samples, prognosis, current status, and cause of death were expressed as percentages. The distribution of patient status according to the follow-up time was analyzed using the Kruskal-Wallis test. The association of prognosis, current event, and cause of death with tumor grade and pTNM was assessed using Pearson's chi-square test. Survival analysis was performed using the Kaplan-Meier test, and the association between the variables was assessed using the logrank test. The independent predictors of survival were evaluated using the Cox regression model. The statistical significance level was set at 5% ($p \leq 0.05$).

3. Results

3.1 Study sample

Twenty-eight patients were excluded from the initial sample of 78 patients due to inadequate information relevant to the study. The characteristics of the patients,

tumor location, clinical staging, and histopathological grading, as well as the type of treatment undergone by the final sample of 50 patients, are described in Table 1.

Table 1. Clinical parameters, type of treatment, and specimen evaluation.

Age	**Mean** 58,2	**SD**	9,953
		n	%
Gender	Male	42	84
	Female	08	16
Skin	Caucasian	40	80
	Non-Caucasian	5	20
Tobacco	Current	21	42
	Former	28	56
	Never	1	2
Alcohol	Current	25	50
	Former	22	44
	Never	3	6
Location	**Oral**	35	70
	Tongue	11	22
	Floor	5	10
	Lip	10	20
	Palate	6	12
	Cheek mucosa	3	6
	Oropharynx	3	6
	Hypopharynx	4	8
	Larynx	8	16
Size	T1/T2	32	64
	T3/T4	18	36
Regional metastases	N0	32	64
	N1/N2/N3	18	36
Treatment	Surgery	34	68
	Surgery/ radiotherapy	13	26
	Surgery/radiotherapy/chemotherapy	2	4
	Radiotherapy/chemotherapy	1	2
Histological Grading	Good	24	48
	Moderate	23	46
	Poor	3	6
Total		50	100

3.2 Clinical parameters

The patients had a mean age of 58.2 years, and the majority were men (84%) and Caucasian (80%). The most frequent tumor location was the mouth (70%), with a predominance of the tongue (22%) and lip (20%). At the time of diagnosis, 42% of patients reported smoking, and 98% of individuals had used tobacco at some point before diagnosis. With regard to alcohol consumption, 94% of patients had used alcohol at some point, and 25% of patients were still consuming it at the time of diagnosis.

3.3 Treatment

Particularly, 68% of patients underwent surgery, and 30% of patients received some form of adjuvant treatment. One patient (2%) had a tumor that was considered inoperable and received a combination of radiotherapy and chemotherapy.

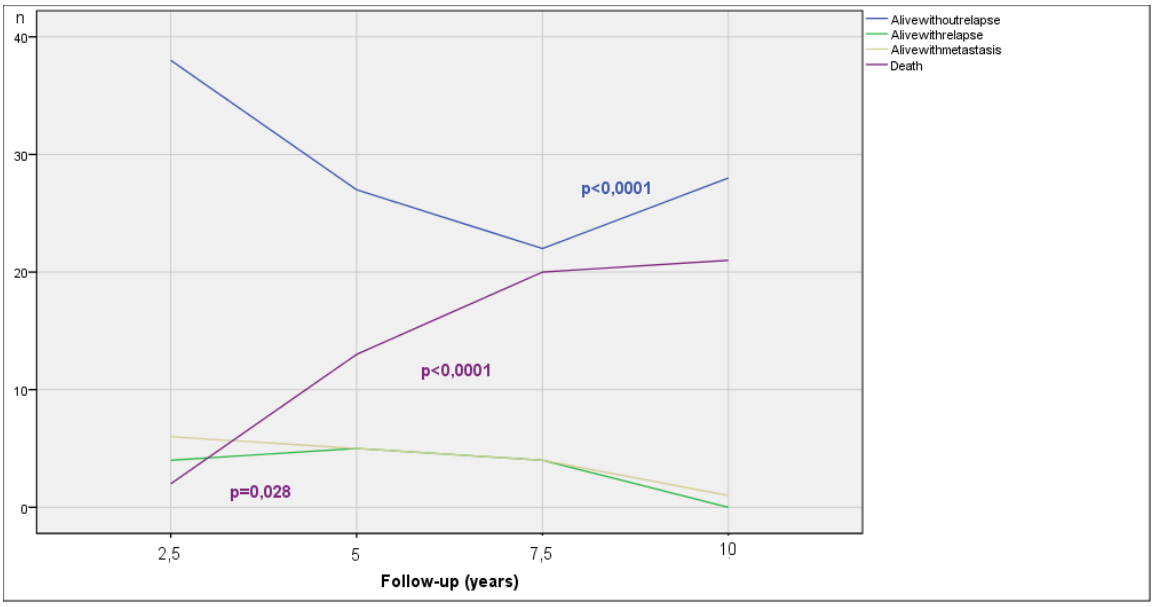
3.4 Clinical staging and histological grading

Most patients in the sample had tumors with size < 4 cm (64%) and no regional metastasis (64%), and no patient had distant metastases at the time of diagnosis. The majority of the individuals (94%) had tumors with good (48%) and moderate (46%) degree of malignancy.

3.5 Survival analysis

The proportion of patients alive (without recurrence, with recurrence, and with metastasis) gradually decreased up to 7.5 years (F1, 48/50=96%; F2, 37/50=74%; F3, 30/50=60%; F4, 29/50=58%) (Figure 1). In the first 2.5 years of follow-up, there was no significant change in the clinical status of the patients ($p=0.070$) (data not shown); however, there was a significant increase in the number of deaths after 2.5 years ($p=0.028$) and 5 years ($p<0.0001$) of follow-up. The number of patients alive without recurrence increased ($p<0.0001$) between 7.5 years of follow-up and the end of the 10-year follow-up.

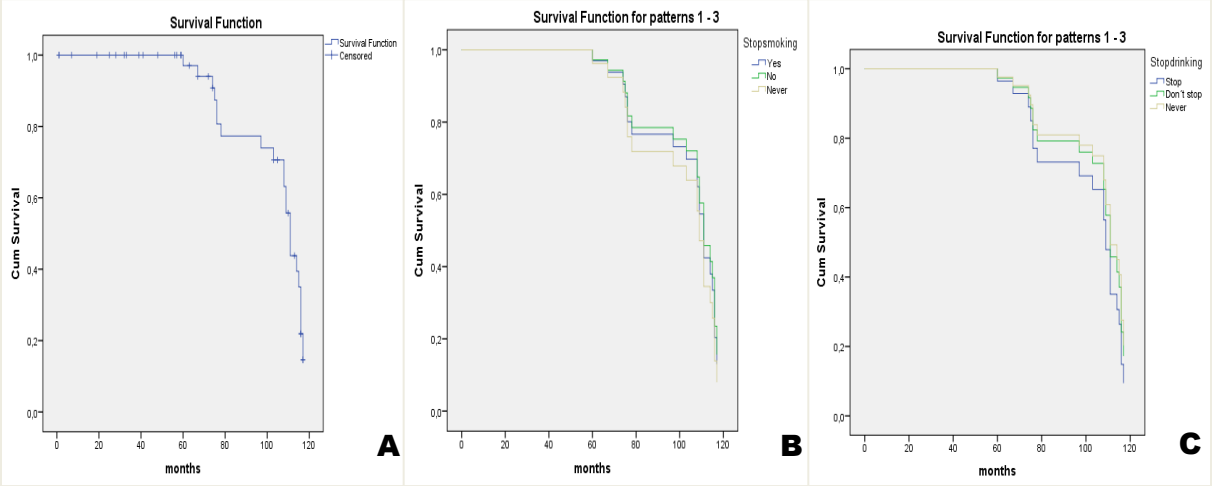
Figure1. Evaluation of patients at the four time points of data collection



P values from Kruskal-Wallis test, p ≤ 0,05.

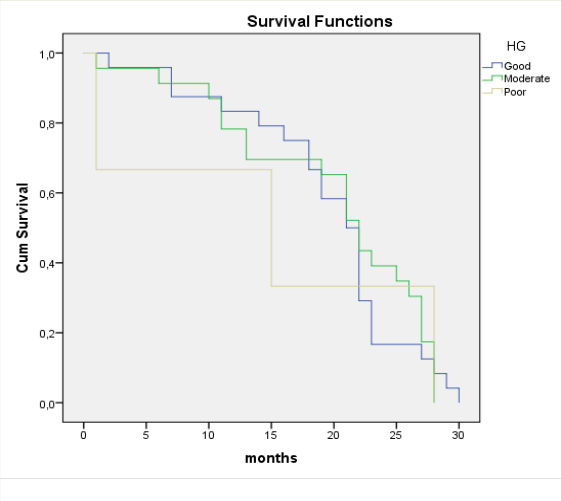
The overall analysis shows that the events related to patient survival occurred from the first month up to 9 years and 9 months of follow-up (Figure 2A). An interesting finding was that cessation of smoking (p= 0.946) and alcohol consumption (0.796) did not change the survival rates (Figures 2B and 2C).

Figure 2. Kaplan-Meier analysis of global survival (A) and Cox regression analysis with adjustment for previous smoking (B) and alcohol consumption (C).

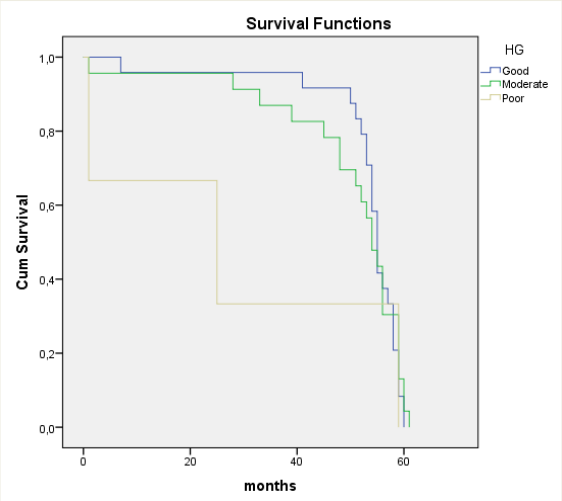


The histopathological grading did not influence the survival of patients up to 5 years of follow-up (F1, p=0.955, and F2, p=0.699). However, after 5 years of follow-up, patients with tumors with a good degree of malignancy had higher survival rate (F3, p=0.001, and F4, p=0.033)(Figure 3).

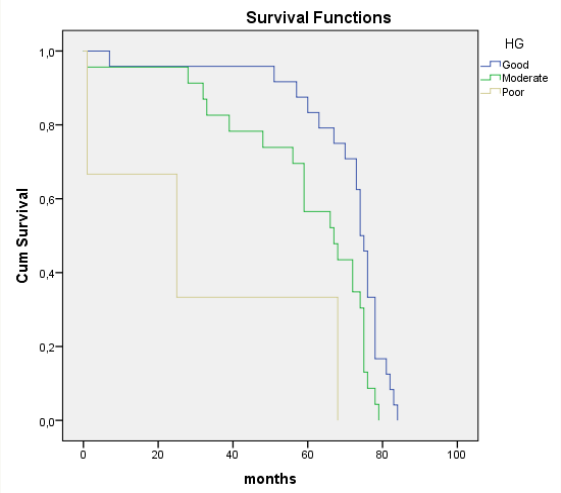
Figure 3. Kaplan-Meier survival curves at different follow-up time points according to the histopathological grade.



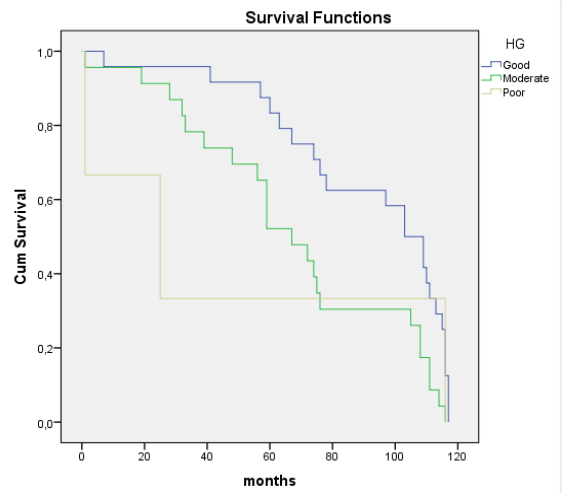
F1: 0,884 standart deviation, 95%



F2: 0,698 standart deviation, 95%



F3: 2,57 standart deviation, 95% confidence

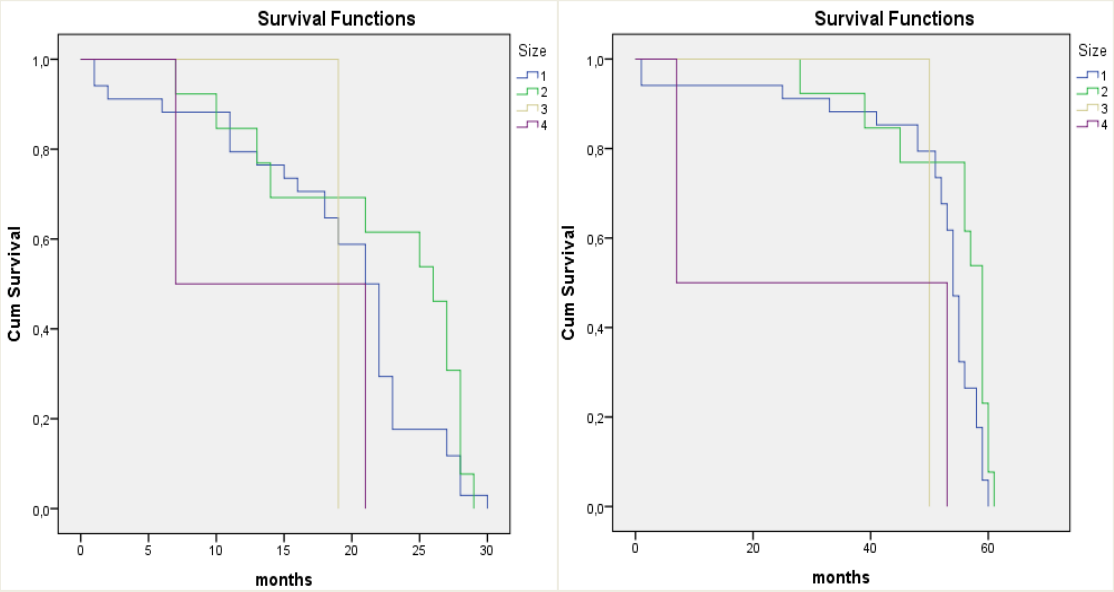


F4: 5,052 standart deviation, 95% confidence

HG= histological grading

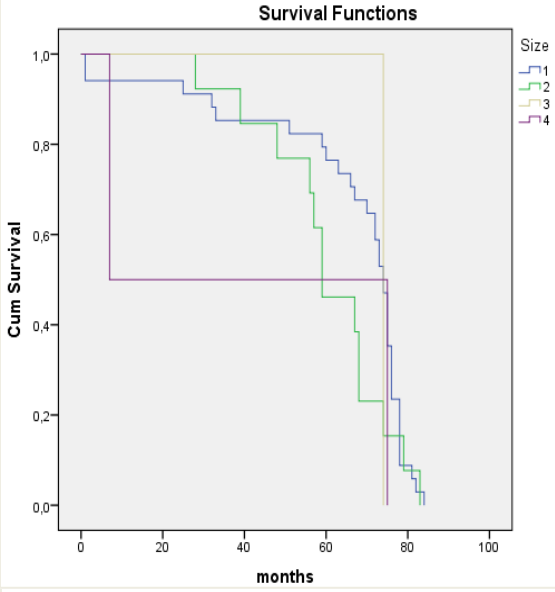
At 5 years of follow-up, there was an association between tumor size < 4 cm (T1/T2) and higher patient survival rate (F2, p=0.010). This association was not observed at the other follow-up time points (F1, p=0.217; F3, p=0.514; F4, p=0.439) (Figure 4).

Figure 4. Kaplan-Meier survival curves at different follow-up time points according to the size of the primary tumor.

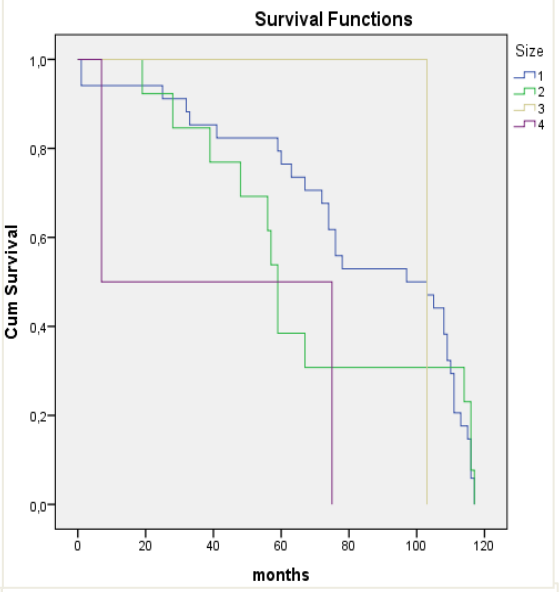


F1: 0,004 standart deviation, 95% confidence

F2: 0,698 standart deviation, 95% confidence



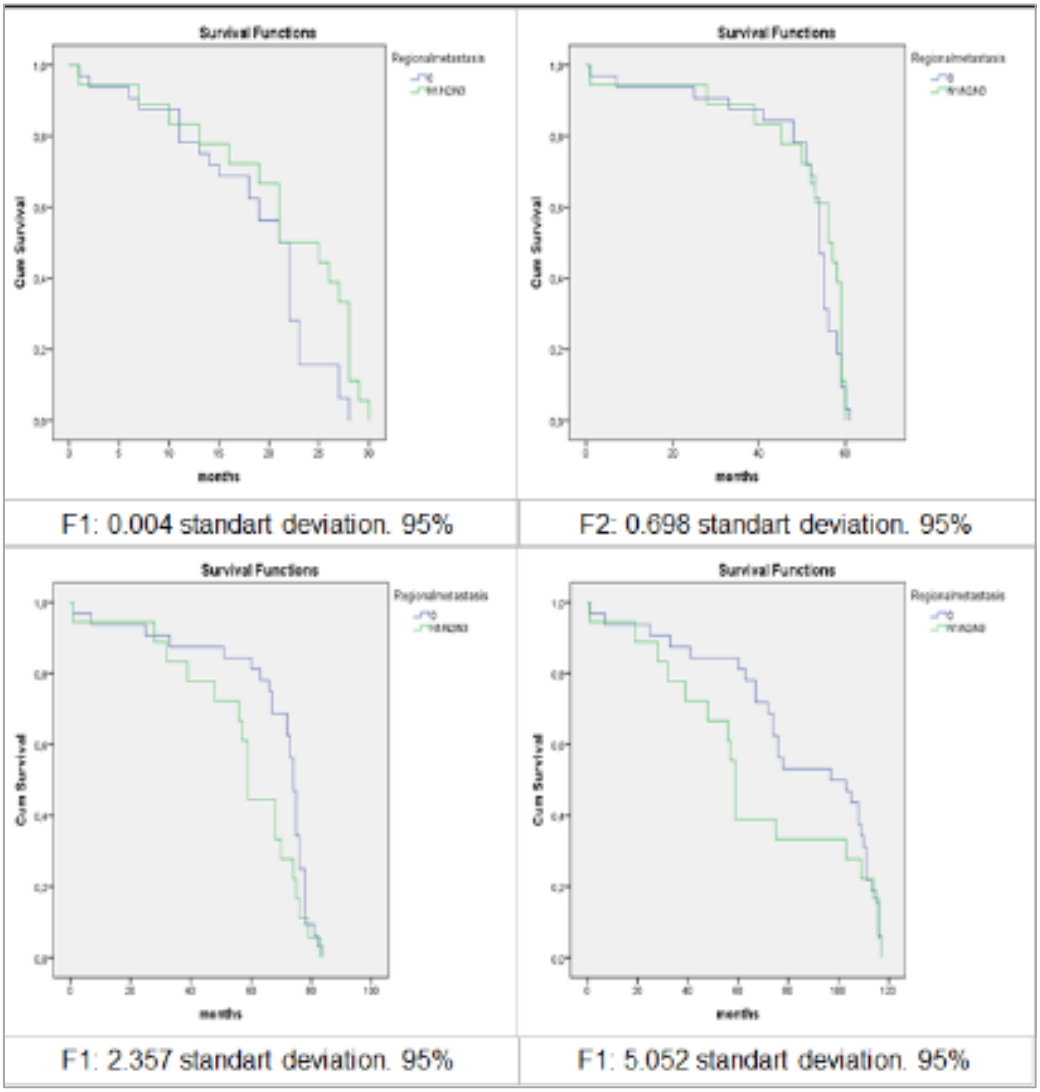
F3: 2,357 standart deviation, 95% confidence



F4: 5,052 standart deviation, 95% confidence

Regional metastases was associated with higher patient survival in the follow-up period of up to 2.5 years (F1, p=0.046). This relationship was not observed at the other follow-up time points (F2, p=0.378; F3, p=0.084; F4, p=0.311) (Figure 5),

Figure 5. Kaplan-Meier survival curve showing the influence of a positive lymph node on the final clinical status of patients after a 10-year follow-up period.



The distribution of the sample according to patient prognosis over the follow-up period, final status, and cause of death is represented on table 2. The percentages of patients with good and poor prognoses were similar, 44% and 42%, respectively. At the end of the study, only 14% of patients were discharged, whereas 44% of patients remained under follow-up due to presence of a potentially

malignant disorder (PMD) at the primary tumor site, treatment for another tumor, and presence of metastases.

Table 2. Classification of patient prognosis over the follow-up period, final status after 10 years of follow-up and cause of death.

		n	%
Evolution	Good	22	44
	Reserved	7	14
	Bad	21	42
Total		50	100
Final status	Hospital discharge	7	14
	Alive without relapse	19	38
	Alive with PMD	2	4
	Alive with metastasis	1	2
	Death	21	42
Total		50	100
Cause of death	Primary tumor	3	14,3
	Relapse	3	14,3
	Relapse and regional metastasis	4	19
	Relapse and distant metastasis	3	14,3
	Relapse, regional and distant Metastasis	1	4,8
	Regional metastasis	4	19
	Regional and distant metastasis	3	14,3
	Total	21	100

An association was observed between cases with good prognosis and treatment with surgery alone (p=0.022), tumor size < 4 cm (p=0.005), absence of regional metastases (p=0.026), and tumors of low degree of malignancy (p=0.016) (Table 3).

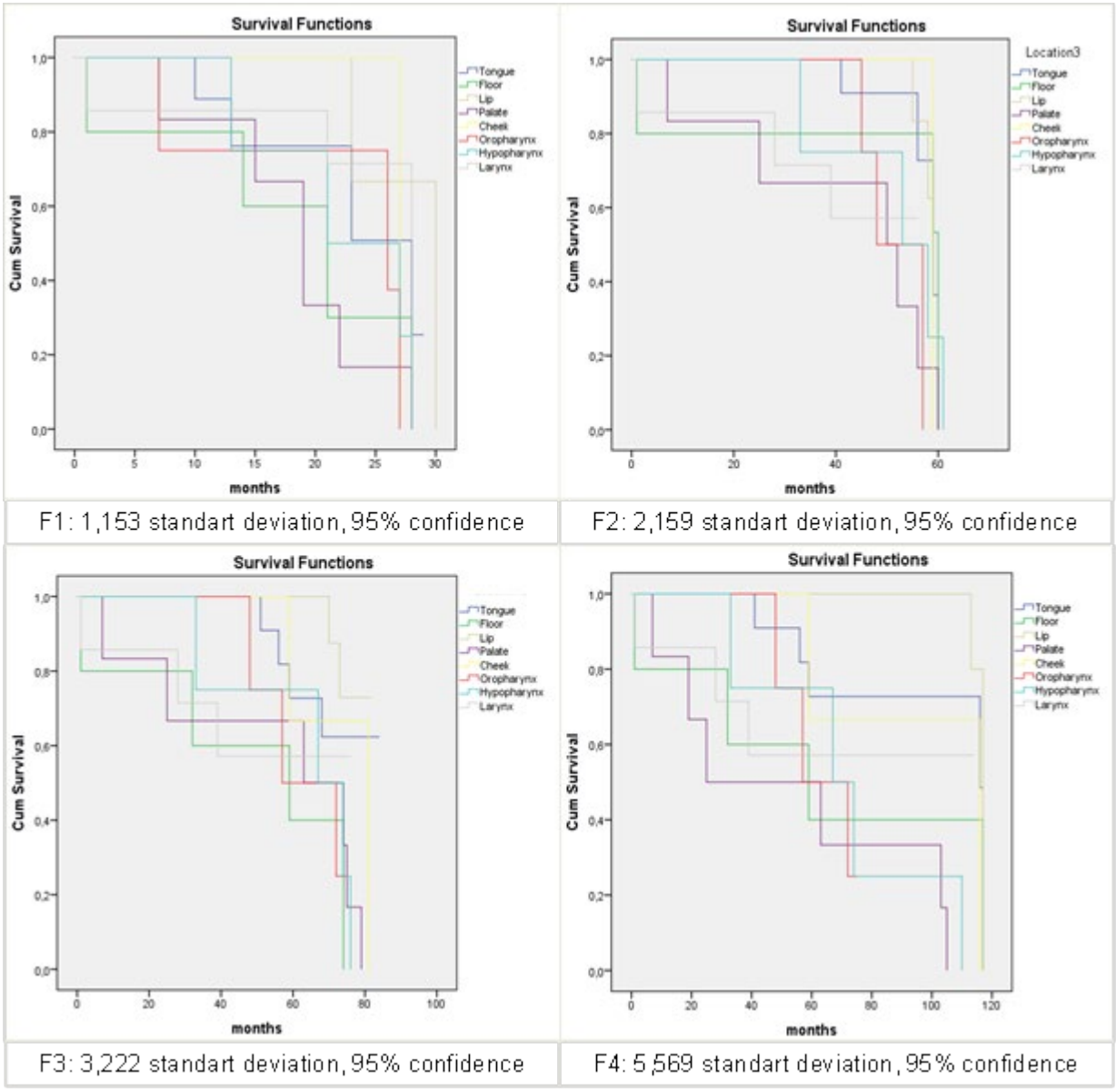
Table 3. Association between patient prognosis over the 10-year follow-up period and clinical parameters, treatment modalities, and tumor histological grading.

Evolução		Good	Reserved	Bad	Total	*p*
Size	T1/T2	18	6	8	32	
	T3/T4	4	1	13	18	
Total		22	7	21	50	0,005
Regional	N0	18	5	9	32	
metastasis	N1/N2/N3	4	2	12	18	
Total		22	7	21	50	0,026
Treatment	Surgery alone	19	6	9	34	
	Other treatment	3	1	12	16	
Total		22	7	21	50	0,022
Histopathological	Good	13	6	5	24	
grading	Moderate	9	1	13	23	
	Poor	0	0	3	3	
Total		22	7	21	50	0,016

Pearson's chi-square test, $p \leq 0,05$.

According to tumor location, the patients had moderate and poor prognoses after 7.5 years of follow-up. The prognosis of patients with tumors located in the hypopharynx, buccal mucosa, palate, and mouth floor worsened after F3 (p=0.045). At F4, the prognosis of patients with lip tumors also worsened (p=0.002). Tumor location had no influence on prognosis up to 7.5 years of follow-up (F1, p= 0.105, and F2, p=0.100)(Figure 6).

Figure 6. Kaplan-Meier survival curve showing the influence of tumor location on patient prognosis in the 10-year follow-up period.



4. Discussion

We present the clinical prognosis of a sample of 50 patients diagnosed with HNSCC in the 10-year follow-up to determine how traditional prognostic factors correlate with the survival of this sample at four distinct time points and contribute to the understanding of a disease with a heterogeneous clinical course.

The mean age of the patients in the sample was slightly lower than the overall mean age for HNSCC [10, 11]. Among the reasons for young patient disease development, the increase of earlier exposed to risk factors such as alcohol [12] stands out. It is also considered that 22% of the patients had tumors of the oropharynx and larynx, which HPV is currently described as a causative factor, also affecting young individuals[10, 13, 14].

The ratio of men to women with HNSCC is 2:1[15], and the mortality rate is higher among men[10]. In the present study, there was a predominance of men in the sample; however, sex did not influence the survival and final status of the patients. Generally, the higher mortality rate among men is explained in the literature by the higher prevalence rate of the disease and higher exposure to tobacco and alcohol among men[7], but there is evidence that differences in genetic and molecular mechanisms between men and women, such as protein regulation and gene mutations, contribute to this disparity[16].

The patients in this study were mostly Caucasian (80%), which is consistent with the sample of other studies. Several hypotheses have been proposed to explain these differences in incidence among ethnic groups, including socioeconomic and environmental factors, HPV infection[17-20], and lower protection at the cellular level from damage caused by UV radiation[21].

Most patients were exposed to tobacco (98%) and alcohol (94%) at the time of primary tumor diagnosis. Smoking and alcohol consumption have been established as causal factors and potentiators of the development of HNSCC[10, 22]. Moreover, prolonged exposure to these factors increases the likelihood of relapse or even development of a second primary tumor[23].

In this study, 56% and 44% of individuals were ex-smokers and former alcohol users, respectively; however, cessation of these habits did not affect the survival of patients, probably because of the cumulative damage caused by these substances[23]. This was observed in patients where moderate and poor prognoses were observed over a long follow-up period, and the prognosis of tumors of the hypopharynx and mouth that were strongly associated with smoking worsened after 7.5 years of follow-up.

An association between good prognosis and treatment with surgery alone, tumor size < 4 cm, absence of regional metastases, and low degree of malignancy was observed, which suggests that patients with less advanced clinical stage of the primary tumor were treated with a single therapeutic modality (surgical removal of the tumor) and did not develop secondary tumors. This finding indicates that better patient prognosis is a consequence of early diagnosis than of the therapeutic modality. Patients with HNSCC in the neck area have been treated exclusively with radiotherapy and have had good results in terms of quality of life because avoiding mutilating surgery is a positive factor for patients[1, 24]. An important aspect of therapy selection is the bias in sample selection, because studies on adjuvant therapies include patients who cannot be treated surgically at advanced stages of the disease, which does not allow a comparison between modalities[24].

The literature discusses the issue of ideal follow-up time because 45% of patients will experience some type of second event after the primary curative treatment. The traditional 5-year follow-up period is based on a consensus on guideline standardization and not on scientific evidence[25, 26]. In line with this, we observed changes in the clinical status of patients up to 9 years and 9 months after the initial treatment.

The data obtained in this study showed a disparity between the percentage of patients diagnosed in the early stages of the disease (T1/T2=64%, N0=64%) and final outcome at 10 years of follow-up; that is, only 14% of patients were discharged. We can considered that, due to the difficulties of the public health system, the long waiting time between diagnosis and the curative treatment wich

contribute to worsening the prognosis. Also it can be inferred that 38% of patients alive and without recurrence who were not discharged developed a second event near the end of or after the 5-year period (considering this period as ideal) that followed the primary treatment, which reinforced the idea that there is no ideal follow-up duration and cases should be analyzed on an individual basis.

In this context, the presence of regional metastases at the time of the initial diagnosis was associated with higher patient survival at 2.5 years of follow-up. This apparent contradiction suggests that a more aggressive therapeutic approach was used for this clinical stage, to avoid the occurrence of second events, and that the development of undetectable regional metastases increased the number of deaths between 2.5 and 5 years.

In the present study, there was a relationship between tumor size < 4 cm and prolonged patient survival in the 5-year follow-up period, which is in line with the literature; however, due to the heterogeneous clinical behavior of patients with HNSCC, the major cause of death was the development of regional metastases in 10 years. No predictive factor for this complication is reported in the literature. Predicting the occurrence of a positive lymph node may be essential in terms of monitoring and survival improvement. This has led to a wide discussion about individualized care based on precision therapy and biomarkers with improved prognostic ability[1, 25].

A follow-up of 2 years after primary curative treatment has been proposed, based on the risk of recurrence of oral SCC[25]; however, in the present study, important changes in the health status of patients were observed after 5 years of follow-up, with a gradual increase in deaths in up to 7.5 years of monitoring and an increase in the number of patients alive without recurrence after this time point, indicating that the disease may be controlled in cases of moderate prognosis. In these cases, the success of the oncological treatment strategy depends on patient age and presence of associated comorbidities[27]. This study included a sample that was relatively young at diagnosis, which may have contributed to the control of the disease (even if prognosis worsening was detected at specific time points).

Many authors have considered the use of a single follow-up routine for the entire head and neck area. However, although there are common etiological factors for the entire anatomical structure, those that differ between sites affect both the course and prognosis of the disease[26]. In line with this, in the present study involving a sample with mouth and neck SCC, patient prognosis changed from good to moderate or poor after 7.5 years of follow-up in cases of hypopharynx, cheek mucosa, palate, mouth floor, and lip tumors.

According to the literature, histological grading has a high prognostic value, with prognosis being worse with degree of tumor undifferentiation[9]. We observed an association between well-differentiated tumors and good prognosis; that is, after 5 years of follow-up the survival rate was higher among patients with tumors with a lower degree of malignancy. However, we did not find an association between undifferentiated tumors and worse prognosis.

Some authors have discussed the development of HNSCC in increasingly younger patients[12, 28], as was observed in the present sample. A longer follow-up period is suggested for young adults because, while the cure rate and, consequently, survival rate of these patients are higher because of their higher tolerance to treatment and their sequelae, the risk of a second event increases as a result of the longer life expectancy after diagnosis[12]. Thus, even if histological grading is relevant in the prediction of a good prognosis in the long term, its predictive ability in patients with a poor histopathological grade still needs improvement. This difficulty was encountered in the present study because only 14% of the patients were discharged after 10 years of follow-up.

The histopathological grading system originally validated for mouth tumors, based on degree of keratinization[9], presents a contradiction as regards oropharyngeal cases because HPV-positive tumors with little or no keratin are not associated with poor prognosis[29], indicating that the heterogeneity of head and neck tumors is a result of other factors associated with this complex. In SCCs of the tongue, this method does not reflect the biological behavior of the disease[30]. Given the evidence of the existence of several etiopathogenic mechanisms, the

subareas of HNSCC involvement should be considered as separate entities rather than collectively[31].

HPV status is an important factor in guiding therapeutic decision-making with respect to patient monitoring and predicting patient survival. Although the literature refers to HNSCC as a single disease, HPV-positive tumors of the oropharynx have distinct microscopic and molecular characteristics and definite clinical course[32]. This study had a bias: HPV infection was not evaluated in the initial data collection because it was only in 2017 that the new TNM classification considering its important role in the staging of patients was published[33].

Although the TNM classification has been improved, paradoxical results were obtained in a retrospective study comparing the current edition with the previous one, in which several patients migrated from less advanced to more advanced stages and had similar or better survival rates. The groups with N stage higher than N1 had improved 5-year survival rates and extranodal extension of the disease as an inconstant indicator of poor prognosis, which suggests that accurate prognostication requires the TNM classification to be improved in the next editions[34]. Although this fact reinforces the idea that the TNM system should be used cautiously for prognosis, it does not exclude its importance in therapeutic decision-making.

Currently, the literature shows that the therapeutic approaches and patient follow-up protocols have been based on the criteria that do not take into account the biology of the tumor[35]. From this perspective, the analysis of the 10-year follow-up showed that traditional prognostic factors are not accurate in detecting subclinical changes or predicting the prognosis of patients, a fact that is even more evident when other clinical-pathological data are added to the traditional prognostic system and the latter fails in predicting survival over periods longer than 8 years[6].

In the last three decades, the panorama of HNSCC etiopathogenesis has changed. Early exposure to risk factors have made the disease more common among young adults[12], and the differences in prognosis between anatomical areas[29, 30] and the advance in knowledge of tumor molecular biology[17] support the

idea that the disease should be considered on an individual basis. Because the prognosis of HNSCC is difficult to predict[3], the knowledge of factors that reflect the biological profile of the disease and changes in its behavior over time is an important tool for improving patient survival.

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Artigo 2

Artigo científico de acordo com as normas do periódico Future Medicinal Chemistry (Qualis Capes 2016 - A2, ISSN 1756-8919, Fator de impacto: 3.617).

The exosomes role in head in neck squamous cell carcinoma: a systematic review

Abstract

Background: traditional methods used to obtain the molecular characteristics of solid tumors like head and neck squamous cell carcinoma do not provide a reliable profile about this disease. Exosomes are found in body fluids carrying different contents, representing the cellular origin of various disorders. Thus the objective of this research was to present a systematic review of literature about the role of exosomes in head and neck squamous cell carcinoma. **Methodology/ Results**: 25 original articles are independently identified by three examiners using several electronic databases. **Conclusion:** exosomes may play a role in promoting tumor growth and metastasis, making them promising to better understand the pathogenesis of head and neck squamous cell carcinoma, being potential targets for diagnosis, treatment and prognosis.

Keywords: extracellular vesicles, cell-derived microparticles, exosome multienzyme ribonuclease complex, head and neck cancer.

Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC), an anatomically heterogeneous group arising most often from the oral cavity (OSCC), oropharynx (OPSCC), hypopharynx (HPSCC), larynx (LSCC) and nasopharynx (NSCC) with different etiological factors [1], is the sixth most frequent malignant tumor in the world accounting for about 6% of all cases [2] and it represents approximately 95% of head and neck cancers [3]. Due to its unpredictable and aggressive behavior,

even with the latest advances in treatment and improvement in the quality of life of patients, the mortality rate has remained high [4]. Currently, incisional biopsy followed by the histopathological analysis of the surgical specimen is used to obtain the molecular profile of solid tumors such as HNSCC. However, such methods present a sampling bias as they only provide an instant snapshot of the disease, without characterizing heterogeneity or the risk of malignancy [5,6].

Exosomes are small extracellular vesicles (EVs) with 30–150 nm in diameter that are secreted by different cell types and originate from multivesicular bodies. Exosomes are founded in body fluids and contain proteins, lipids, metabolites, DNA, microRNA (miRNA) and several species of non-coding RNA, which are able to modulate crucial functions during pathophysiology. These vesicles of endocytic origin are released by multiple cell types under physiological and pathological conditions and can be isolated to detect proteins, nucleic acids and membrane markers that represent the cellular origin of various diseases [7-11].

The study of the exosomes can be useful to identify potential biomarkers and factors involved in tumorigenesis [12]. Tumor progression, resistance to chemotherapy and immunological evasion occur through horizontal transfer of bioactive molecules; studies suggest that exosomes released by neoplastic cells can modify the tumor itself and the tumor microenvironment [13]. The HNSCC exosomes can function as carriers of miRNAs between hypoxic and normoxic tumor cells, remodeling the environment [14] and the association of your molecular profile with disease progression supports the exosome potential as future cancer biomarker. It is possible to simultaneously gain insights into relative levels and functions of immunoregulatory proteins carried by the exosome subsets derived from different immune and non-immune cell populations in patients with cancer [15]. Based on this information, the aim of this study is to present a systematic review of the literature regarding the exosomes role in HNSCC.

Methods

This study follows the criteria recommended by PRISMA (Items of Preferential Reports for Systematic Reviews and Meta-Analyzes) [16] and is included in PROSPERO (International Prospective Record of Systematic Reviews in Progress) with the following record: CRD42018081639.

Main goal

Establish the relationship between exosomes and HNSCC.

Secondary goal

Present the relevance of exosomes and your clinical applicability to knowledge for the HNSCC.

Search strategy and data sources

These search used the electronic databases of the National Library of Medicine of the United States (MEDLINE/PUBMED; www.pubmed.gov), Cochrane Library (www.cochranelibrary.com), Biomedical Research Database of Elsevier (EMBASE; www.elsevier.com/solutions/embase-biomedical-research) and Virtual Health Library (BIREME; bvsalud.org/). Keywords were defined according to the specific descriptors: MEDLINE (MeSH), Cochrane Library (MeSH), Embase (Emtree) and Bireme (DeCS), with combinations of terms listed: exosome [MeSH] OR exosomes [MeSH] OR extracellular vesicle [MeSH] OR extracellular vesicles [MeSH] OR cell-derived microparticle [MeSH] OR cell-derived microparticles [MeSH] OR exosome [Emtree] OR exosome multienzyme ribonuclease complex [Emtree] OR exosome [DeCS] OR exosomes [DeCS] OR secretory vesicle [DeCS] OR secretory vesicles [DeCS] AND head and neck squamous cell carcinoma [MeSH] OR head and neck squamous cell carcinoma [Emtree] OR head and neck cancer [DeCS] OR squamous cell carcinoma [DeCS]. In addition to the electronic search, a manual search of references cited in identified articles was performed.

Inclusion criteria

Completed studies published in English up to March 2019, regarding the relationship of exosomes with HNSCC were considered eligible for inclusion. Non-original articles (reviews, abstracts, editorials, letters, comments, and book chapters were excluded.

Data extraction and analysis

Initially, selection was based on evaluation of titles and abstracts, followed by full-text analysis in cases of uncertainty. Using a standardized instrument, two authors (KBD, ND) independently extracted the following data: first author, year, and clear definition about the aims of the research, study design, main results and limitations, type of carcinoma, source and method of isolation of the exosomes. Discrepancies regarding their inclusion/exclusion were evaluated by a third examiner (MLL). Any articles that met the inclusion criteria and did not meet any of the exclusion criteria were retained. Before the final selection the studies was classified according their possible clinical applicability.

Data synthesis

A qualitative synthesis was conducted since the heterogeneity found across studies no not made possible to realize the meta-analysis.

Results

Trials included

Of the 286 articles identified, 158 were excluded based on the title, leaving 128 abstracts for evaluation. After analysis of the abstracts, 33 articles were retained for full-text analysis, of which 25 met all of the eligibility criteria and were included in the study. A flowchart depicting the selection of articles is shown in Figure 1.

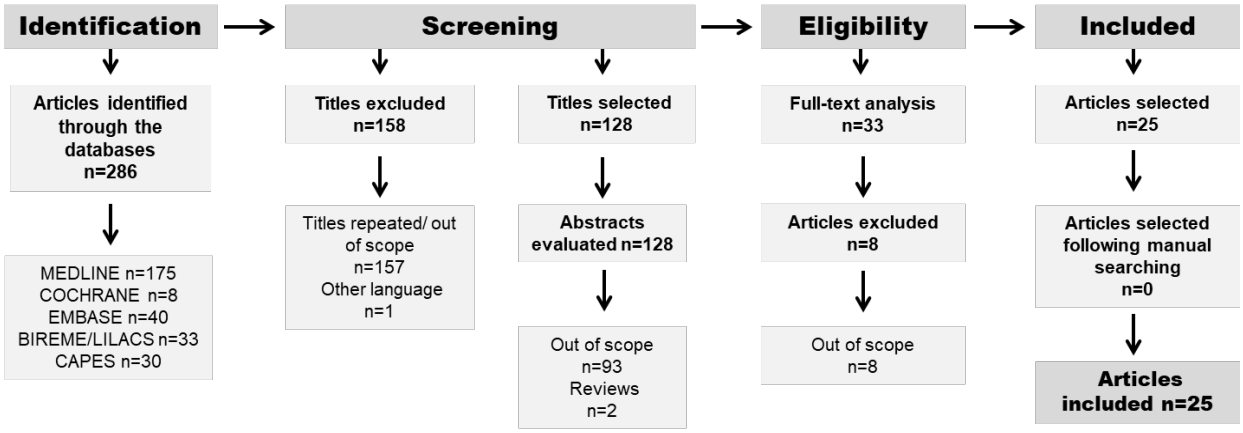

Fig. 1: flowchart of the screening and selection process, based on the PRISMA format.

Qualitative synthesis

There were 25 articles included in this study; 5 experimental studies with in vitro and in vivo data; 8 studies that were exclusively in vitro; 3 cross-sectional studies; 1 cohort study, 7 controlled clinical trials and 1 longitudinal study. The data from each study is presented in table 1.

Table 1: data collected from selected articles.

Author	Design	Cancer anatomical site	Source of exosomes	Isolation and purification
Rodriguez Zorrilla et al., 2019 [19]	Longitudinal	Oral	Plasma	Differential centrifugation
Zlotogorski-Hurvitz et al., 2019 [18]	Controlled clinical trial	Oral	Saliva	Differential centrifugation
Fujiwara et al., 2018 [25]	*In vitro*	Oral	HSC3, HT7	PBP
Ludwig and Whiteside, 2018 [24]	*In vitro, In vivo*	Oral, Head and neck, Murine Head and neck	PCI-13, UM-SCC-47, UM-SCC-90, SCC-7 Plasma	SEC
Peacock et al., 2018 [22]	*In vitro*	Hypopharynx, Oral, Tonsil	SCC2, SCC90, SCC72, SCC89	SEC
Raulf et al., 2018 [37]	Cross-sectional	Oral, Pharynx, Larynx, Hypopharynx	HN5, HSC3 , H357, HN30, UT-SCC-34, UT-SCC-6A, SCC-22B	Ultracentrifugation
Theodoraki et al., 2018 [35]	Controlled clinical trial	Oral, Pharynx, Larynx	Plasma	SEC
Theodoraki et al., 2018 [14]	Controlled clinical trial	Oral Head and neck	PCI13, SCC90 Plasma	SEC
Theodoraki et al., 2018 [26]	Controlled clinical trial	Oral, Pharynx, Larynx	Plasma	SEC
Xiao et al., 2018 [29]	*In vitro*	Oral	HIOEC, Leuk1, SCC25, Cal27	Filtration
Dickman et al., 2017 [30]	*In vitro, In vivo*	Oral	SCC-4, SCC-9, SCC-25, Cal 27, DOK	Ultracentrifugation
Langevin et al., 2017 [20]	*In vitro, In vivo*	Oral, Pharynx, Hypopharynx Oral Oral	H413, Cal 27, Detroit 562 , FaDu Primary culture of oral epithelial cells Saliva	Ultracentrifugation

Mutschelknaus et al., 2017 [15]	*In vitro*	Oral, Hypopharynx	BHY, FaDu	Ultracentrifugation
Overmiller et al., 2017 [21]	*In vitro, In vivo*	Skin, Head and neck	A431, Serum	Ultracentrifugation, PBP
Languino et al., 2016 [31]	*In vitro*	Oral	Primary culture of keratinocytes and fibroblastos	Ultracentrifugation
Li et al., 2016 [13]	Cross-sectional	Oral Oral	SCC-9, Cal-27 Serum	PBP
Mutschelknaus et al., 2016 [27]	*In vitro*	Oral Hypopharynx	BHY FaDu	Ultracentrifugation
Sakha et al., 2016 [33]	*In vitro*	Oral	HOC313-P, HOC313-LM	SEC, Ultracentrifugation, PBP
Sento et al., 2016 [32]	*In vitro, In vivo*	Oral	OSC-3, OSC-4	PBP
Zlotogorski-Hurvitz et al., 2016 [17]	Controlled clinical trial	Oral	Oral fluids	Ultracentrifugation
Jelonek et al., 2015 [28]	*In vitro*	Hypopharynx	FaDu	PBP, Differential centrifugation
Vered et al., 2015 [38]	Cohort	Oral	HSC-3	PBP
Wang et al., 2014 [34]	Controlled clinical trial	Larynx	Serum	PBP
Gourzones et al., 2013 [23]	Controlled clinical trial	Nasopharynx	Plasma	Density gradient centrifugation
Kim et al., 2005 [36]	Cross-sectional	Oral, Lymphoid cells Oral	PCI-13, Jurkat Serum	SEC, Ultracentrifugation

There were observed three possible clinical applications for exosomes in head and squamous cell carcinoma: diagnosis, therapeutic target and prognostic marker. Selected studies are presented in table 2.

Diagnosis

Exosomes derived from oral fluids of patients with OSCC differ morphologically and molecularly from the ones obtained from healthy individuals [17]. Salivary exosomes, providing a detecting subtle change in the conformations of proteins, lipids and nucleic acids, can do early detection of in high-risk patients with no evidence of clinical signs of oral lesions [17,18]. In patients with OSCC, the level of CD63 positive plasmatic exosomes decrease immediately after surgery procedure, confirming that the tumor releases these EVs [19]. Numerous miRNA secreted by cell cultures of HNSCC were detected at high levels in saliva of HNSCC patients, compared to the healthy individuals. The RNA splicing miR-486-5p was able to identify an early HNSCC stage case (stage I), being an interesting tool for early detection [20].

Exosomes isolated from HNSCC patients' sera were enriched in Desmoglein 2 (Dsg2) C-terminal fragment and EGFR compared to that observed in healthy volunteer defining a mechanism by which Dsg2 expression in cancer cells can modulate the tumor microenvironment, a step critical for tumor progression and tumorigenesis [21].

HPV (-) cells produced more exosomes than HPV (+) cells. The miRNA signature in these EVs is indicative of the HPV status of the parent cell. This may provide a platform from which to validate salivary or blood-based biomarkers with utility for early detection and stratifying risk in OPSCC patients [22].

The miR-BART17 was significantly more abundant in plasma samples from NPC patients compared to non-NPC donors. Above a threshold of 506 copies/mL, detection of miR-BART17 was highly specific for NPC patients [23].

Therapeutic target

Tumor-derived exosomes can induce angiogenesis in vitro and in vivo through functional reprogramming and phenotypic modulation of endothelial cells. They can carried angiogenic proteins promoting angiogenesis and driving HNSCC progression [24].

Exosomes derived from OSCC patients can transform epithelial cells into a mesenchymal phenotype, being a mode of carcinogenesis and tumor progression; Anti-EGFR antibody cetuximab inhibits internalization of the OSCC-EGRF exosomes into epithelial cells. Cetuximab captured OSCC-EGRF exosomes and blocked internalization of this exosomes into epithelial cells, inhibiting the epithelial-mesenchymal transition (EMT) initiation effect in epithelial normal cells [25].

Tumor conversion from the mesenchymal to epithelial phenotype umpired from photo dynamic therapy (PDT) is mediated by exosomes. These EVs in plasma of HNSCC patients undergoing PDT on day 7 or 4-6 weeks after PDT carried E-cadherin, restored epithelial morphology and epithelial cell adhesion molecule (EpCAM) expression in tumor cells, down-regulated expression of mesenchymal genes and inhibited proliferation, migration and invasion [14].

CD3 (+) T cell-derived exosomes and CD3 (–) tumor derived exosomes obtained from the plasma of the patients with HNSCC represents the functional potential of their parent cells. CD3 (–) exosome fraction is enriched significantly in HNSCC patients with advanced stages (III/IV). T cell-derived CD3 (+) exosomes, in patients with early stages (I/II) HNSCC, carry a significantly higher levels of ADA/CD26. These exosomes have a potential to serve as biomarkers of tumor activities and also of patient immune competence/suppression [26].

Exosomes transmit pro tumor survival effects by promoting the proliferation and radioresistance of HNSCC cells. They can mediate cell-to-cell communication acting as potential drivers of metastatic HNSCC progression during tumor

radiation. Proteomic data suggest a subset of radiation-regulated exosomal proteins as candidates to induce the pro-migratory effects [15,27]. Among the proteins specifically over-represented in exosomes of irradiated HNSCC cells, were those involved in transcription, translation, division and cell signaling. The exposure to ionizing radiation resulted in important changes in exosomal cargo. It was 148 “common” proteins before and after exposure, 236 proteins detected specifically after irradiation and 69 proteins not detected after irradiation. This indicates that the exosomal content may reflect changes induced by radiation in cellular processes such as transient suppression of transcription/translation or stress-induced signaling [28].

The point adjustment of tumor-associated macrophages type 1 (M1-like) polarized by tumor exosomes shows great potential as therapeutic target for control of cancerous cell migration in OSCC. Macrophages were activated by taking up exosomes released from OSCC cells through at the early phase. The conditioned media from exosomes induced M1-like tumor-associated macrophages and significantly promoted migration of OSCC cells. It is suggested a cycle between cancer cells and macrophages when OSCC exosomes activate factors derived from macrophages and these activate migration of the tumor cells [29].

Exosomes can selectively retain miRNAs in vitro and that miR-142-3p overexpression is associated with carcinogenesis decrease by the TGFβR1 reducing [30]. Exosomes obtained from the tumor microenvironment can enhance TGFβ1 signaling in tumor cells and induces reactivation of TGFβ signaling in OSCC tumors that no longer respond to the TGFβ ligand, maintaining the carcinogenesis activated [31]. OSCC cells can promote tumor progression through the secretion and absorption of their own exosomes. The use of heparin can inhibit the uptake of exosomes by OSCC cells and constrain the tumor progression, being useful for the disease treatment [32].

Prognostic marker

The detection of miRNA signatures present in circulating exosomes in biological fluids may be an adequate method for the surveillance of patients with OPSCC who have metastatic recurrence [22]. There is evidence that exosomes can be remodeled to promote tumor migration and invasion under hypoxia. The hypoxia increases miR-21 levels in tumor-derived exosomes and these would be retained by normoxic cells causing these cells to acquire a pro-metastatic phenotype. Results suggest that exosomes derived from circulating cells containing miR-21 were associated with tumor size (T) and regional metastasis [13].

Overexpression of miR-342-3p and miR-1246 in tumor-derived exosomes is associated with malignancy. The Domain containing 2D gene (DNN D2D) is regulated by miR-1246 and, following its suppression, migration and invasion of tumor cells is promoted [33]. Expression of exosomal miR-21 and HOX Transcript Antisense RNA (HOTAIR) was significantly higher in patients with LSCC than with vocal cord polyps and correlated with clinical parameters like lymph node metastasis [34].

Exosomes of OSCC patients with late stages of disease had higher levels of programmed death – ligand 1 (PD-L1) and COX-2 than early stages patients [35]. It is suggested that exosomes containing Fast Ligant (FasL) in patients with OSCC have a prognostic value. They can induce death of T cells, providing compelling evidence for the study of the tumor-based immune system [36].

Annexin 1 (ANXA1) was downregulated in HNSCC tissues in mRNA and protein level. Its anti-proliferative effects were mediated through the intracellular form of the protein acting as a tumor suppressor [37]. In vitro, Caveolin 1(CAV1) can promote the epithelial-mesenchymal transition of tumor cells, as well as the differentiation of normal fibroblasts into cancer-associated fibroblasts. It is suggested that CAV1 derives from exosomes released by tumor cells because your expression in OSCC is higher in the tumor microenvironment compared to

neoplastic cells and these fact suggest a negative impact on recurrence and survival of patients [38]. Patients with lower exosome CD63 (+) levels after surgery lived a higher average of months then those with higher plasmatic levels of exosome CD63 (+). When exosome CAV1 (+) levels after surgery were lower, the average overall survival was better, in contrast when the exosome CAV1 (+) levels were higher [19].

Table 2: exosomes and your possible clinical applications according collected data.

Diagnosis	Therapeutic target	Prognostic marker
Rodriguez Zorrilla et al., 2019 [19]	Fujiwara et al., 2018 [25]	Rodriguez Zorrilla et al., 2019 [19]
Zlotogorski-Hurvitz et al., 2019 [18]	Ludwig and Whiteside, 2018 [24]	Raulf et al., 2018 [37]
Peacock et al., 2018 [22]	Theodoraki et al., 2018 [14]	Peacock et al., 2018 [22]
Langevin et al., 2017 [20]	Theodoraki et al., 2018 [26]	Theodoraki et al., 2018 [35]
Overmiller et al., 2017 [21]	Xiao et al., 2018 [29]	Xiao et al., 2018 [29]
Zlotogorski-Hurvitz et al., 2016 [17]	Dickman et al., 2017 [30]	Dickman et al., 2017 [30]
Gourzones et al., 2013 [23]	Mutschelknaus et al., 2017 [15]	Li et al., 2016 [13]
	Languino et al., 2016 [31]	Sakha et al., 2016 [33]
	Mutschelknaus et al., 2016 [27]	Vered et al., 2015 [38]
	Sento et al., 2016 [32]	Wang et al., 2014 [34]
	Jelonek et al., 2015 [28]	Kim et al., 2005 [36]

The included studies may present more than one proposal for exosomes utility.

Discussion

Although the aim of this systematic review was to clarify the relationship between exosomes and HNSCC and we select a relative high number of studies, the evidence presented is promising but limited, since only 10 studies analyzed patient´s exosomes, some present limited sample [13,14,17-21,23,24,26,34-36] and others do not present in vivo analyses [15,22,25,27,29,33]. The heterogeneity of studies designs also does not allow for meta-analysis. The seven controlled clinical trials presented different purposes to be compared.

Another limitation is the absence of control groups [30-32,38]. Li et al. (2016) and Zlotogorski-Hurvitz et al. (2016) evaluated the presence of exosomes in patients without the disease but did not thoroughly analyze exosomal content. The Theodoraki et al., (2018) studies [14,26,35] appear to be more complete in relation to this issue.

Other studies do not appear to have representative samples, such as exosomes characterized in size [25] as described in the literature [7] or limited volume of sample collected from patients, causing a bias in the purification of them [18]. The cell lines used were also not fully representative for the stated objective [14,15,24,27,28,37] with many studies used oral cancer cell lines like HNSCC.

The HNSCC affects several anatomical sites, with different clinical courses. Certain sites have a predilection for early metastasis and treatment planning depends almost entirely on the anatomical staging of the disease at diagnosis, like OSCC [1]. Although HNSCC are part of an anatomical group and share some etiological factors, each entity itself has its peculiarities. Studying tumors of the head and neck as unic disease doesn´t allow extrapolating data.

There identified the nature and extent of genomic alterations specific to the OSCC subset within the wider anatomical set of HNSCC. This different genes and mutationally enriched pathways in relation other head and neck neoplams [39] may provide a divergent role about exosomes action, just like this present study.

Several estimates suggest that 70–90% of new OPSCC have evidence of HPV. Overall 2-year and 5-year survival for HPV (+) patients is significantly greater than for negative, likely due to HPV(+) cancers being more responsive to treatment [40]. Peacock B et al. (2018) [22] were observed that HPV (-) cells produced more EVs than HPV (+) cells and there is a difference in miRNA content according to HPV status of the cell line. Clearly we have a different course of the disease, suggesting diverse way of pathogenesis when HPV (-) cancers seems to be more aggressive and it`s may be related to the exosomal behavior. Recently, a new TNM edition was published, considering a different staging for HPV positive patients, confirming this condition as influential in HNSCC comportment [41].

One remarkable particularity of the NPSCC is its constant association with the EBV. The EBV genome is present in the nuclei of all malignant cells which are in their overwhelming majority latently infected. Despite the absence of viral replication, several viral genes are active in latently infected cells, driving production of a few viral proteins and noncoding RNAs, including viral miRNAs [23]. Exosomes are recently discovered to be involved in the transfer of viral miRNAs as part of a mechanism through which cancer and virus infected cells can manipulate the tumor microenvironment and influence tumor growth and spread [42].

There are many methods to purify exosomal material. Our studies demonstrated several methods like different types of centrifugation [15,17-20,23,27,30,31,37], size and mini-size-exclusion chromatography (SEC)[14,22,24,26,35] and Polymer-based precipitation (PBP) [13,25,32,34,38]. Some articles related the combination of one or more method [21,28,33,36].

The most critical problem in characterization of the content of exosomes is purity and homogeneity of preparation for further proteomics or genomics analyses. Jelonek K et al. (2015) [28] comparing ultracentrifugation with PBP concluded that the PBP is relatively simple and quick but was observed lower purity of the material. Fujiwara el at, (2018) [25] used the PBP method and the exosomes extracted were bigger than the literature preconceived. In fact this study

presents EVs and in this content exosomes.

The method of exosome purification based on differentiating centrifugation provided an exosomal preparation consisted of microvesicles with the average diameter of 70–80 nm, which was verified by the transmission electron microscopy (TEM) ultrastructure analysis [28] and is compatible with the described size of the exosomes in literature [7,10,11]. However, centrifugation-based methods can isolate non-exosomal materials, cause damage to the vesicle membrane structure and generate non-standard parameters, leading to qualitative and quantitative variability that can produce misleading results [43]. SEC appears to be a better alternative for exosome isolation, since there is no loss of exosomes or no damage to the vesicle structure. Most studies support the finding that immunoaffinity capture can isolate both total exosomes and pathology-specific exosomes, while maintaining vesicle integrity and cargo [44]. However actually standard analytic and isolation techniques haven't been achieved and the exosomes characterization and subtype couldn't be established [45].

Importantly, Zlotogorski-Hurvitz et al. (2016) observed morphological and molecular differences between exosomes derived from healthy patients and those from patients with OSCC [17]. We suggest that this difference may relate to specific exosomal content. The specific spectral signature for OSCC exosomes based on detecting subtle changes in the conformations of proteins, lipids and nucleic acids represent a non-invasive method that should be further investigated [17,18]. The most frequently reported exosomal component across all studies was miRNA; other studies reported the presence of the TGFβ receptor and its variants, EGFR, cadherins and proteins such as CAV 1, c-Src, THBS1, ANXA1, Dsg and FasL. Although there is no consensus on exosomes content, EVs carry a large and variable load [7-10] and due to this variability, diverse roles have been suggested for exosomes in tumor pathogenesis.

Malignant tissues exhibit distinct miRNA signatures compared to normal tissue and miRNA signatures are more specific to cancer-type than mRNA profiles. Overexpression of oncogenic miRNAs and loss of tumor suppressor miRNAs have

been associated with tumorigenesis, progression and metastasis in several cancers [46].

Regarding exosomal miRNA, there is a consensus that miR-21 and miR-181 [47-49] among other species are associated with OSCC since it was shown that they interfere with the repression of translation and gene pairing [50].

Studies of exosomes and their contents are still incipient and the data presented in this review highlights some controversies. Li et al. (2016) [13] suggested that exosomes containing miR-21 cause cells to acquire pro-metastatic phenotypes. Dickman et al. (2017) [30] demonstrated that overexpression of miR-142-3p leads to a decrease in TGFβ1 and thus to decreased growth of neoplastic cells, whereas exosomes that retain and release miR-142-3p from tumor cells lead to increased TGFβ1 signaling, causing growth of the tumor and the associated stroma [51]. These processes may be considered ambiguous, however, structures such as miRNAs can have hundreds of different targets, presenting function in almost all cellular pathways, which critically impacts various biological and metabolic processes such as proliferation and apoptosis, [48] making it difficult to understand their role in tumor evolution and metastasis.

FasL is a type-II transmembrane protein that induces apoptosis and plays an important role in the regulation of the immune system and the progression of cancer. Its presence in exosomes seems to interfere with the host immune response, specifically T cells, leading to spontaneous apoptosis and abnormal signaling, including deregulation of T-cell receptors [52]. Kim et al. (2005) also suggest that there is a significant association between the presence of FasL in exosomes in the serum and the size of the tumor at the time of diagnosis, suggesting it has a role in the immune system during tumorigenesis.

Zlotogorski-Hurvitz et al. (2016) sought to identify biomarkers for the early detection of OSCC. They observed that exosomes derived from oral fluids of patients with OSCC have a higher concentration and larger size of nanoparticles compared to patients without the disease [17]. Rodriguez Zorrilla et al. (2019)

found out that the plasma exosomes levels decrease after surgery procedure in OSCC patients confirming the fact that this structures are released by the tumor in blood, being useful to identification the disease. With the same objective, Langevin et al. (2017) suggested that the detection of an specific exosomal content (miR-486-5p) correlated within early stage case (stage I) of OSCC, which makes the exosomes an interesting tool for early diagnostic.

There are more evidences that suggest that exosomes can induce EMT [38] and influence the immune system [20,36]. While the exosomes transform epithelial cells into a mesenchymal phenotype, the anti-EGFR therapeutic antibody cetuximab is able to inhibit the internalization of these exosomes in normal epithelial cells and thereby the EMT, making the study of the exosomes a new model of understanding the carcinogenesis and tumor progression [25]. Jelonek et al. (2015) [28] also suggested that the presence of transcription/translation-related factors in cargo of exosomes secreted from irradiated HPSCC cells could reflect dynamic adaptation of cells to stress-induced conditions, fact that maybe can explain the resistance in some patients about certain types of treatment.

Proteomic data suggest a subset of proteins as candidates to induce the pro-migratory effects, representing an attractive target to improve radiation therapy strategies [15,27]. Another option in treatment is the use of heparin to inhibit the uptake of OSCC-derived exosomes by OSCC cells; it was observed that multiple administrations of heparin inhibit proliferation, migration and invasion [32]. Tumor-derived exosomes can carried angiogenic proteins promoting angiogenesis and driving HNSCC progression. Eliminate or silencing this vesicles can improve existing anti-angiogenic therapies [24].

While CAV1 is a protein responsible for homeostasis of normal and pathological tissues, may acts in a tumor-dependent manner. CAV1 can positively regulates hypoxia inducible factor (HIF-α) in some malignancies, increasing its oncogenic potential and increasing proliferation, migration and invasion capacities cumulating both number of EVs and the EV protein content [19,26,53,54].

The presence of CAV1 in the tumor microenvironment has negative prognostic impact [38], confirming that this protein promotes oncogenesis. In vitro evidence suggests that CAV1 is derived from exosomes released by tumor cells and can promote EMT and the differentiation of normal fibroblasts into cancer associated fibroblasts (CAFs) [38]. As such, it is a potential prognostic marker of OSCC. Through stimulation of CAFs, proliferation rates and migration profiles may increase in neoplasm and would cause reactivation of TGFβ signaling in the tumor microenvironment of OSCC patients. There are suggestions that exosomal exchange with the tumor microenvironment induces reactivation of TGFβ signaling [13,31].

The CD63 (+) exosome levels decrease significantly after tumor resection indicating that the tumor release those Evs, turning the identification of these exosomes as a future diagnostic tool. Patients with lower plasmatic levels of CD63 (+) and CAV1 (+) exosomes before and after surgery showed a longer overall survival reinforcing the use of these vesicles for prognosis. CAV1 has been shown to be directly related to the disease progression increasing proliferative, migratory and invasive capacities [19].

Alterations in Dsg2 secretion during malignant transformation, when SCC EVs were enriched with the C-terminal fragment of Dsg2, release a mitogenic content including epidermal growth factor receptor and c-Src (proto-oncogene), regulating the exosomal biogenesis. In SCC cells, Dsg2 down-regulated CAV1 levels, suggesting that Dsg2 may enhance endocytosis of CAV1 representing a type of paracrine cycle between Dsg2 e CAV1. With this cycle we may define how the tumor microenvironment can be modulated and this information would be useful to understand the SCC tumorigenesis for diagnoses and prognostication [21]. Although it is possible that the CAV1 present in tumor microenvironment may be absorbed by cells and provoke EMT through Dsg2, these results are controversial; CAV 1 have a negative prognostic value in OSCC patients and according Fang et al. (2014) data, Dsg2 cannot be used to clinical outcome of

esophageal SCC patients [55] and these diseases have very similar risk factors and pathogenesis.

ANXA1, target of the oncogenic miR-196a, was found to be downregulated in HNSCC acting as a tumor suppressor [37]. ANXA 1 is higher expressed in well-differentiated HNSCCs in keratinization areas while undifferentiated tumors present poorly expression. This type of regulation happens at the transcriptional level and there is an inverse correlation between miR-196a and ANXA 1 [56]. The modulation of ANXA 1 could affect the exosome production and influence your release, being an important prognostic biomarker [37].

Conclusion

The study about exosomes in HNSCC is promisor but still incipient; even if the selected studies analyzed the relationship between exosomes and HNSCC in a variety of several ways, the methods of isolation and purification must be standardized.

Studies of exosomes for diagnostic purposes tends to takes into account their morphology, quantity and content. Others that discuss their role in cell-cell communication, promotion, growth and metastasis, tumor microenvironment, immunological response and in different pathways involved in carcinogenesis may serve to therapeutic targets and prognostic markers.

Future perspective

Understanding the role of exosomes in all processes involving head and neck squamous cell carcinoma will assist in the development of new diagnostic strategies, definition of prognosis and treatment of this pathology in order to increase the survival of patients.

Executive summary

Head and neck squamous cell carcinoma

- Head and neck squamous cell carcinoma can affect several anatomical sites.
- The anatomical difference between sites may result in several clinical courses.
- Incisional biopsy followed by the histopathological analysis of the surgical specimen can fail in characterize the molecular profile of solid tumors like head and neck squamous cell carcinoma.

Exosomes

- Exosomes are small extracellular vesicles secreted by different cell types.
- Exosomes are founded in body fluids.
- These vesicles are released by multiple cell types under physiological and pathological conditions.
- They can be isolated to identify the cellular origin of various diseases.
- The most critical problem in characterization of the content of exosomes is purity and homogeneity of preparation for further proteomics or genomics analyses.
- Isolation and purification of exosomes is a complex method and the literature doesn´t provide a golden standard technic.
- The most frequently reported exosomal component is miRNA because these structures can have hundreds of different targets, presenting function in almost all cellular pathways.

Exosomes role in head and neck squamous cell carcinoma

- In future, the analysis of exosomes morphology, quantity and content may be a diagnostic tool.

- Exosomes play a role in different pathways involved in carcinogenesis being useful like therapeutic targets and prognostic markers.

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Conflict of interest

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Artigo 3

Artigo científico nas normas do periódico Oral Diseases (Qualis Capes 2016 – A1, ISSN 1601-0825, Fator de impacto: 2,613).

Vesículas extracelulares e carcinoma espinocelular bucal: uma abordagem *in vitro* sobre migração celular

RESUMO

Objetivo: Recidiva e metástase são as principais causas de baixa sobrevida dos pacientes acometidos pelo carcinoma espinocelular bucal (CECB). O processo metastático é regulado por diversas alterações moleculares que alteram o perfil migratório das células tumorais e do seu microambiente. Sugere-se que as vesículas extracelulares (VEs) sejam responsáveis pelo transporte de moléculas bioativas capazes de influenciar o comportamento da célula receptora. Neste estudo objetivamos avaliar *in vitro* a influência das VEs de CECB sobre a migração celular. **Materiais e métodos:** cultivo celular de linhagens de queratinócitos (HaCat), CECB (Cal27, SCC25) e fibroblastos primários. Caracterização das VEs coletadas das linhagens de Hacat e CECB por análise de nanopartículas e ensaio de migração celular com Hacat e fibroblastos expostos à estas vesículas. **Resultados:** as VEs de CECB apresentaram maior variabilidade de tamanho e concentração em relação às VEs de Hacat. Houve um aumento significativo da velocidade de migração celular das células expostas as VEs de CECB. Os queratinócitos migraram em velocidades maiores quando expostos as VEs de SCC25 do que de Cal27. **Conclusão:** sugere-se que as VEs de CECB

podem induzir um comportamento pró-metastático nas células epiteliais e no microambiente tumoral.

Palavras chave: câncer bucal, metástase, micropartículas derivadas de células.

1. INTRODUÇÃO

O carcinoma espinocelular compreende 90% de todas as neoplasias malignas bucais, sendo as recidivas e metástases regionais os principais fatores responsáveis pela sobrevida limitada dos pacientes (Tanaka & Ishigamori, 2011). Ainda que a capacidade de detecção desta doença tenha sido aprimorada, sendo o diagnóstico precoce imprescindível para o seu manejo, a contenção do processo de disseminação tem sido o atual desafio terapêutico, onde pacientes com doença recorrente e/ou metastática têm uma sobrevida média de apenas 10 meses (Saloura et al., 2019).

O processo metastático é regulado por alterações moleculares que modificam a adesão e mobilidade celular, permitindo o seu deslocamento a partir do sítio primário. Este processo, caracterizado como transição epitélio-mesenquimal permite, através da reprogramação celular, a saída da célula do tumor primário e orquestra os primeiros passos para a disseminação tumoral (Paolillo & Schinelli, 2019).

O preparo de um novo microambiente para o estabelecimento das células malignas é suportado pela reprogramação das células do estroma receptor através de evasão e supressão imunológica, angiogênese, fibroblastos associados ao câncer e macrófagos tumorais, permitindo a progressão do tumor pela

formação do chamado nicho pré-metastático (Paolillo & Schinelli, 2019, Zhao et al., 2018). Dentre as células do estroma que povoam o microambiente tumoral, os fibroblastos são os mais abundantes e estão criticamente envolvidos na progressão da doença (Chen & Song, 2019).

Células de diferentes subtipos de tumor com potencial metastático podem influenciar outros tipos celulares por meio de vesículas extracelulares (VEs). Estas estruturas carregam conteúdos de origem específica e proporcionam troca de informações e modulação epigenética, alterando o perfil das células receptoras e participando das etapas críticas de evolução da doença (de Andrade et al., 2018, D'Souza-Schorey & Schorey, 2018) tornando o estudo das VEs na progressão do carcinoma espinocelular bucal (CECB) interessante para o desenvolvimento de novos biomarcadores e alvos terapêuticos, especialmente na predição e prevenção do desenvolvimento metastático. Neste estudo objetivamos avaliar *in vitro* a influência das VEs de CECB sobre a migração celular.

2. MATERIAIS E MÉTODOS

2.1 Meio livre de vesículas extracelulares (MLV)

Um meio de cultura livre de VEs foi preparado para incubação das células e para controle do ensaio de migração celular: Dulbecco's Modified Eagle Medium (Gibco), suplementado com 10% de soro fetal bovino (Gibco) e 1% de antibiótico penicilina estreptomicina (Gibco) foi centrifugado a média força-g, 100.000 x g por 18 horas e filtrada em 0,22μm.

2.2 Cultivo celular

As linhagens de CECB foram obtidas na ATCC (Manassas, EUA). Células HaCat e Cal27 (ATCC® CRL-2095 ™) foram cultivadas em DMEM suplementado com 10% de soro fetal bovino (FBS), 1% de antibiótico penicilina estreptomicina (P/S) e 1% de glicose. A linhagem SCC25 (ATCC® CRL-1628 ™) em DMEM / F12 com HEPES 15 mM e piruvato de sódio 0,5 mM suplementado com FBS 10%, hidrocortisona (400 mg / mL) e 1% de antibiótico penicilina estreptomicina. Fibroblastos primários (approved by the Ethical Committee of UFRGS CAE#59124916.6.0000.5327) foram cultivados em DMEM com baixa glicose suplementado com FBS 10% e P/S 1 %. Todas as linhagens foram mantidas em incubadora à 37ºC e com 5% de CO2. Os respectivos meios foram trocados por MLVE e as células incubadas por 24 horas. Após este período o meio foi coletado e processado para separação das VEs.

2.3 Separação das VEs

2.3.1 Ultracentrifugação (UC)

Os meios retirados de cada cultivo celular foram ultracentrifugados a máxima força-g em duas etapas: 17.000 x g, 20 minutos e 100.000 x g, 120 minutos. Na primeira etapa o sobrenadante foi transferido de tubo e o *pellet* formado descartado. Na segunda etapa o sobrenadante foi descartado e o *pellet* diluído em 1 ml de PBS.

2.3.2 Cromatografia por exclusão de tamanho (SEC)

Para cada linhagem celular, foi preparada uma coluna Telos SPE (Kinesis, Cambridgeshire, UK) repleta de Sepharose CL-2B (GE Healthcare, Uppsala, Suécia) até um volume final de 10 mL, equilibrada com PBS. Amostras de 1ml provenientes da UC foram aplicadas às colunas e eluídas em PBS, totalizando a coleta de 30 frações. As frações de 7 a 10 foram armazenadas e chamadas de meio concentrado de VEs (MCV)(Karimi et al., 2018).

2.4 Análise de rastreamento de nanopartículas (NTA)

2.4.1 Tamanho das vesículas extracelulares

As VEs derivadas das linhagens celulares foram caracterizadas utilizando o espalhamento dinâmico do sistema Malvern Zetasizer ZS90. Após a diluição em PBS (1:25), as amostras foram introduzidas na câmara de laser com fluxo controlado e constante, em temperatura ambiente. A captura de vídeo e dados foi realizada em triplicatas com o software Malvern Zetasizer v7.13 PSS0012 – 39 EM JP e o tamanho das partículas individuais foi determinado pela velocidade de movimento browniano. Partículas com dimensões na faixa de 100 a 1000 nm foram caracterizados como VEs (de Andrade et al., 2018).

2.4.2 Concentração das vesículas extracelulares

As amostras foram diluídas em PBS 1:50. As concentrações de medição ideais foram definidas de acordo com estudo anterior (Bachurski et al., 2019). Para cada medição, três ciclos foram realizados examinando 11 posições de células cada um e capturando 60 quadros por posição nas seguintes configurações: foco automático; sensibilidade da câmera para todas as amostras = 92; obturador = 70;

intensidade de dispersão = 4; temperatura da célula = 20° C. Depois da captura, os vídeos foram analisados pelo Software ZetaView 8.02.30.02 com parâmetros de análise específicos: tamanho máximo de partícula = 1000 nm, tamanho mínimo da partícula = 5 nm e brilho mínimo de partícula = 25. Hardware: laser incorporado = de 40 mW a 488 nm e câmera CMOS. O número de trilhas concluídas em medições NTA sempre foi maior do que o mínimo proposto de 1000, a fim de minimizar a distorção de dados com base em partículas grandes únicas (Bachurski et al., 2019).

2.5 Ensaio de migração celular

Para o ensaio de migração, as células foram suspensas em meio sem soro CCM1 (Thermo) e plaqueadas em placas de 6 poços (1 x 105) em condições de promoção de migração (Ramos Gde et al., 2016). Após 1 hora do plaqueamento, as linhagens de queratinócitos (HaCat) e fibroblastos primários foram incubadas com meio concentrado em vesículas (MCV) proveniente de Cal27 e SCC25, tendo como controle o MLV. O MCV foi normalizado em $1x10^8$ VEs provenientes das duas linhagens celulares. As células foram transferidas para um microscópio Zeiss Axion Vision Z1 equipado com um controlador de temperatura (37 ºC). A aquisição das imagens foi realizada como descrito na literatura (Lamers et al., 2011) em intervalos de 10 minutos por 20 horas. O núcleo de cada célula migratória foi marcado utilizando a função "*manual tracking*" do *Image J*. A velocidade da migração foi calculada com a razão entre a distância total percorrida (distância) pelo tempo de migração. Para direcionalidade, as coordenadas X e Y da célula migratória foram transferidas para o gráfico polar.

2.6 Análise estatística

A análise estatística foi realizada com o Software Statistical Package for the Social Sciences 21 (SPSS Inc, Chicago, IL, EUA). Diferenças estatisticamente significantes foram consideradas quando $p \leq 0,05$.

3. RESULTADOS

3.1 Caracterização das VEs

O NTA sugere que as linhagens celulares de CECB apresentam heterogeneidade em relação à HaCat**.** A distribuição de tamanho das VEs e concentração média de partículas sugere maior variabilidade de tamanho e concentração de VEs provenientes das linhagens tumorais (Figura 1).

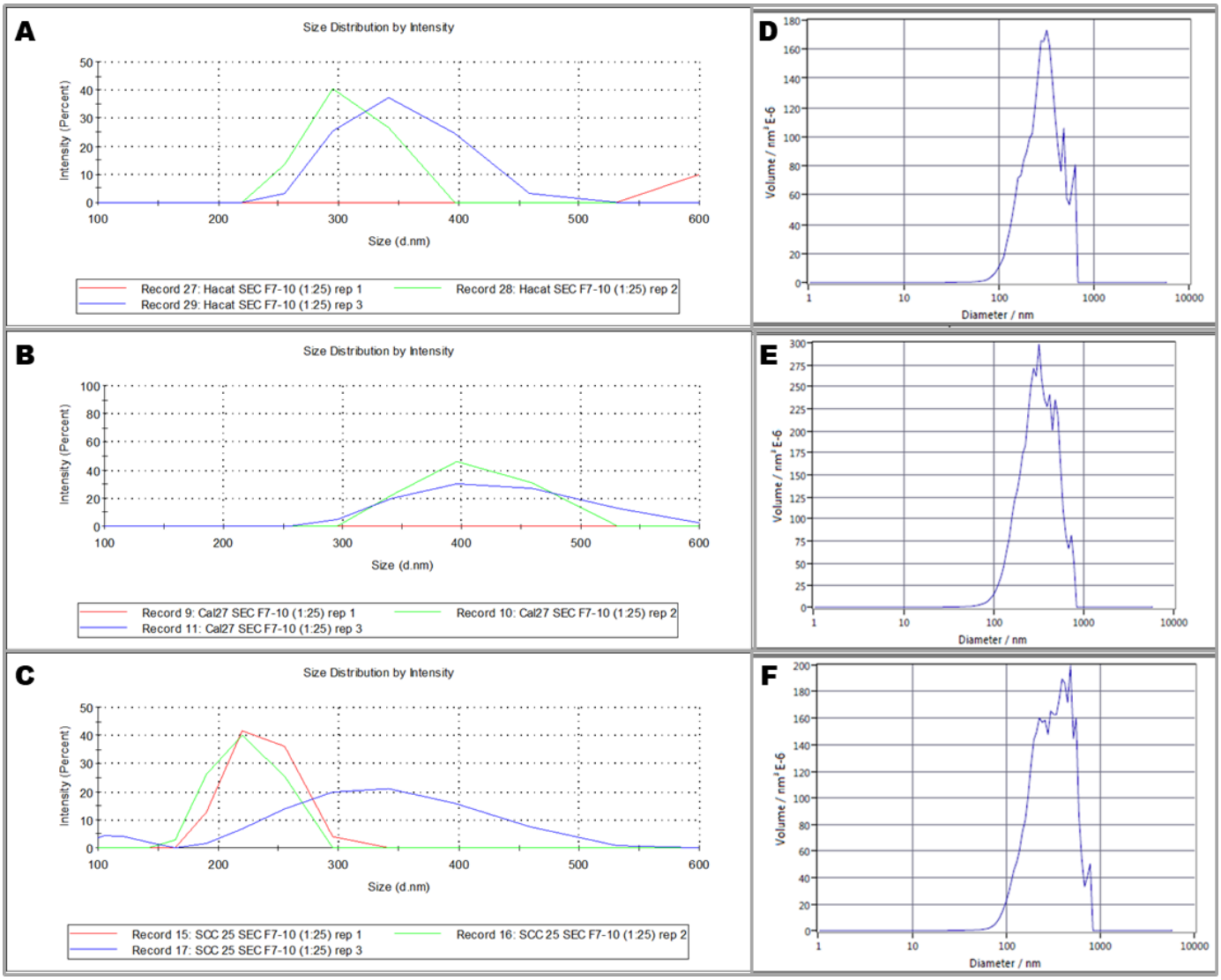

Figura 1: A análise de rastreamento de nanopartículas para tamanho das VEs demonstra picos de 93,6% (344,4nm ± 45,33) para HaCat e 94,4% (415 nm ±65,7) para Cal 27 (A e B). A SCC25 apresenta dois picos: 87,% (327,2nm ± 71,75) e 12,5% (114,9nm ± 17,02) (C). O desvio padrão sugere maior heterogeneidade das VEs tumorais. A concentração de partículas demonstra que linhagens tumorais apresentam maiores concentrações de vesículas sendo 3.7^{7}, 6.1^{7} e 5.8^{7} partículas/ml para HaCat, CAL 27 e SCC25 respectivamente (D, E e F).

3.2 Efeito das VEs de CECB na migração e direcionalidade dos queratinócitos

As VEs de CECB aumentaram a velocidade média de migração dos queratinócitos em relação ao MLV (Tabela 1). A migração dos queratinócitos apresentou média de velocidade significativamente maior quando expostos as VEs de SCC25 em relação às de Cal 27 (Figura 2).

Tabela 1: velocidade média de migração dos queratinócitos expostos as VEs de CECB.

	Cal 27	**SCC25**	**MLV**
Velocidade média (µm/min)	0,274826	0,402024	0,26339
Desvio padrão	0,077773	0,09375	0,125619
Aumento da velocidade (%)	4,43	52,63	-
	***p* = 0,726**	***p* = 0,022**	

Valores de *p* referentes ao Teste t de Student.

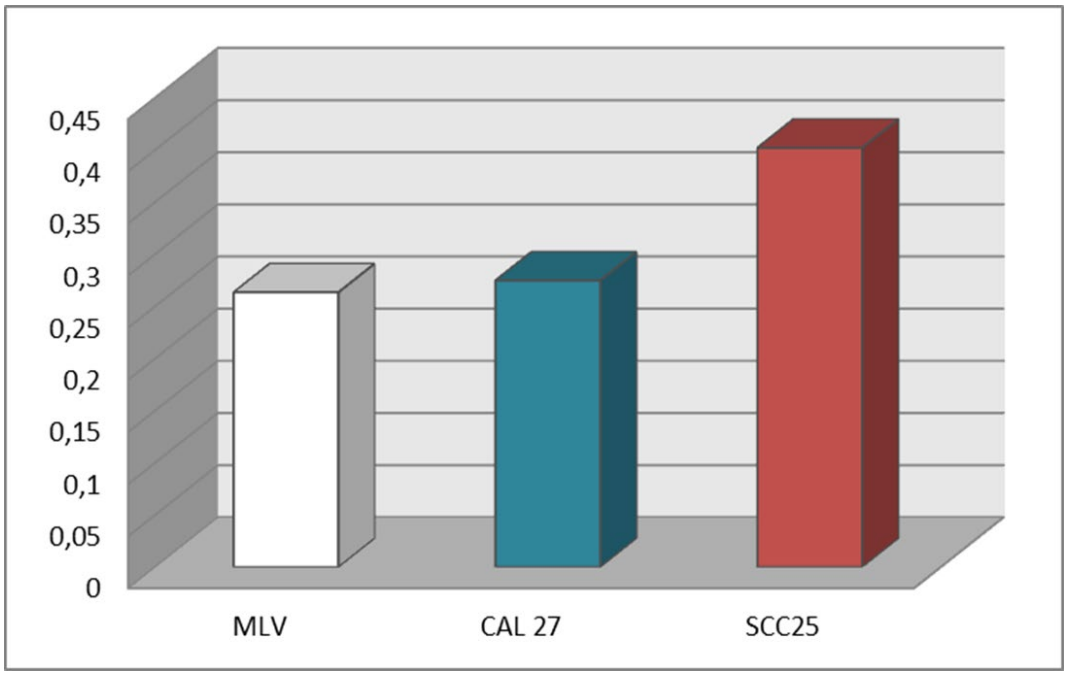

Figura 2: Relação do aumento das médias de velocidade. As VEs de SCC25 aumentaram significativamente a velocidade de migração dos queratinócitos em relação às VEs de Cal 27 (p=0,026). Teste *t* de Student.

Um aumento de 67,8% e 69,64% na direcionalidade dos queratinóticos quandos expostos as VEs de Cal27 (0,0004203;± 0,0022423) e de SCC25 (0,004248 ± 000018667) foi observado em relação ao controle (0,0002504; ± 0,0019923)(Figura 3).

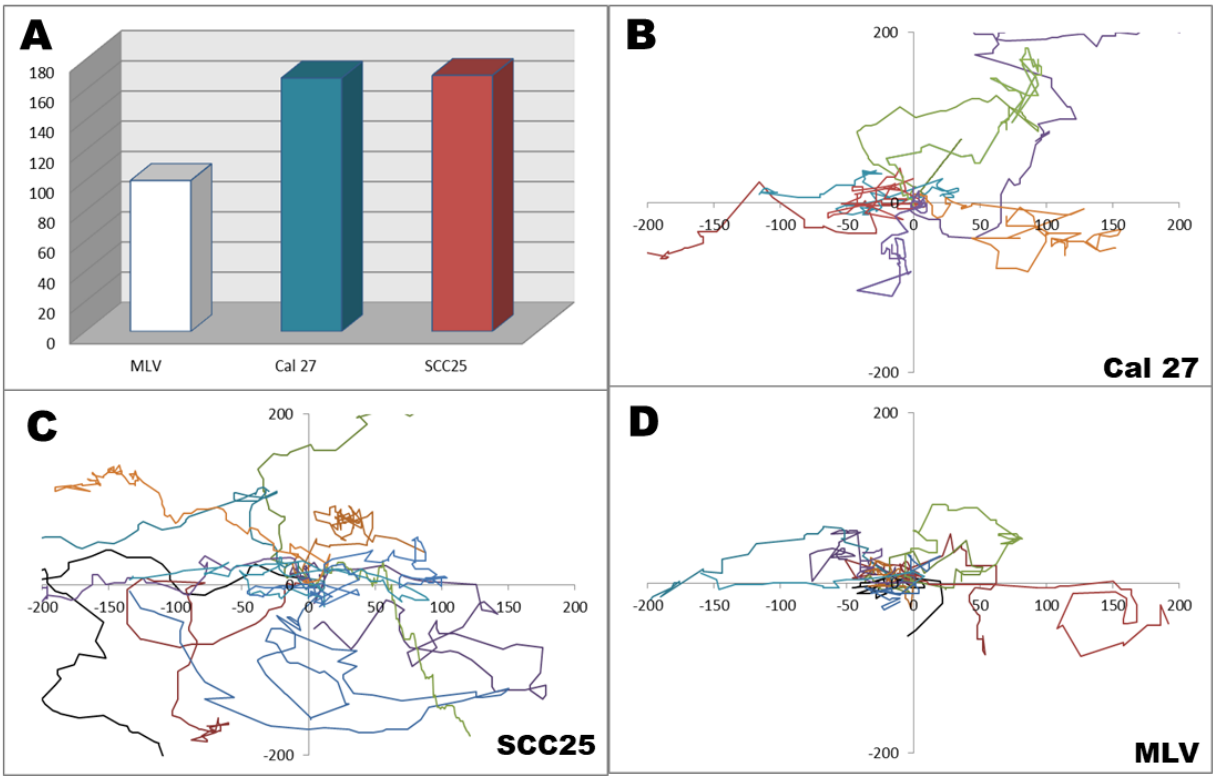

Figura 3: (A) Relação do aumento percentual de direcionalidade dos queratinócitos expostos às VEs de CECB. No *Roseplot,* em relação ao MLV (D), observa-se uma tendência à maior direcionalidade das células expostas as VEs de SCC25 (p = 0,056) (C) do que de Cal 27 (p=0,075) (B). Cada linha representa uma única célula migratória. Valores de *p* referentes ao Teste *t* de Student.

3.3 Efeito das vesículas extracelulares na migração e direcionalidade dos fibroblastos

As VEs de CAL 27 aumentaram significativamente a velocidade de migração dos fibroblastos em relação ao MLV (Tabela 2; Figura 4).

Tabela 2: velocidade média de migração dos fibroblastos expostos as VEs de Cal27.

	Cal 27	MLV
Velocidade média (µm/min)	0,26339	0,216869
Desvio padrão	0,125619	0,081786
Aumento da velocidade (%)	60,56%	-
	***p* < 0,001**	

Valores de *p* referentes ao Teste *t* de Student.

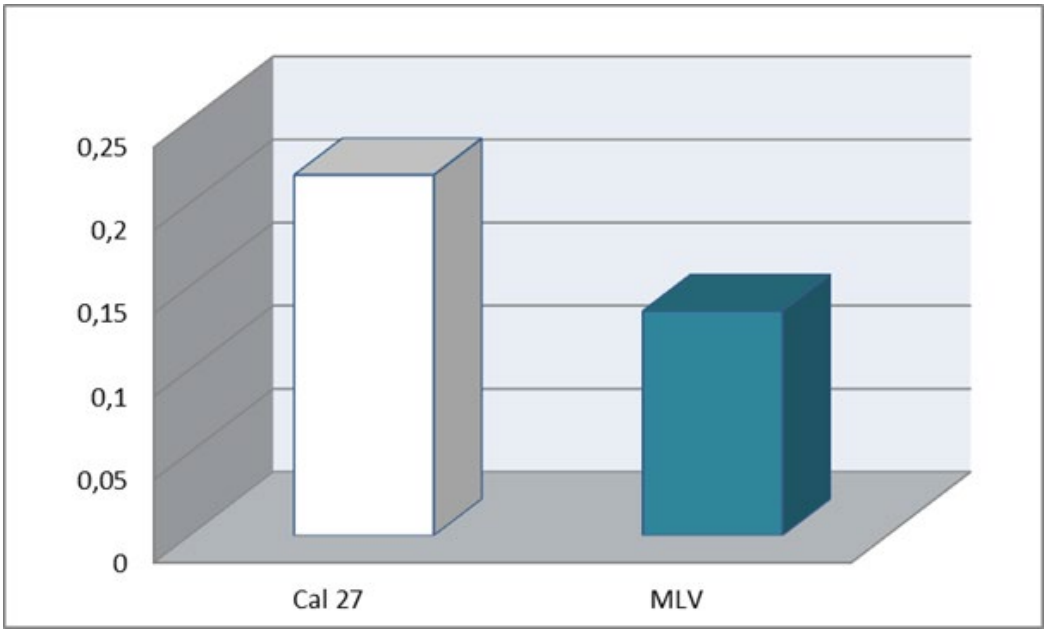

Figura 4: Relação do aumento das médias de velocidade. As VEs de Cal 27 aumentaram significativamente a velocidade de migração dos fibroblastos em relação às VEs de Cal 27 (p<0,001). Teste *t* de Student.

Um aumento de 22,8% na direcionalidade dos fibroblastos expostos as VEs de Cal 27 (0,0004574;± 0,00076430) foi observado em relação ao controle (0,0002053; ± 0,00011223). (Figura 5).

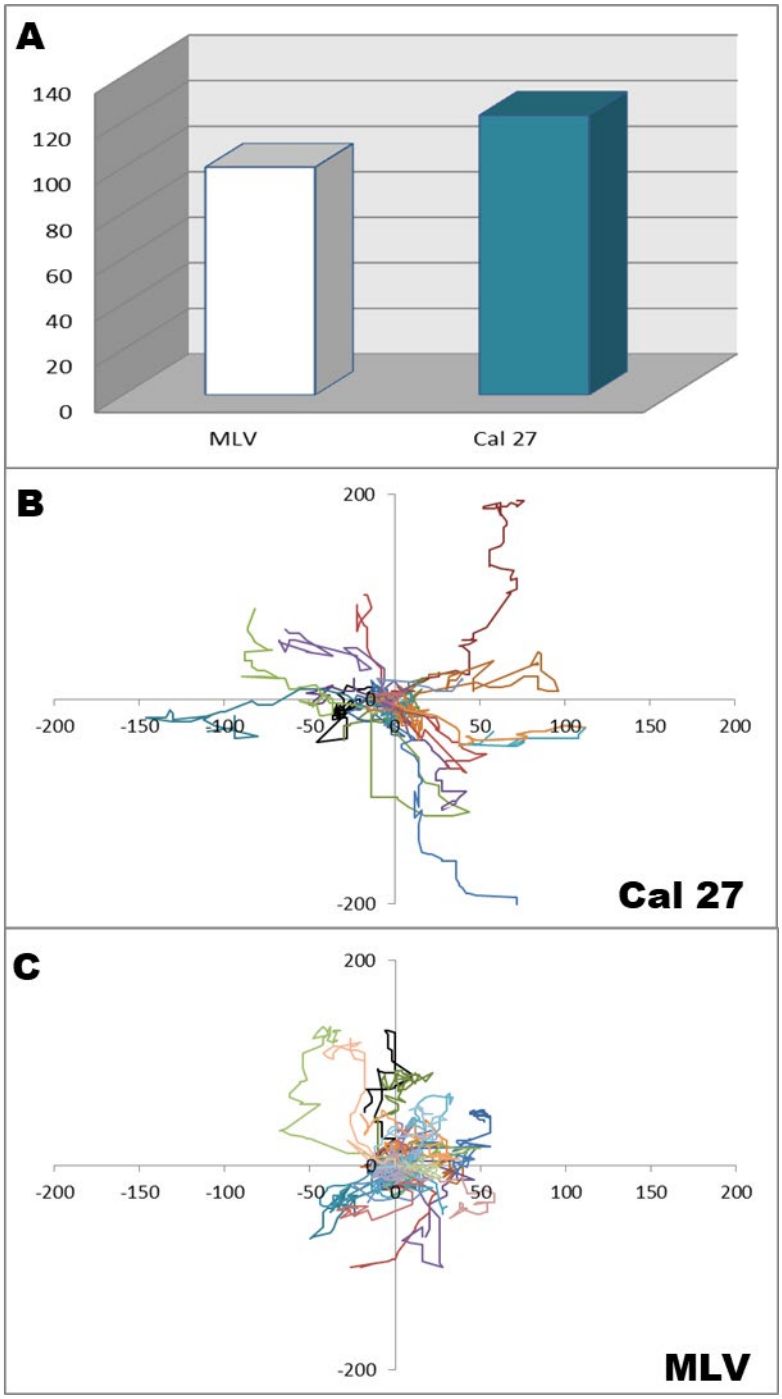

Figura 5: (A) Relação do aumento percentual de direcionalidade dos fibroblastos expostos às VEs de Cal 27. O gráfico *Roseplot* sugere uma maior direcionalidade dos fibroblastos expostos as VEs de Cal 27 (B) em relação ao controle (C), no entanto, não houve significância estatística (p=0,141). Cada linha representa uma única célula migratória. Valor de *p* referente ao Teste t de Student.

4. DISCUSSÃO

As VEs são definidas por um grupo heterogêneo de nanoestruturas esféricas (50-1000nm) que contém componentes solúveis hidrofílicos e proteínas transmembrana, delimitados por bicamada lipídica, secretadas pelas células em condições fisiológicas e patológicas (de Andrade et al., 2018, Thery et al., 2009). Evidências têm sugerido que células malignas liberam quantidades maiores de VEs em comparação com células não malignas, o que torna os mecanismos da biogênese dessas estruturas e/ou seus componentes alvos interessantes para diagnóstico e terapias anticâncer, no entanto, os métodos de isolamento utilizados atualmente, resultam em perda substancial de amostras e geração de artefatos, impedindo a sua precisa detecção e análise, bem como a padronização dessas estruturas para o uso clínico (Lim et al., 2020).

Com a intenção de isolar precisamente, utilizamos a UC combinada com SEC como técnica de eleição para a separação das VEs, visando eliminar lipoproteínas que podem se assemelhar estruturalmente ao seu tamanho e superfície (Karimi et al., 2018). Embora o método utilizado apresente resultados satisfatórios na literatura, ainda não foi estabelecida uma técnica padrão para separação dessas vesículas (Whitford & Guterstam, 2019).

Neste estudo, observamos que a distribuição das VEs em relação a tamanho e concentração média de partículas sugere maior variabilidade comparada à linhagem epitelial não tumoral. De acordo com a literatura, pacientes

com CECB apresentam diferenças morfológicas entre as VEs em comparação aos indivíduos saudáveis (Zlotogorski-Hurvitz et al., 2016) e as células tumorais, em sua maioria, tem a capacidade de liberar populações heterogêneas de VEs (Zomer et al., 2015). Traçando um paralelo, tumores sólidos como CECB apresentam heterogeneidade intra-tumoral como resultado de diferenças genéticas individuais em sua população celular (Zomer et al., 2015), o que poderia explicar a produção dessas vesículas de forma heterogênea e tal fato, estar ligado à progressão da doença e metástase (Steenbeek et al., 2018).

Concentrações maiores de VEs já foram observadas no plasma de pacientes com neoplasias malignas em relação aos pacientes hígidos (Matsumoto et al., 2016, Silva et al., 2012). Nos pacientes com câncer colorretal, a concentração maior de VEs está associada a parâmetros de mal prognóstico e sobrevivência global curta (Silva et al., 2012), no entanto, embora a comparação de tamanho e concentração de VEs represente um norte no estudo do câncer, há a necessidade de padronização de parâmetros corretos para o seu uso.

Em estudo *in vitro* para câncer de mama, observou-se que células não tumorais podem excretar 2,8 vezes mais VEs do que as células tumorais individualmente (Chiu et al., 2016), corroborando com estudos que sugerem resultados equivocados pela incapacidade de diferenciação entre VEs liberadas por processos fisiológicos e patológicos (Taylor & Shah, 2015, Tauro et al., 2012). Altas taxas de mitose e um microambiente favorável ao tumor (Tanaka & Ishigamori, 2011) podem explicar porque as células malignas produzem mais vesículas de forma coletiva quando comparadas às células normais.

A identificação das VEs através de marcadores específicos pode ser uma solução frente às limitações técnicas que impedem a sua aplicabilidade clínica. As proteínas transmembrana chamadas tetraspaninas (CD63, CD81 e CD9), são recomendadas pela Sociedade Internacional de Vesículas Extracelulares (Thery et al., 2018) na identificação dessas estruturas. Enquanto sugere-se que todos os tipos de VE expressam CD63, as proteínas CD81 e CD9, têm sido associadas à malignidade (Chiu et al., 2016). Técnicas de captura por imunoafinidade têm sido estudadas onde, nesta abordagem, pela seleção positiva proteínas direcionadas se ligam em populações específicas de VEs ou pela seleção negativa, a ligação remove VEs irrelevantes (Taylor & Shah, 2015).

Observamos que as VEs de CECB aumentaram a velocidade de migração de células epiteliais; VEs de tumor menos diferenciado (SCC25) aumentaram significativamente a velocidade de migração das células epiteliais em relação às VEs da linhagem mais diferenciada (Cal27). Células malignas de diferentes subtipos de tumor com potencial metastático distinto influenciam umas as outras pela troca de biomoléculas através das VEs, no entanto, as vias que possibilitam que pequenas quantidades de carga, com diferenças moleculares sutis produzam a heterogeneidade metastática não estão bem estabelecidas (Steenbeek et al., 2018).

É sugerido que as células podem copiar o fenótipo migratório através das VEs (Steenbeek et al., 2018) e que a troca dessas vesículas tem o potencial de acelerar drasticamente a progressão do tumor se células altamente metastáticas compartilham seu comportamento maligno com células menos agressivas (Becker

et al., 2016). Esse tipo de comportamento pôde ser observado neste estudo onde, além do aumento de velocidade, observamos maior direcionalidade dos queratinócitos quando expostos as VEs de CECB, com tendência maior das células expostas as VEs de SCC25. Especula-se ainda que, VEs oriundas de células tumorais carregam uma carga pró transição epitélio-mesenquimal, aumentando a migração e invasão celular por meio de regulação negativa de marcadores epiteliais como E-caderina e regulação positiva de marcadores mesenquimais como a Vimentina, de maneira dependente do tipo histológico do tumor (Rocha et al., 2019).

A resposta do hospedeiro em relação à neoplasia é a produção de um tecido tumoral que contenha elementos normais (Kalluri, 2016). No presente estudo, constatamos que as VEs de Cal 27 aumentaram significativamente a velocidade de migração dos fibroblastos, e de maneira discreta, a direcionalidade dessas células. Assim como as VEs derivadas de células tumorais remodelam o estroma circundante e apoiam a progressão de tumores gástricos através da migração dos fibroblastos locais e formação do nicho pré-metastático (Becker et al., 2016), sugerimos que da mesma forma, as VEs provenientes de CECB podem estar envolvidas na progressão do câncer de boca.

Os paradigmas atuais da terapêutica centrada no câncer geralmente não são suficientes para erradicar a malignidade, pois o estroma tumoral pode causar recidiva e resistência terapêutica (Chen & Song, 2019). Atualmente está claro que a progressão e metástase são controladas pelo microambiente, não sendo eventos autônomos da célula neoplásica (Kalluri, 2016), fazendo sentido que para

o estudo do CECB, observe-se em conjunto as vias de progressão da doença através das células epiteliais e de componentes do microambiente tumoral.

Ainda que limitações sobre os métodos de isolamento e caracterização existam e o estudo do conteúdo das VEs seja crucial para o entendimento da real relação dessas estruturas com o CECB, sugerimos que as VEs podem possuir um papel crítico na migração celular através da transferência de possíveis conteúdos funcionais, com a possibilidade de influenciar comportamentos pró-metastáticos.

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CONSIDERAÇÕES FINAIS

O CECCP é uma doença maligna que causa sequelas clinicamente importantes e evolui em curtos períodos de tempo para o óbito, decorrente do processo de disseminação da doença.

Anualmente, 300.000 novos casos são constatados em nível mundial, com um aumento recente e significativo em indivíduos jovens. Até o presente momento, não existem diretrizes baseadas em evidências científicas que determinem com precisão antecipada os eventos de recidiva e metástase, fazendo com que apenas a parcela da população diagnosticada precocemente com a doença tenha sucesso no tratamento e prognóstico positivo, o que não é a realidade atual do nosso sistema de saúde.

A medicina de precisão é um novo foco de estudo mundial, que tem por objetivo a identificação de biomarcadores que podem refletir as propriedades das doenças malignas, incluindo alterações bioquímicas que sinalizem o processo de disseminação e metástase tumoral.

As VEs são nanoestruturas originadas por brotamento da membrana celular que estão presentes em diversos flúidos corporais, carregando informações que podem refletir as características da célula mãe, auxiliando na detecção e monitoramento de diversas doenças, como o câncer. A facilidade de acesso a estas estruras através de coletas de sangue, saliva ou urina, entre outros, faz do seu uso para a identificação de biomarcadores específicos um método promissor no manejo clínico do CECCP, pois a compreensão do papel dessas vesículas no

processo de progressão da doença auxiliará no desenvolvimento de novas estratégias de diagnóstico, definição de prognóstico e tratamento da patologia, com intuito de aumentar a sobrevida dos pacientes.

Embora tenhamos observado e a literatura também relate resultados promissores sobre a relação das VEs com o CECCP bem como outros tipos de câncer, a necessidade de padronização e sintetização das técnicas de isolamento bem como a definição de conteúdos específicos destas estruturas faz com que este campo de pesquisa continue sendo explorado.

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