UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

MARISOL MIRANDA GALVIS

IMPACTO DO HPV NO PERFIL PROTEÔMICO E RESPOSTA À IMUNOTERAPIA NO CÂNCER DE CABEÇA E PESCOÇO

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

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Oral Medicine and Oral Pathology, in Pathology area.

Orientador: Prof. Dr. Luiz Paulo Kowalski
Coorientadora: Prof[a]. Dra. Adriana Franco Paes Leme

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In memoriam de **Pedro Curioso.**

Uma vida belamente vivida,
e um coração eternamente amado,
sentiremos sua falta todos os dias,
mas sua lembrança continua sempre viva**.**

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RESUMO

Os mecanismos moleculares de oncogênese do vírus do papiloma humano de alto risco (HR-HPV, do inglês *High risk - human papillomavirus*) nos tumores de orofaringe estão bem estabelecidos e descritos na literatura. Em relação à cavidade oral, um estudo recente que determinou a prevalência de 17 subtipos de HR-HPV, revelou uma alta frequência de HPV ADN 16 no carcinoma de células escamosas (CCE) oral em pacientes jovens, e o papel do vírus transcricionalmente inativo nesses tumores ainda é inexplorado. Complementarmente, tem sido demostrado que a infecção pelo HPV nos tumores de cabeça e pescoço influencia a resposta às modalidades terapêuticas, apresentando melhores taxas de sobrevida. Portanto, a presente tese se propôs avaliar o impacto do HPV no perfil proteômico com o intuito de explorar o papel do HR-HPV no desenvolvimento e progressão do CCE oral, assim como a sua influência na resposta à imunoterapia no câncer de cabeça e pescoço. Com esta finalidade, foram micro dissecadas a laser ilhas neoplásicas de 20 tumores de CCE oral de pacientes menores de 40 anos em estádio clínico avançado, para sua análise por espectrometria de massa. Posteriormente, os resultados foram validados com *tissue microarray*, e ensaios *in vitro* com linhagens de células de CCE oral HPV positivas e negativas possibilitaram pesquisar a atividade biológica das proteínas previamente identificadas. Em adição, foi realizada uma revisão sistemática e meta-análise de *clinical trials* avaliando a eficácia e segurança da imunoterapia no câncer de cabeça e pescoço, enfatizando a resposta nos tumores associados ao HPV. Os resultados demonstraram que o perfil proteômico dos CCE oral HR-HPV ADN positivos apresenta diferenças dos HR-HPV ADN negativos. Foi identificada a superexpressão da proteína S100A8 nos tumores associados ao HPV, e a ativação da via dessa proteína levou a uma reposta pró inflamatória exclusivamente nos tumores HPV positivos, sugerindo que a presença do vírus pode levar a uma modificação no microambiente tumoral com uma presumível influência na carcinogênese oral. Por outro lado, a revisão de literatura e meta-análise demostraram que a imunoterapia melhora as taxas de resposta e sobrevida com redução nas toxicidades nos pacientes com câncer de cabeça e pescoço, e um maior benefício foi observado nos tumores associados ao HPV.

Palavras-chave: Câncer de cabeça e pescoço; HPV; proteômica; S100A8; prognóstico; inflamação; imunoterapia.

ABSTRACT

Molecular mechanisms of high risk-human papillomavirus (HR-HPV) oncogenesis in oropharyngeal tumors are already established and described in the literature. A recent study proposed to determine the prevalence of 17 subtypes of HR-HPV, revealed a high frequency of HPV DNA 16, in oral squamous cell carcinoma (OSCC) in young patients. The role of the HR-HPV transcriptionally inactive in these tumors is unexplored. In addition, it has been demonstrated that HPV infection influences the response to head and neck cancer treatment, with better survival rates. Therefore, this thesis aimed to evaluate the impact of HPV on the proteomic profile in order to explore the role of HR-HPV in the development and progression of OSCC, as well as, its influence on the response to immunotherapy for head and neck cancer. For this purpose, islands of neoplastic epithelial cells of 20 OSCC affecting patients younger than 40 years old in advanced clinical stage were laser microdissected for mass spectrometric analysis. Subsequently, the results were validated in a tissue microarray, and in vitro assays with OSCC cell lines HPV positive and negative allowed to research the biological function of previously identified proteins. In addition, we conducted a systematic review and meta-analysis of clinical trials evaluating the efficacy and safety of immunotherapy for head and neck cancer, with focus on the response of HPV-associated tumors. The results showed that the proteomic profile of HR-HPV DNA positive OSCC differs from HR-HPV DNA negative. HPV-associated tumors exhibited overexpression of S100A8 protein, and activation of the protein pathway led to a pro-inflammatory response only in HPV-positive tumors, suggesting that the presence of the virus may lead to a modification in the tumor microenvironment with a supposed influence in tumor progession. On the other hand, the systematic review and meta-analysis demonstrated that immunotherapy improves overall response and survival rates with a reduction in the toxicities in patients treated for head and neck cancer, and a greater benefit was observed in HPV-associated tumors.

Keywords: Head and neck cancer; HPV; proteomics; S100A8; prognosis; inflammation; immunotherapy.

SUMÁRIO

1 INTRODUÇÃO

1.1 Epidemiologia do câncer

Segundo a Agência Internacional de Pesquisa em Câncer – IARC (do inglês *International Agency for Research on Cancer*) no ano de 2018 foram estimados 18,1 milhões de novos casos e 9,6 milhões de mortes, observando-se um aumento nas taxas de mobidade e mortalidade ao ser comparado com as estatísticas de 2014 (Bray et al. 2018; Stewart BW 2014). O câncer de cabeça e pescoço, afetando a cavidade oral, orofaringe e laringe, representa 3% de todos os tipos de câncer, e também apresenta números alarmantes, onde metade dos novos pacientes diagnosticados morreram em cinco anos devido à progressão da doença (Bray et al. 2018). O Instituto Nacional de Câncer – INCA do Brasil, através do programa de epidemiologia e vigilância do câncer e seus fatores de risco, reportou no ano de 2017 uma estimativa de 14.700 novos casos de câncer de cavidade oral para os anos 2018 e 2019, ocupando a quinta posição dos tumores malignos mais frequentes entre os homens, e o decimo segundo nas mulheres (INCA 2017).

1.2 Fatores etiológicos para o câncer de cabeça e pescoço

O Carcinoma de Células Escamosas – CCE representa 90% dos tumores malignos localizadas na cavidade oral e 40% das neoplasias de cabeça e pescoço (Kowalski LP 2000), sendo o subtipo histológico mais comum. O CCE é o resultado de múltiplos eventos moleculares que se desenvolvem na combinação de predisposições genéticas individuais, exposição a agentes carcinogênicos ambientais e/ou uma função reduzida dos fatores intrínsecos de proteção do ADN, os quais em interação promovem um comportamento maligno das células epiteliais com consequentes modificações fenotípicas (Vairaktaris et al. 2008).

O consumo sinérgico de tabaco e álcool são considerados os principais fatores externos associados ao desenvolvimento de CCE de cabeça e pescoço. O aumento do risco depende da intensidade e da duração do hábito (Hashibe et al. 2009). O cigarro contém, mais de 40 sustâncias carcinogênicas que causam danos e mutações no ADN. Por outro lado, o álcool atua como co-fator, mediante seu mecanismo facilitador da absorção de substâncias cancerígenas para dentro das células, além de alguns de seus metabolitos interferirem no reparo do ADN (Hashibe et al. 2007).

1.2.1 O vírus do HPV

O vírus do papiloma humano – HPV (do inglês *Human papillomavirus*), é o único vírus oncogênico relacionado ao câncer de cabeça e pescoço, especificamente ao de orofaringe (Gillison et al. 2000). O HPV é um vírus de ADN, da família Papillomaviridae e aproximadamente 200 subtipos foram descobertos até hoje, os quais são classificados como de baixo risco (envolvidos nas lesões benignas), de alto risco (conhecidos carcinogênicos) e potencialmente cancerígenos (de Villiers et al. 2004).

O HPV de alto risco possui capacidade de infectar os queratinócitos da camada basal e posteriormente multiplicar o seu ADN viral nos estratos mais superiores (Egawa et al. 2015). O genoma viral contém três regiões: **i)** região E (early) constituída por oito genes (E1-E8) que codificam as proteínas que irão interagir e alterar a função das proteínas das células hospedeiras responsáveis pelo controle do ciclo celular, apoptose, sinalização celular e expressão gênica. **ii)** região L (Late) onde são codificadas as proteínas (L1-L2) que proporcionarão ao vírus os fatores de virulência, tais como o capsídeo. **iii)** região regulatória que controla a transcrição dos genes (Duensing et al. 2000).

A oncoproteína E7 tem a capacidade de se unir e formar um complexo de alta afinidade com várias proteínas, entre elas a proteína do retinoblastoma - pRb a qual ativa os fatores de transcrição E2F, importante no controle da transição da fase G1 à fase S do ciclo celular. Por outro lado, E6 pode formar um complexo ou degradar o gene supressor de tumor p53, responsável da proteção da integridade do genoma celular. Como resultado destas interações se suprimem *checkpoints* no ciclo celular favorecendo a multiplicação do ADN danificado, imortalização das células, transformação das linhagens celulares, inibição da apoptose e por último carcinogênese (Egawa et al. 2015).

O papel do HPV no CCE oral ainda não foi esclarecido, devido à baixa prevalência do vírus na cavidade oral em comparação com alta frequência nos tumores de orofaringe. Esta diferença pode ser explicada em parte pelas características histológicas. As membranas da cavidade oral e da orofaringe são cobertas por epitélio escamoso estratificado, porém a mucosa da orofaringe não é queratinizada e o epitélio reticulado das criptas invaginadas facilitam que o HPV acesse aos queratinócitos da camada basal. No entanto, Kaminagakura et al. relatou a presença do HPV-ADN de alto risco em 68,2% dos pacientes menores de 40 anos, sugerindo a possibilidade de ser um fator contribuinte na carcinogênese oral nesta faixa etária

(Kaminagakura et al. 2012). Além disso, um recente *clinical trial* fase II que avaliou a eficácia do durvalumab como imunoterapia em pacientes com câncer de cabeça e pescoço recorrente e/ou metastático, mostrou taxas de resposta mais altas em pacientes HPV positivos, independentemente da localização do tumor; sugerindo que a infecção pelo HPV em tumores não orofaríngeos pode gerar uma resposta imune que permite uma melhor eficácia à imunoterapia (Zandberg et al. 2019).

1.3 Características clínicas e histopatológicas do câncer de cabeça e pescoço

Na última classificação da Organização Mundial da Saúde – OMS em 2017 (EI-Naggar et al. 2017), os CCE de cabeça e pescoço foram classificados segundo a sua localização em: **i)** tumores de hipofaringe, laringe, traquéia e espaço parafaríngeo, **ii)** tumores da cavidade oral e os 2/3 anteriores da língua, e **iii)** tumores da orofaringe (base de língua, amígdalas, palato mole e parede posterior da orofaringe). Na sua vez os tumores de orofaringe foram subclassificados segundo o status do HPV, devido à importantes diferenças reconhecidas nos fatores de risco, na biologia tumoral, características clínicas, histopatologicas e prognóstico.

1.3.1 Carcinoma de células escamosas HPV negativo

Na cavidade oral, o CCE HPV negativo afeta principalmente pacientes na sétima década de vida e as localizações mais comumente acometidas são a língua e o assoalho de boca. Apresenta-se como lesões eritroleucoplasicas assintomáticas em fases iniciais, e como úlceras ou nódulos doloridos com margens endurecidas e irregulares em estágios mais avançados. Observa-se alta propensão para numerosas metástases locais em linfonodos cervicais e escassas metástases à distância, as quais, quando presente, afetam o pulmão, linfonodos não loco regionais, pele, ossos e fígado. O CCE HPV negativo tem origem no epitélio de superfície com invasão ao tecido conjuntivo subjacente, onde as células malignas apresentam diferentes graus de diferenciação escamosa (EI-Naggar et al. 2017). O grau de diferenciação, considerando a semelhança do tecido tumoral com o de origem, é o fator avaliado para a classificação histológica dos tumores em: **i)** bem diferenciado, assemelha-se morfológica e funcionalmente ao epitélio normal, **ii)** moderadamente, pleomorfismo celular com moderada atividade mitótica, e **iii)** pobremente diferenciado, células imaturas, numerosas mitoses atípicas e escassa queratinização (EI-Naggar et al. 2017).

1.3.2 Carcinoma de células escamosas HPV positivo

O CCE HPV positivo afeta principalmente pacientes jovens com tumores localizados na base de língua e as tonsilas (Duensing et al. 2000). Clinicamente se apresenta como tumores pequenos, porém com uma alta propensão para metástases linfonodales as quais são geralmente císticas. O aspecto histopatológico demostra um padrão basaloide com crescimento lobular, um estroma altamente infiltrado por linfócitos e ausência de displasia epitelial. As células neoplásicas demostram alta imunoreatividade para o anticorpo p16, podendo ser um biomarcador para o status do HPV exclusivamente para os tumores de orofaringe. Os pacientes com CCE associados ao HPV apresentam um menor risco de desenvolvimento de recorrências e segundos tumores primários ao ser comparados com os HPV negativos, o que resulta em melhores taxas de sobrevida (Gillison et al. 2000).

1.4 Tratamento

O tratamento do CCE de cabeça e pescoço consiste em cirurgia com ou sem esvaziamento cervical, radioterapia e/ou quimioterapia. A escolha da terapia é guiada pela classificação TNM, a localização do tumor e avaliação microscópica da peça cirúrgica reportando a relação do fronte tumoral com as margens cirúrgicas, a espessura da neoplasia, e a presença ou não de invasão perineural e/ou angiolinfática. Pacientes com tumores em estádio inicial (T1-T2) são tratados com cirurgia ou radioterapia, enquanto aqueles com tumores avançados (T3-T4) recebem mais de uma modalidade de tratamento (Kowalski 2002).

O tratamento de primeira linha para pacientes com doença recorrente e/ou metastática que não são elegíveis para cirurgia de resgate ou radioterapia é o regime EXTREME, composto por quimioterapia a base de platina e taxanos. Para indivíduos com doença progressiva, as opções terapêuticas de segunda linha são limitadas à monoterapia, com metotrexato, docetaxel ou cetuximabe (Steinbichler, 2018). Apesar das multimodalidades empregadas, o controle da doença em longo prazo é desafiador, com uma taxa de resposta de 4% e uma sobrevida global de 10 meses ou menos (Vermorken, 2008).

A imunoterapia surgiu como uma opção terapêutica promissora, com a finalidade de restaurar o microambiente tumoral imunossupressor no CCE de cabeça e pescoço (Mandal, 2016). As alterações imunológicas encontradas nestes tumores compreendem: **i)** redução no número e na atividade das células *natural killer*, **ii)** falha na apresentação de antígenos e **iii)** disfunção nas células T que expressam moléculas de ponto de verificação, como a proteína de

morte celular programada 1 (PD-1) e/ou linfócitos T citotóxicos associada à proteína 4 (CTLA-4) (Ferris, 2015). Assim, *checkpoint inhibitors* e *costimulatory agonists* são atualmente as modalidades de imunoterapia mais pesquisadas, com *clinical trials* fase I, II e III em andamento ou concluídos e já com resultados promissores (Chan, 2015).

1.4.1 Tratamento de carcinoma de células escamosas associado ao HPV

Devido à melhor reposta ao tratamento que apresentam os pacientes com tumores associados ao HPV, c*linical trials* procuram a desintensificação terapêutica neste grupo específico de pacientes. Em geral, os novos protocolos propõem modificações nas três modalidades terapêuticas, com abordagens cirúrgicas menos agressivas, redução da dose da radioterapia e substituindo a cisplatina por cetuximab na quimioterapia. Estas estratégias levam a uma diminuição nas toxicidades agudas e crônicas com uma subsequente melhora na qualidade de vida, mas mantendo a eficácia. Porém, alguns estudos apresentam resultados contraditórios o que faz necessário mais pesquisas com alta evidencia cientifica para adoptar estes protocolos (Deschuymer, Mehanna, and Nuyts 2018; Gillison et al. 2019; Mehanna et al. 2019). Por outro lado, tem sido reportado um maior benefício à imunoterapia nos tumores HPV positivos, independente da sua localização anatômica, presentando melhores taxas de resposta e sobrevida (Zandberg et al. 2019; Ferris et al. 2018).

Neste cenário onde não está bem estabelecido a função biológica do HPV ADN em tumores extra orofaringe, esta tese se propôs avaliar o impacto do HPV no perfil proteômico com o intuito de explorar o papel do HR-HPV no desenvolvimento e progressão do CCE oral, assim como a sua influência na resposta à imunoterapia no câncer de cabeça e pescoço.

2 ARTIGOS

2.1 New insights in the impact of HR-HPV DNA in oral cancer: Proteomic approach reveals a novel role for S100A8

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ABSTRACT

Background: Molecular mechanisms of high risk-human papillomavirus (HR-HPV) oncogenesis in oropharyngeal tumors are already established and described in the literature. A recent study also revealed a high frequency of HPV DNA 16, in oral squamous cell carcinoma (OSCC) in young patients, and the role of the HR-HPV transcriptionally inactive in these tumors is unexplored. **Objective:** To evaluate the impact of HPV on the proteomic profile in order to recognize the role of HR-HPV in the development and progression of OSCC. **Methods:** Liquid chromatography coupled to mass spectrometry was carried out in microdissected neoplastic cells from surgical specimens of OSCC affecting young patients (≤40 years). The identified proteins were validated in a tissue microarray, and in vitro assays with the OSCC cell lines UPCI-SCC154, UM-SCC104, HN6 and HN13 allowed to assess patterns of HPV-related inflammatory response. **Results:** The proteomic profile of HR-HPV DNA positive OSCC differs from HR-HPV DNA negative. HPV-associated tumors exhibited overexpression of S100A8 protein and it is correlated with a worse prognosis. In addition, activation of S100A8 and NF-Kb pathway led to a pro-inflammatory response exclusive in HPV-positive tumors. **Conclusion:** Our findings indicate that HPV DNA in OSCC may lead to a modification in tumor microenvironment with a supposed influence in tumor progression through the NF-Kb and S100A8 pathway.

Keywords: Head and neck cancer; HPV; proteomics; S100A8; prognosis; inflammation.

INTRODUCTION

Human papillomavirus (HPV) is a small, non-enveloped DNA virus that presents a tropism for basal keratinocytes of stratified squamous epithelium, often detected in mucosal and cutaneous tissues (1). More than HPV 200 genotypes have been identified so far and classified into five major genera, ∝-HPV, β-HPV, γ-HPV, mu-HPV and nu-HPV(2). ∝-HPV gender also is categorized in low or high risk for development of malignant lesions. Low-risk HPVs (HPV6, 11, 42, 43, 44, 54, 61, 70, 72, and 81) are etiologic factors for benign lesions (3); while HPV26, 53, and, 66 have been classified as potentially carcinogenic (4).

High-risk HPV (HR-HPV) comprises the subtypes HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82, and its viral genes have a crucial role in tumorigenesis (4). HPV viral genome is composed of early (E), late (L), and regulatory regions. L region codifies the structural proteins (L1-L2), whereas E region encodes 8 oncogenes (E1-E8). E6 and E7 oncoproteins inactivate and degrade the tumor suppressor protein p53 and retinoblastoma protein (pRb), respectively, which regulate the cell cycle. As such, the transcription of HPV oncoproteins leads to suppression of cell cycle checkpoints, favoring the replication of damaged DNA, transformation and immortalization of epithelial cells (5).

Recently, HPV 16 and 18 have been linked to a subset of head and neck cancer, particularly affecting the oropharynx. The so-called HPV-related nonkeratinizing squamous cell carcinoma of the oropharynx (6) comprise a distinct disease with specific risk factors, tumor biology, clinical features, histopathology, and, prognosis (7). The role of HPV in non-oropharyngeal squamous cell carcinoma has not been clarified due to the low prevalence of the virus in extra oropharyngeal locations (7, 8) and contradictory reports regarding its prognosis impact (9-12). Nevertheless, a previous study (13) assessing the prevalence of HPV in oral squamous cell carcinoma (OSCC) according to age, reported that 68.2% of the patients younger than forty years old were positive for HR-HPV DNA. Furthermore, a phase II clinical trial (14) evaluating the efficacy of durvalumab monotherapy in patients with recurrent and/or metastatic head and neck cancer, showed higher response rates in HPV-positive tumors, regardless of localization; suggesting that HPV infection in non-oropharyngeal tumors may generate an immune response that allows a better efficacy to the immunotherapy.

In order to clarify the impact of HR-HPV in OSCC development and progression, we aimed to identify and analyze the proteome profile of HPV-related OSCC. We further assessed patterns of HPV-related inflammatory response in OSCC cell lines.

MATERIALS AND METHODS

Patients and samples

Patients diagnosed for OSCC were retrospectively retrieved from the archives of the A.C. Camargo Cancer Center (São Paulo - Brazil). The proteomic analysis was performed in cohort 1, composed for twenty cases of OSCC affecting patients younger than forty years old in advanced clinical stage. Cohort 2 with ninety-one OSCC was used to validate the proteomic results. HPV status of all tumor samples was conducted and published by Kaminagakura et al (13). The medical records were examined to obtain sociodemographic characteristics (Gender, age, habits) and clinicopathological features (TNM) (15), histological grade (16), as well as, survival rates. Eligibility criteria included previously untreated patients, without a second primary tumor and submitted for treatment in the same institution. Tumors affecting lips and oropharynx were excluded. This study was approved by the Research Ethics Committees of A.C. Camargo Cancer Center (2199/16). Ethical guidelines were followed and samples and clinicopathological data were handled in a coded fashion.

Laser-capture microdissection and protein extraction

Histological sections with 10-μm-thick obtained from OSCC paraffin-embedded surgical specimens of cohort 1 were placed on Arcturus PENmembrane glass slide (Thermo fisher Scientific, MA, USA), deparaffinized with xylene and stained with hematoxylin. Neoplastic epithelial cells were microdissected using the LMD CTR 6500 equipment (Leica Microsystems, Wetzlar, Germany), obtaining a mean area of ~ 4000 μm^2 per sample, which was deposited in microtubes previously identified for each case and then stored under -80°C.

Proteins were extracted in the form of tryptic peptides as previously described (17). In brief, the samples were treated with urea in the concentration of 1.6 M, reduced with 5 mM dithiothreitol for 25 minutes at 56°C, alkylated with 14 mM iodoacetamide for 30 minutes at room temperature protected from light and the proteins were digested with trypsin at 37°C for

16 hours. Formic acid at 0.4% was added to stop the reaction and then the samples were dried in a vacuum concentrator and stored under -80°C.

Liquid chromatography–mass spectrometry (LC-MS/MS)

An aliquot containing 4.5µL of peptide mixture was analyzed on a LTQ Orbitrap Velos (Thermo Fisher Scientific, MA, USA) mass spectrometer coupled to nanoflow liquid chromatography on EASY-nLC system (Proxeon Biosystems, Odense, Dinamarca) through a Proxeon nanoelectrospray ion source. Peptides in 0.1% formic acid were separated by a 2-90% acetonitrile gradient in a PicoFrit analytical column (20 cm x ID75, 5 µm particle size, New Objective), with a flow rate of 300 nL/min over 212 minutes and a gradient of 35% acetonitrile at 175 min. Nanoeletrospray voltage was set to 2.2 kV and source temperature to 275°C. Instrument methods for LTQ Orbitrap Velos were set up in the data dependent acquisition mode and full scan MS spectra (m/z 300–1600) were acquired in the Orbitrap analyzer after accumulation to a target value of $1e^6$. Resolution in the Orbitrap was set to r = 60,000 and the 20 most intense peptide ions with charge states ≥ 2 were sequentially isolated to a target value of 5,000 and fragmented in the high-pressure linear ion trap by CID (collision-induced dissociation) with normalized collision energy of 35%. Dynamic exclusion was enabled with exclusion size list of 500 peptides, exclusion duration of 60 s duration and repetition count of 1. An activation Q of 0.25 and activation time of 10 ms were used.

Data analysis

The raw files were processed using the MaxQuant v1.3.0.3 software (18) and MS/MS spectra were searched against the Human UniProt database (release January 7th, 2015, 89,649 sequences, 35,609,686 residues) using the Andromeda search engine (19). A tolerance of 20 ppm was considered for precursor ions (MS search) and 0.5 Da for fragment ions (MS/MS search), with a maximum of 2 missed cleavage. Oxidation of methionine and protein N-terminal acetylation was set as variable modifications and carbamidomethylation of cysteine as fixed modification. Label-free quantification (LFQ) was used for protein quantification; with a 2 min window for matching between runs and minimal ratio count set as 1. A maximum of 1% peptide and 1% protein FDR was considered. Statistical analysis was performed with Perseus v1.2.7.4 software (18), available at MaxQuant package. Protein dataset were processed excluding reverse sequences and only identified by site entries. A filter of minimum valid values (3 valid

values) was applied in at least one group and Student *t*-test assessed significance's to identify differentially expressed proteins ($p<0.05$).

Bioinformatics analysis

Proteins with differential expression between the HR-HPV positive (+) and HR-HPV-negative (-) groups were submitted to an enrichment analysis in order to gain biological information from this list of the identified proteins. Using the Integrated Interactome System (IIS) platform (20), Uniprot IDs of differential proteins were submitted to the Integrated Interactome System (IIS) platform (20) to perform the enrichment analysis for the GO (Gene Ontology) (21) database. Only significantly biological processes (p value < 0.05) were considered in the results. Canonical pathways were obtained using Ingenuity Pathway Analysis software (IPA; v8.0; Ingenuity® Systems, Redwood City, CA, USA; http://www.ingenuity.com).

The immune cells infiltrate composition was estimated through a deconvolution method based on gene expression panels to determine the proportion of B cells, CD4+ T cells, CD8+ T cells, Natural Killer (NK) cells, Macrophages, Dendritic cells (DC) and Neutrophils in the samples from TCGA-HNSC selecting Oral cavity samples (N = 301). The reference signature matrix was constructed using TIMER pipeline (22) and LM22 marker genes proposed by Newman et al., (23). The deconvolution algorithm CIBERSORT R code (23) was then used for estimate relative proportions of immune cells above with 1000 permutations and disabled quantile normalization as set parameters. Proportion of each immune was compared between "top" and "bottom" samples classified by third and first quartile according S100A8 expression. Wilcoxon Test with statistical significance was set at $p <0.05$. A second approach proposed by Aran et al., (24) known as xCell, based on gene set enrichment analysis, was used to reinforce the findings about immune cells comparison between "top" and "bottom" samples.

Cell Lines and MPLAs administration

The tongue squamous cell carcinoma cell lines positive for HPV-16, UPCI-SCC154 and UM-SCC104, were grown in Minimum Essential Medium (MEM; ThermoFisher scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Fisher scientific, Hampton, NH, EUA), 50 mg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA), 200 mM L-glutamine (ThermoFisher scientific, Waltham, MA, USA) and Mem non-essential amino acid solution (ThermoFisher scientific, Waltham, MA, USA).

The tongue squamous cell carcinoma cell lines negative for HPV-16, HN6 and HN13 were cultured in Dulbelco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA). The medium was supplemented with 10% fetal bovine serum, FBS (Fisher scientific, Hampton, NH, USA), 1% Antibiotic Antimycotic Solution (10000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL; Sigma-Aldrich, St. Louis, MO, USA).

The cells lines were maintained at 37 °C in a humidified incubator of 5% CO_2 and 95% air, cultured to a maximum of 40% confluence, starved with 0% FBS for 24 hours and administered a Synthetic monophosphoryl lipid A, MPLAs at a final concentration of 10 ng/ml during 24 hours.

Immunofluorescence

Immunofluorescence staining was performed on all cell lines and tissue sections obtained from a tissue microarray (TMA) containing 91 OSCC (cohort 2), as previously described (25). The cells were seed on glass coverslips in 6-well plates and fixed with 3% paraformaldehyde at room temperature for 20 minutes. Standard protocol was performed to deparaffinize and re-hydrate the tissues through graded ethanol solutions, followed by antigen retrieval with citric acid. Cells and tissues were blocked with 1% bovine serum albumin (BSA) in Phosphatase Buffer Saline (PBS) and incubated with Anti-MRP8 antibody (Abcam Plc, Cambridge, UK) and PCRP-RELA-2B6 (The Developmental Studies Hybridoma Bank, IA, USA) at 4°C. After incubation overnight, slides were washed 3 times with PBS and incubated with a secondary antibody Alexa Fluor 568 anti-rabbit or Alexa Fluor 488 anti-mouse (ThermoFisher scientific, Waltham, MA, USA) for 60 minutes at room temperature. DNA was stained with Hoechst 33342 (ThermoFisher scientific, Waltham, MA, USA) and the slides were mounted with Fluoroshield (Sigma-Aldrich, St. Louis, MO, USA). Images were visualized under a Nikon Eclipse 80i Microscope (Nikon, Melville, NY, USA), and took representative pictures using NIS Element software to further quantification. The cases from tissue sections were divided into low (≤ 50% positives cells) and high expression (> 50% positives cells), and associated with clinicopathological features and survival. Cell assays were performed in triplicate and in all cases included positive and negative controls.

Flow cytometry

The four cell lines were submitted to flow cytometry assay. Cell suspensions were adjusted to a concentration $1x10^6$ cells/mL in PBS. After that, the cells were fixed with cold

1% paraformaldehyde, washed and blocked with 0.5% BSA-PBS. The cells were incubated with Anti-TLR4 antibody [76B357.1] (ab22048) (Abcam Plc, Cambridge, UK) for 45 minutes using a rotor at room temperature. The same conditions were used to incubate with the secondary antibody in dark, Alexa Fluor 488 anti-goat or Alexa Fluor 633 anti-mouse (ThermoFisher scientific, Waltham, MA, USA). Finally, the cells were washed and suspended in 0.5% BSA-PBS to be analyzed using a BD Accuri C6 plus flow cytometer (BD Biosciences, USA). All assays were performed in triplicate and included negative controls.

Quantitative reverse transcription-PCR

RNA was isolated from cell lines using Quick-RNA Microprep Kit (Zymo research, Irvine, CA, USA), followed by reverse transcribed to cDNA with high capacity cDNA reverse transption kit (ThermoFisher scientific, Waltham, MA, USA). Quantitative reverse transcription PCR was performed using SYBR™ Green PCR Master Mix (Th ermoFisher scientific, Waltham, MA, USA). The analyses were performed in a Sequence Detection System RT-PCR (ABI Prism 7900HT, Applied Biosystem, Foster City, CA, USA), following thermo cycling conditions (1 cycle of 10 min at 95 °C, 40 cycles of 15 seconds at 95 °C, 20 seconds at 58 °C, 30 seconds at 72 °C and 1 cycle of 15 seconds at 95 °C, 15 seconds at 60 °C and 15 seconds at 95 °C). The oligonucleotide sequences are listed in **supplementary appendix S1**. The assays were performed in sextuplicate for each sample and GAPDH was used as a control.

Statistical analysis

Statistical analyses of associations between variables were performed by the Fisher's exact test and for continuous variables the non-parametric Mann–Whitney U test. The Kaplan–Meier method, analyzed survival probabilities while the multivariate analysis was performed using Cox proportional hazards model. The log-rank test was applied to assess the significance of differences among actuarial survival curves with a 95% confidence interval. A significance set for $p< 0.05$ was adopted. Analyses were performed using the statistical software package the Software SPSS statistics version 23.0 (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.) and GraphPad Prism 5.0 (San Diego, CA, USA).

RESULTS

Twenty-six proteins were differential expressed and enriched for biological processes between HR-HPV+ and HPV- OSCC

Nine patients younger than 40 years old with HR-HPV+ OSCC in advanced clinical stages were retrieved and matched for age and stage with eleven HR-HPV- **(Figure 1A)**. There were no significant differences between the groups in the other sociodemographic and clinicopathological features **(Supplementary appendix S2)**. Briefly, in both groups, males (66.7% in HPV+ and 54.5% HR-HPV-, p=0.67) with tobacco consumption (62.5% in HPV+ and 90.9% HR-HPV-, p=0.262) and tumors involving the tongue (37.5% in HPV+ and 40% HR-HPV-, p=0.84), histologically well differentiated (55.6% in HPV+ and 54.5% HR-HPV-, p=0.05), were most frequently affected. Surgery associated with radiotherapy was the most common treatment performed (55.6% in HPV+ and 45.5% HR-HPV-, p=1) and with negative surgical margins (85.7% in HPV+ and 63.6% HR-HPV-, p=0.59). Regarding survival, recurrences (77.8% of HR-HPV+ and 60% HR-HPV-, p=0.628), and the 5-year disease-free survival rate (22.2% in HR-HPV + patients and 37.5% in HR-HPV-, p=0.58), were similar in both groups. Seven patients with HR-HPV+ and 6 with HR-HPV tumors died due to tumor recurrence and the 5-year specific survival rate was 44.4% in the first group and 29.2% in the second group (p=0.83). The 5-year overall survival rate was 44.4% in the group with HR-HPV+ tumors and 0% in the HR-HPV- group (p=0.36) **(Supplementary appendix S3)**.

Proteomic analysis carried out in islands of neoplastic epithelial cells of the above samples, identified a total of 1,030 proteins and 39 were differentially expressed between HR-HPV+ and HR-HPV- patients **(Figure 1B, C)**. After the enriching for biological processes, 26 proteins were recognized of which 13 proteins were down regulated and 13 were up regulated in HR-HPV+ OSCC **(Supplementary appendix S4)**. The enrichment for biological process revealed that these proteins were principally associated with gene expression (34.7%), cell death (11.5%), viral process (7.7%), cytokine-mediated signaling pathway (7.7%), platelet degranulation (7.7%) and other functions (30.8%).

We also examined the connectivity degree between the identified proteins with proteins previously described in the literature as possible regulatory mechanisms in oral cavity tumors, as well as, the relation with miRNAs reported in The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/tcga). In doing so, NFkB pathway and Akt proteins were found in

relation with most of the proteins recognized in proteomic analysis **(Figure 1D)**. Results from miRNA prediction and connectivity degree are presented in **supplementary appendix S4**.

High expression of S100A8 is correlated with advanced clinical stage and worse survival

Notably, the proteomic findings suggested that the proteins differentially expressed between HPV+ and HPV- may have a possible role in oral carcinogenesis and tumor progession; however, to explore the clinical relevance of the proteins we next performed the Kaplan-Meier survival analysis to evaluate their prognostic impact. To our surprise, of the 26 differential expressed proteins only five were associated with survival outcomes. High expression of A2M and Serpine1 were correlated with an increase in the DFS **(Figure 2A)**. Otherwise, high expression of COPS3, DYHC1 and S100A8 decreased the DFS **(Figure 2B)**. S100A8 was the only protein that influenced CSS and OS **(Figure 2C)**. Furthermore, the multivariate Cox proportional regression analysis indicated A2M, Serpine1, COPS3, DYHC1 and S100A8 to be significant independent predictors for DFS. Once again, S100A8 was significant for CSS and OS in Cox proportional hazard model. OS was longer in the group with low expression of this protein, patients with high expression of S100A8 had a risk of death 3.68 higher than those with low expression **(Figure 2D)**.

S100A8 protein was selected for further investigation due to it presented the most relevant correlation with survival probability, a miRNA fold change of 1.97, and high connectivity degree **supplementary appendix S4**. Moreover, until now there is no study in the literature showing the biological function of this protein in HPV-related OSCC.

In order to validate the prognosis impact of S100A8 in a larger sample, an immunofluorescence assay for S100A8 protein was performed in a second set of 91 OSCC **(Figure 2E)**. The results indicated that higher levels of S100A8 were correlated with tumor size *(p*=0.030*),* clinical stage *(p*=0.007*)* and surgical margins *(p*=0.015*)* **(Figure 2F) (Supplementary appendix S5)**. Furthermore, the prognosis impact of S100A8 was confirmed for this sample, patients with high expression of S100A8 showed worse DFS (*p*=0.020), CSS (*p*=0.023) and OS (*p*=0.001) **(Figure 2G)**.

OSCC with high levels of S100A8 mRNA exhibit lower amount for macrophages M1 and iDC

S100A8 is a protein member of S100 family, that plays a role in the regulation of inflammatory processes and immune response (26). We next explored whether upregulation of S100A8 influences the inflammatory cell infiltration of tumors using the TCGA database and sorting the patients in top and bottom for S100A8 gene expression (using quartiles classification, top: ≥ 75% (Q3), bottom, ≤ 25%(Q1)).

Considering the samples with RNA-Seq data, a total of 301 patients were identified. Quantiles of 0.75 and 0.25 were used to separate top and bottom patients for the gene of interest (S100A8). This classification generated groups with 75/76 patients, respectively. The analysis considered 7 cells including lymph. B, lymph. TCD4, lymph. TCD8, NK cells, Neutrophil, Macrophage and Dendritic cells (DC) **(Supplementary appendix S6)**. The data indicated that patient's top for *S100A8* have fewer amount of macrophages and higher count of DC. Further, we assessed the subtypes of macrophages and DC altered. In doing so, M1 and iDC were identified **(Figure 3)**.

HPV related-OSCC exhibit high levels of S100A8

Collectively, our results demonstrated that S100A8 could impact the inflammatory profile and have an important role in tumor progression regardless of HPV status. However, our previous results of the proteomic analysis identified higher expression of S100A8 in HPV-related tumors (Fold change 1,8, p=0.004) **(Figure 4A) (supplementary appendix S4).** Thus, we decided to validate it and to study the impact of HPV in the inflammatory response.

In search for it, immunofluorescence for S100A8 was performed in a TMA containing 59 OSCC with known HPV status. Immunostaining was observed in the cytoplasm of tumor cells and few cases expressed a nuclei staining. Moreover, it was detected a positive staining in inflammatory cells located in the stroma **(Figure 4B)**. Quantification of positive tumor cells showed a higher expression in the HPV positive set. Although a significant p value could not be established (p=0.089), the simple logistic regression showed that tumors HPV positives has a 3.68 chance of higher expression of S100A8 than HPV negative **(Figure 4C)**.

Stimulation of TLR-4 through MPLAs activates NF-κB and S100A8 pathway in HPV positives cell lines but not in HPV negatives.

Overexpression of S100A8 has been reported in breast cancer and associated with tumor growth and invasion, through of the activation of RAGE, TLR4 and NF-κB pathway (27). To understand the role of S100A8 in HPV-related OSCC, first we evaluated the expression of TLR4 in the HNSCC cells lines HPV positives and negatives. As revealed by flow cytometry, all the cell lines expressed any levels of TLR4 **(supplementary appendix S7)**.

Next, we aimed to examine the levels of S100A8 and NF-κB after the stimulation of TLR4 exposing our four cell lines to MPLA, a synthetic monophosphoryl Lipid A that activates TLR4. Interestingly, we found that the activation of TLR4 resulted in different responses among the cell lines according to the HPV status. The HPV positive cell lines, UPCI-SCC154 and UM-SCC104 significantly increased the levels of NF-κB and S100A8 **(Figure 5A, B, C)**. On the other way, MPLAs administration in HPV negative cell lines only increase NF-κB protein in the cell line HN13, however, it did not alter the expression of S100A8 in both HPV negatives cell lines **(Figure 5D, E, F)**.

Activation of NF-κB and S100A8 pathway results in pro-inflammatory response in HPV positives cell lines.

NF-κB is a protein complex with a crucial role in the inflammatory response and immune cells function, through the production of pro-inflammatory cytokines, chemokine, adhesion molecules, and inflammatory mediators (28). Following our previous results that stimulation of TLR-4 through MPLAs activates NF-κB and S100A8 pathway in HPV positives cell lines but not in HPV negatives, we hypothesize that the inflammatory response also could be different according HPV status. In search of it, we analyzed the mRNA levels of the pro-inflammatory cytokines IL-6, IL-1B, and TNF-a after the administration of MPLAs to activate NF-κB and S100A8 pathway. Not surprisingly, we found an increase in the pro-inflammatory molecules after the stimulation of TLR-4 in the HPV positives cell lines, but did not find significant modifications in the HPV negative cell lines **(Figure 6)**.

DISCUSSION

OSCC has low survival rates worldwide, which is alarming given that half of the newly diagnosed patients will die beyond five years following treatment due to disease progression (29). Treatment protocols for recurrent or metastatic squamous cell carcinoma

comprise platinum-based chemotherapy plus anti-epidermal growth factor receptor monoclonal antibody (EXTREME regimen) (30); nevertheless, some patients are refractory to this therapy. Recently, immunotherapy emerges as second line treatment with a promising approach to reduce the risk of death in 30% (31, 32), and ongoing clinical trials are evaluating its efficacy as adjuvant therapy and first line treatment (ClinicalTrials.gov Identifier: NCT03355560, NCT02741570). Despite the efforts to develop new therapy options, cancer resistance is unavoidable in some cases due to tumor heterogeneity (33). A better understanding of all agents involved in the establishment and progression of oral cancer (i.e etiological and promoting factors, genetic and epigenetic alterations, molecular features, immune profile), will enable to discover new drugs and identify the set of patients that could benefit from a specific therapy (34). In search for that, PD-L1 expression and HPV status has been investigated as biomarkers to predict the response to immunotherapy (14, 35, 36). Of our interest, Zandberg et al. (14) found that immunotherapy doubled overall survival (OS) in oropharyngeal and non-oropharyngeal cancers HPV positives, compared with those negatives, suggesting that the presence of HPV in non-oropharyngeal tumors may has immunological implicances.

The HPV prevalence in OSCC is relatively low compared with rates reported for oropharyngeal cancer (7). This could be explained in part for differences in the histologic features. Although oral cavity and oropharynx membranes are covered for stratified squamous epithelium, the nonkeratinized mucosa and reticulated epithelium of invaginated crypts allow the HPV access to basal layer (13). As a result of that, HPV-related tumors have a prominent basaloid morphology permeated by lymphocytes and with a lobular growth. In addition to histological differences, this group of tumors has distinct tumor biology carrying fewer p53 mutations, and presents a better prognosis. While the HPV negative tumors affect more frequently patients older 60 years old, the profile of patients HPV positive is younger subjects (7). Kaminagakura et al. have also reported the high prevalence of HR-HPV DNA in this age group in OSCC (13) and the role of this oncogenic virus transcriptionally inactive in these tumors is unexplored. Therefore, exploring the proteomic profile of HR-HPV-related OSCC could lead us to identify the ability of the virus to modify the immune and inflammatory microenvironment.

Here, we found twenty-six differentially expressed proteins from microdissected tissue of HR-HPV DNA + OSCC, using LCM coupled mass spectrometry. Despite a few studies have explored the proteome in this set of tumors, to our knowledge, we are the first group to use this technique to this end, and also to combine bioinformatics tools and *in vitro*

assays to gain insights into underlying biological processes. Melle et al. (37) identified 18 proteins-related to HPV positive OSCC, and none was found in our proteome profile, however, the authors used p16 antibody for determinate HPV status and the accuracy of this biomarker in oral tumors is low (6). Subsequently, a quantitative proteomics-based study (38) recognized 155 proteins differentially expressed in three HNSCC cell lines, and five matched with our finding, SON, NDRG1, Serpin 1, RPL14 and 40S ribosomal protein S24. An important point to consider is that the study used 2 cell lines derived from oropharyngeal tumors and our sample was composed exclusively by oral cavity tissues.

In agreement with our results demonstrating overexpression of S100A8 in HPV-related tumors upon validation in an expanded cohort, Lo et al. (39), also identified this protein associate with HPV18+ OSCC. Moreover, we found that high expression of S100A8 is correlated with advanced clinical stage and worse survival. It has been showed that this protein promotes cell proliferation, tumorigenesis, and metastasis in anaplastic thyroid carcinoma, through the interaction with RAGE and activation of p38, ERK1/2, and JNK signaling pathways (40). In line with this, it was stabilized the interaction of S100A8, RAGE, and NF-κB signaling pathway, inducing epithelial-mesenchymal transition (EMT), and subsequent, lymph node and distant metastases in breast cancer (27).

In search to clarify the impact of HPV-DNA in NF-κb and S100A8 pathway, we exposed our four cell lines to MPLAs for 24 hours and our results showed that only HPV positive cell lines increase significantly the levels of both proteins. Abnormal function of NF-κb protein is involved in tumorigenesis stimulating cell proliferation, inhibiting apoptosis and favoring the angiogenesis and metastasis (28). Therefore, S100A8 may be a crucial player in onset and progression of HPV-related OSCC via NF-Kb signaling. Interestingly, S100A8 also influenced the survival in HPV negative patients, suggesting the presence of other pathways that need to be further investigated.

Our last result showed that NF-κb and S100A8 activation led to pro-inflammatory response only in HPV positives cell lines. One of the new generations of hallmark of cancer include the ability of neoplastic cells to promote inflammation. Inflammation provides a favorable microenvironment for tumor progression, invasion and metastasis through the production of growth factors pro-proliferative, survival factors to inhibit cell death, pro-angiogenic factors and extracellular matrix-modifying enzymes to activation of EMT. Thus, S100A8 may be a crucial participant in the initiation and progression of HPV-related OSCC,

through NF-κB and an increased inflammatory response, supporting the hypothesis that this virus infection may generate an immune response in oral cavity tumors (41).

The present study has the limitation of evaluating a restricted number of HPV + OSCC due to the low prevalence of this condition. It influenced to retrieve only formalin-fixed paraffin-embedded tissues, obtain a small area of tumor microdissected, and lack statistical power in the validation phase when compared the positive and negative tumors. We consider that this limitation did not comprise the results obtained, and conversely, they are evidence to continue exploring this field.

Combining our results, we conclude that HR-HPV DNA may be a contributing factor to tumor progession through the NF-Kb and S100A8 pathway. In addition, HPV DNA positivity in oral cancer may have an immunologic and inflammatory impact that might impact the response to cancer therapy.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

None declared.

REFERENCES

1. Egawa N, Egawa K, Griffin H, Doorbar J. Human Papillomaviruses; Epithelial Tropisms, and the Development of Neoplasia. Viruses. 2015;7(7):3863-90.
2. de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. Virology. 2004;324(1):17-27.

3. Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, et al. Mechanisms of human papillomavirus-induced oncogenesis. J Virol. 2004;78(21):11451-60.
4. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med. 2003;348(6):518-27.
5. Duensing S, Lee LY, Duensing A, Basile J, Piboonniyom S, Gonzalez S, et al. The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. Proc Natl Acad Sci U S A. 2000;97(18):10002-7.
6. Fakhry C, Lacchetti C, Rooper LM, Jordan RC, Rischin D, Sturgis EM, et al. Human Papillomavirus Testing in Head and Neck Carcinomas: ASCO Clinical Practice Guideline Endorsement of the College of American Pathologists Guideline. J Clin Oncol. 2018;36(31):3152-61.
7. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000;92(9):709-20.
8. de Abreu PM, Co ACG, Azevedo PL, do Valle IB, de Oliveira KG, Gouvea SA, et al. Frequency of HPV in oral cavity squamous cell carcinoma. BMC Cancer. 2018;18(1):324.
9. Annertz K, Rosenquist K, Andersson G, Jacobsson H, Hansson BG, Wennerberg J. High-risk HPV and survival in patients with oral and oropharyngeal squamous cell carcinoma - 5-year follow up of a population-based study. Acta Otolaryngol. 2014;134(8):843-51.
10. Duray A, Descamps G, Decaestecker C, Remmelink M, Sirtaine N, Lechien J, et al. Human papillomavirus DNA strongly correlates with a poorer prognosis in oral cavity carcinoma. Laryngoscope. 2012;122(7):1558-65.
11. Schwartz SR, Yueh B, McDougall JK, Daling JR, Schwartz SM. Human papillomavirus infection and survival in oral squamous cell cancer: a population-based study. Otolaryngol Head Neck Surg. 2001;125(1):1-9.
12. D'Souza G, Anantharaman D, Gheit T, Abedi-Ardekani B, Beachler DC, Conway DI, et al. Effect of HPV on head and neck cancer patient survival, by region and tumor site: A comparison of 1362 cases across three continents. Oral Oncol. 2016;62:20-7.
13. Kaminagakura E, Villa LL, Andreoli MA, Sobrinho JS, Vartanian JG, Soares FA, et al. High-risk human papillomavirus in oral squamous cell carcinoma of young patients. Int J Cancer. 2012;130(8):1726-32.
14. Zandberg DP, Algazi AP, Jimeno A, Good JS, Fayette J, Bouganim N, et al. Durvalumab for recurrent or metastatic head and neck squamous cell carcinoma: Results from a single-arm, phase II study in patients with >/=25% tumour cell PD-L1 expression who have progressed on platinum-based chemotherapy. Eur J Cancer. 2019;107:142-52.
15. Greene FL PD, Fleming ID, Fritz A, Balch CM. AJCC cancer staging handbook. Springer Verlag. 2002.
16. el-Naggar AK CJ, Grandis JR, Takata T, Slootweg PJ. Tumours of the oral cavity and mobile tongue. Lyon: IARC Press. 2017;4th ed.
17. Carnielli CM, Macedo CCS, De Rossi T, Granato DC, Rivera C, Domingues RR, et al. Combining discovery and targeted proteomics reveals a prognostic signature in oral cancer. Nat Commun. 2018;9(1):3598.
18. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. 2008;26(12):1367-72.
19. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res. 2011;10(4):1794-805.

20. Carazzolle MF, de Carvalho LM, Slepicka HH, Vidal RO, Pereira GA, Kobarg J, et al. IIS--Integrated Interactome System: a web-based platform for the annotation, analysis and visualization of protein-metabolite-gene-drug interactions by integrating a variety of data sources and tools. PLoS One. 2014;9(6):e100385.
21. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000;25(1):25-9.
22. Li B, Severson E, Pignon JC, Zhao H, Li T, Novak J, et al. Comprehensive analyses of tumor immunity: implications for cancer immunotherapy. Genome Biol. 2016;17(1):174.
23. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. Nat Methods. 2015;12(5):453-7.
24. Aran D, Hu Z, Butte AJ. xCell: digitally portraying the tissue cellular heterogeneity landscape. Genome Biol. 2017;18(1):220.
25. Miranda Galvis M, Santos-Silva AR, Freitas Jardim J, Paiva Fonseca F, Lopes MA, de Almeida OP, et al. Different patterns of expression of cell cycle control and local invasion-related proteins in oral squamous cell carcinoma affecting young patients. J Oral Pathol Med. 2018;47(1):32-9.
26. Wang S, Song R, Wang Z, Jing Z, Wang S, Ma J. S100A8/A9 in Inflammation. Front Immunol. 2018;9:1298.
27. Yin C, Li H, Zhang B, Liu Y, Lu G, Lu S, et al. RAGE-binding S100A8/A9 promotes the migration and invasion of human breast cancer cells through actin polymerization and epithelial-mesenchymal transition. Breast Cancer Res Treat. 2013;142(2):297-309.
28. Park MH, Hong JT. Roles of NF-kappaB in Cancer and Inflammatory Diseases and Their Therapeutic Approaches. Cells. 2016;5(2).
29. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.
30. Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med. 2008;359(11):1116-27.
31. Ferris RL, Blumenschein G, Jr., Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab vs investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-year long-term survival update of CheckMate 141 with analyses by tumor PD-L1 expression. Oral Oncol. 2018;81:45-51.
32. Bauml J, Seiwert TY, Pfister DG, Worden F, Liu SV, Gilbert J, et al. Pembrolizumab for Platinum- and Cetuximab-Refractory Head and Neck Cancer: Results From a Single-Arm, Phase II Study. J Clin Oncol. 2017;35(14):1542-9.
33. Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. Nat Rev Clin Oncol. 2018;15(2):81-94.
34. Le Tourneau C, Borcoman E, Kamal M. Molecular profiling in precision medicine oncology. Nat Med. 2019;25(5):711-2.
35. Ferris RL, Saba NF, Gitlitz BJ, Haddad R, Sukari A, Neupane P, et al. Effect of Adding Motolimod to Standard Combination Chemotherapy and Cetuximab Treatment of Patients With Squamous Cell Carcinoma of the Head and Neck: The Active8 Randomized Clinical Trial. JAMA Oncol. 2018;4(11):1583-8.
36. Cohen EEW, Soulieres D, Le Tourneau C, Dinis J, Licitra L, Ahn MJ, et al. Pembrolizumab versus methotrexate, docetaxel, or cetuximab for recurrent or metastatic head-and-neck squamous cell carcinoma (KEYNOTE-040): a randomised, open-label, phase 3 study. Lancet. 2019;393(10167):156-67.

37. Melle C, Ernst G, Winkler R, Schimmel B, Klussmann JP, Wittekindt C, et al. Proteomic analysis of human papillomavirus-related oral squamous cell carcinoma: identification of thioredoxin and epidermal-fatty acid binding protein as upregulated protein markers in microdissected tumor tissue. Proteomics. 2009;9(8):2193-201.
38. Descamps G, Wattiez R, Saussez S. Proteomic study of HPV-positive head and neck cancers: preliminary results. Biomed Res Int. 2014;2014:430906.
39. Lo WY, Lai CC, Hua CH, Tsai MH, Huang SY, Tsai CH, et al. S100A8 is identified as a biomarker of HPV18-infected oral squamous cell carcinomas by suppression subtraction hybridization, clinical proteomics analysis, and immunohistochemistry staining. J Proteome Res. 2007;6(6):2143-51.
40. Reeb AN, Li W, Sewell W, Marlow LA, Tun HW, Smallridge RC, et al. S100A8 is a novel therapeutic target for anaplastic thyroid carcinoma. J Clin Endocrinol Metab. 2015;100(2):E232-42.
41. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

Figure 1. Proteomic analysis was performed in cohort 1composed for twenty cases of OSCC affecting patients younger than forty years old in advanced clinical stage **(A).** Laser-capture microdissection was carried out in histological sections obtained from cohort 1 to evaluate only neoplastic epithelial cells **(B)**. Volcano plot showing identified proteins in the proteomic analysis. Dotted square is magnified on the heatmap indicating differential expressed proteins between HPV + e HPV – ($p < 0.05$) **(C).** Global protein expression profile in OSCC demonstrated a network focused in NF-κB. Up regulated (red) and down regulated (green) proteins in HPV + tumors were correlated with other molecules (white) obtained using Ingenuity Pathway Analysis **(D)**.

Figure 2. Five of the 26 identified proteins were associated with survival outcomes. High expression of A2M and Serpine1 were correlated with an increase in the DFS **(A).** Otherwise, high expression of COPS3, DYHC1 and S100A8 decreased the DFS **(B)**. S100A8 was the only protein that influenced CSS and OS **(C)** (*$p< 0.05$). Cox proportional regression analysis indicated A2M, Serpine1, COPS3, DYHC1 and S100A8 to be significant independent predictors for DFS and S100A8 was significant for CSS and OS **(D)**. Cohort 2 with ninety-one OSCC was used to validate the proteomic results **(E)**. High levels of S100A8 was correlated with T3/T4 tumors, advanced clinical stage **(F),** and decrease DFS, CSS and OS (¨ ¨$p>0.05$, *$p< 0.05$, **$p< 0.01$, *** $p < 0.001$).

Figure 3. The Cancer Genome Atlas database indicated that the patient's top for S100A8 has a fewer amount of antigen presentation cells, macrophages M1 and immature dendritic cells (**$p< 0.01$).

Figure 4. Proteomic analysis carried out in cohort 1 identified a higher expression of S100A8 in HPV-related tumors **(A)**. Immunofluorescence assay for S100A8 performed in cohort 2 showed immunostaining in the cytoplasm of tumor cells and in inflammatory cells located in the stroma **(B)**. Quantification of positive tumor cells showed a higher expression in the HPV positive set **(C)**.

Figure 5. Immunofluoresence staining of S100A8 and NF-κB in HPV + OSCC cell lines increase upon administration of MPLAs (**A, B, C**). MPLAs did not impact S100A8 and NF-Kb protein levels in HPV - OSCC cell lines **(D, E, F)** (¨ ¨$p>0.05$, *$p< 0.05$, **$p< 0.01$, ***$p < 0.001$).

Figure 6. mRNA levels of the pro-inflammatory cytokines IL-1B **(A)**, IL-6 **(B)**, and TNF-a **(C)** increase in the HPV + OSCC cell lines UPCI-SCC154 and UM-SCC104 after the administration of MPLAs but no in the HPV - OSCC cell lines HN6 and HN13.

Figure 7. Schematic representation of our results showing a tumor mass with high expression of S100A8 and low count of macrophages M1 that could impact in a decrease of survival **(A)**. On the other hand, in OSCC HPV infection may contribute to tumor progression through the NF-kB and S100A8 pathway **(B)** leading to a pro-inflammatory microenvironment **(C)**.

Figure 1.

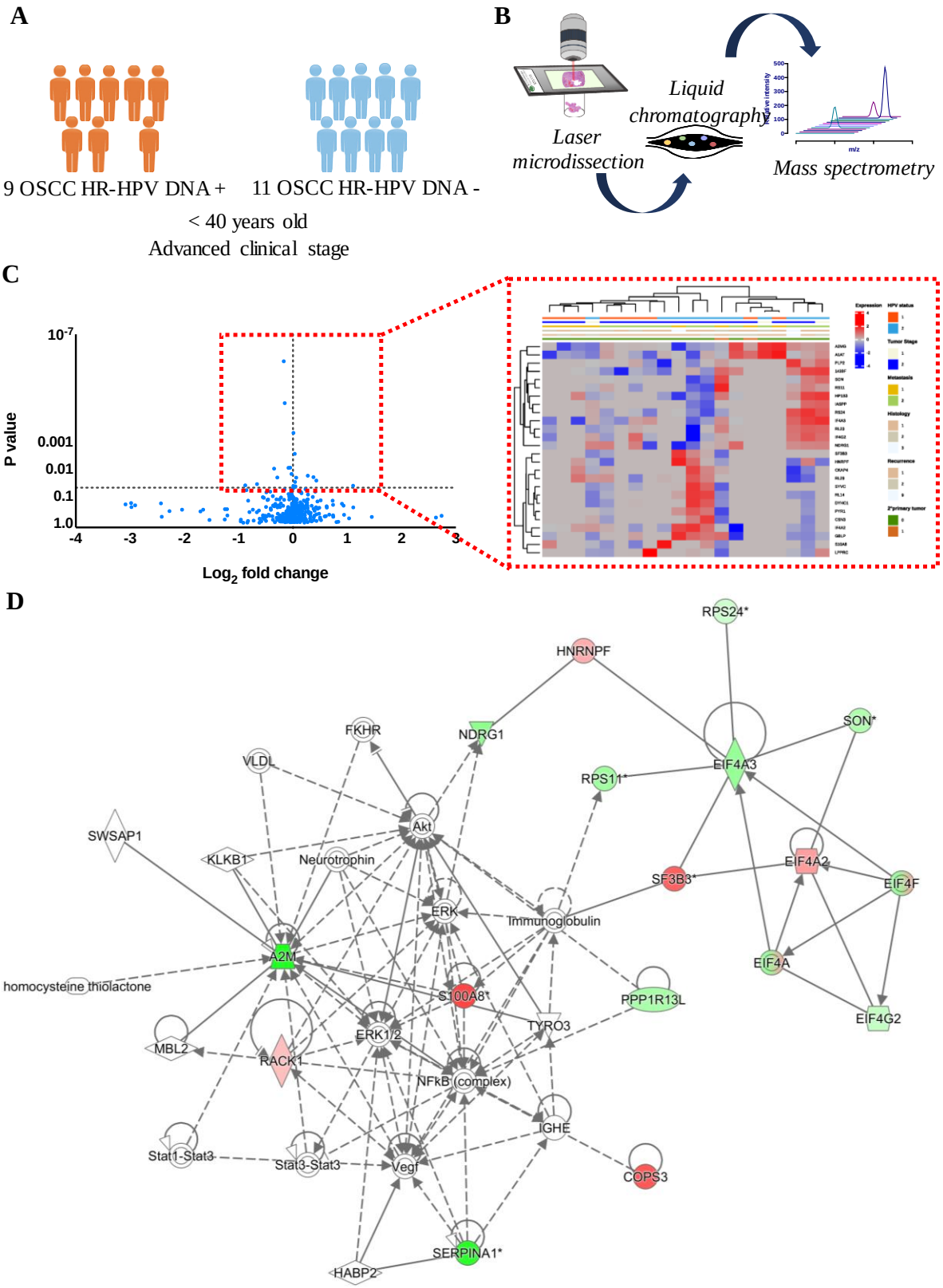

A
9 OSCC HR-HPV DNA +
11 OSCC HR-HPV DNA -
< 40 years old
Advanced clinical stage
B
Laser microdissection
Liquid chromatography
Mass spectrometry
C
P value
Log2 fold change
D
RPS24*
HNRNPF
FKHR
NDRG1
SON*
VLDL
EIF4A3
RPS11*
SWSAP1
Akt
KLKB1
Neurotrophin
SF3B3*
EIF4A2
EIF4F
ERK
Immunoglobulin
A2M
EIF4A
homocysteine thiolactone
S100A8
PPP1R13L
TYRO3
EIF4G2
MBL2
ERK1/2
RACK1
NFkB (complex)
IGHE
Stat1-Stat3
Stat3-Stat3
Vegf
COPS3
SERPINA1*
HABP2

Figure 2.

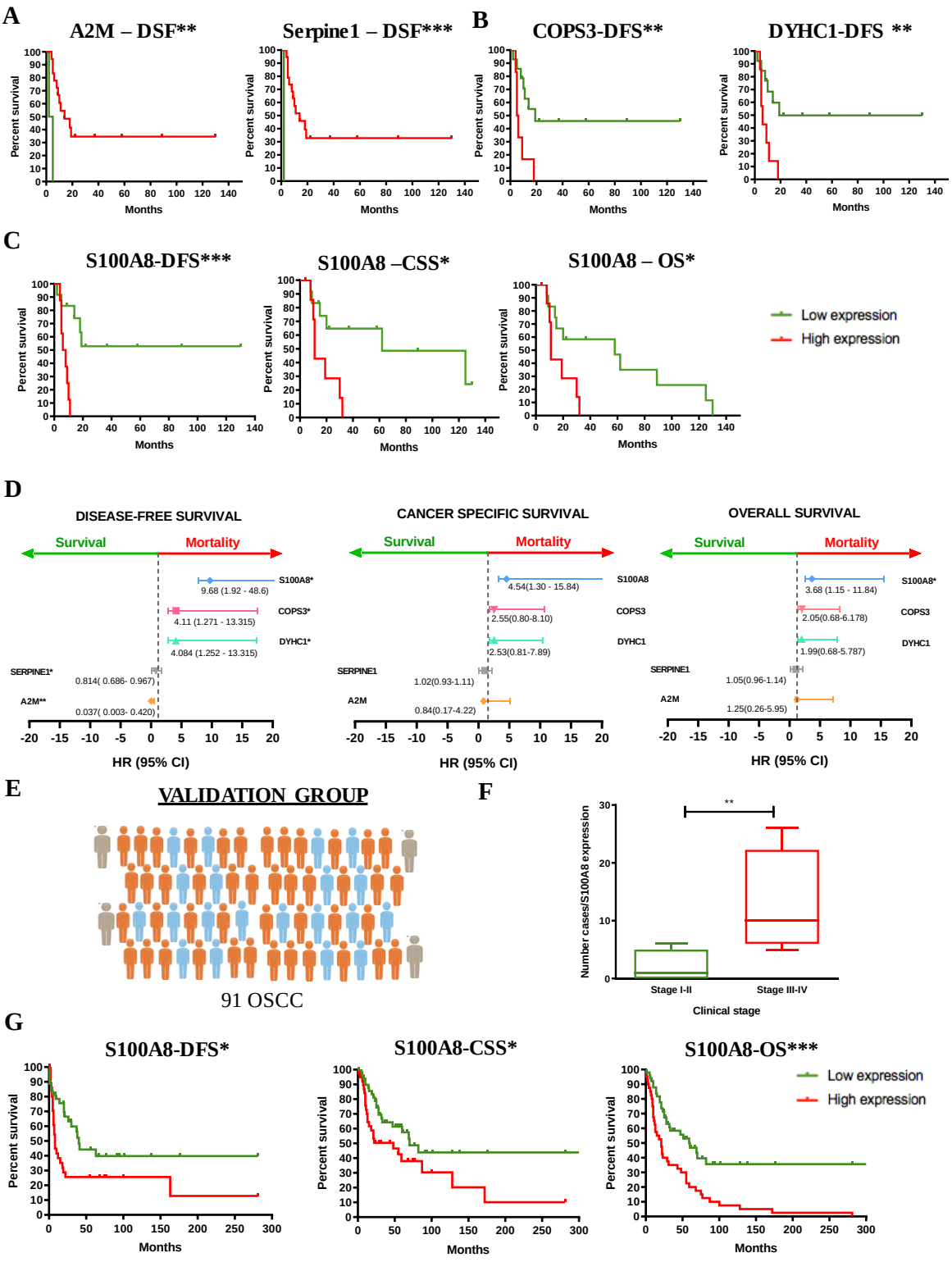

A
A2M – DSF**
Serpine1 – DSF***
B
COPS3-DFS**
DYHC1-DFS **
Percent survival
Months
C
S100A8-DFS***
S100A8 –CSS*
S100A8 – OS*
Low expression
High expression
D
DISEASE-FREE SURVIVAL
CANCER SPECIFIC SURVIVAL
OVERALL SURVIVAL
Survival
Mortality
S100A8*
9.68 (1.92 - 48.6)
COPS3*
4.11 (1.271 - 13.315)
DYHC1*
4.084 (1.252 - 13.315)
SERPINE1*
0.814(0.686- 0.967)
A2M**
0.037(0.003- 0.420)
S100A8
4.54(1.30 - 15.84)
COPS3
2.55(0.80-8.10)
DYHC1
2.53(0.81-7.89)
SERPINE1
1.02(0.93-1.11)
A2M
0.84(0.17-4.22)
3.68 (1.15 - 11.84)
2.05(0.68-6.178)
1.99(0.68-5.787)
1.05(0.96-1.14)
1.25(0.26-5.95)
HR (95% CI)
E
VALIDATION GROUP
91 OSCC
F
Number cases/S100A8 expression
Stage I-II
Stage III-IV
Clinical stage
G
S100A8-DFS*
S100A8-CSS*
S100A8-OS***

Figure 3.

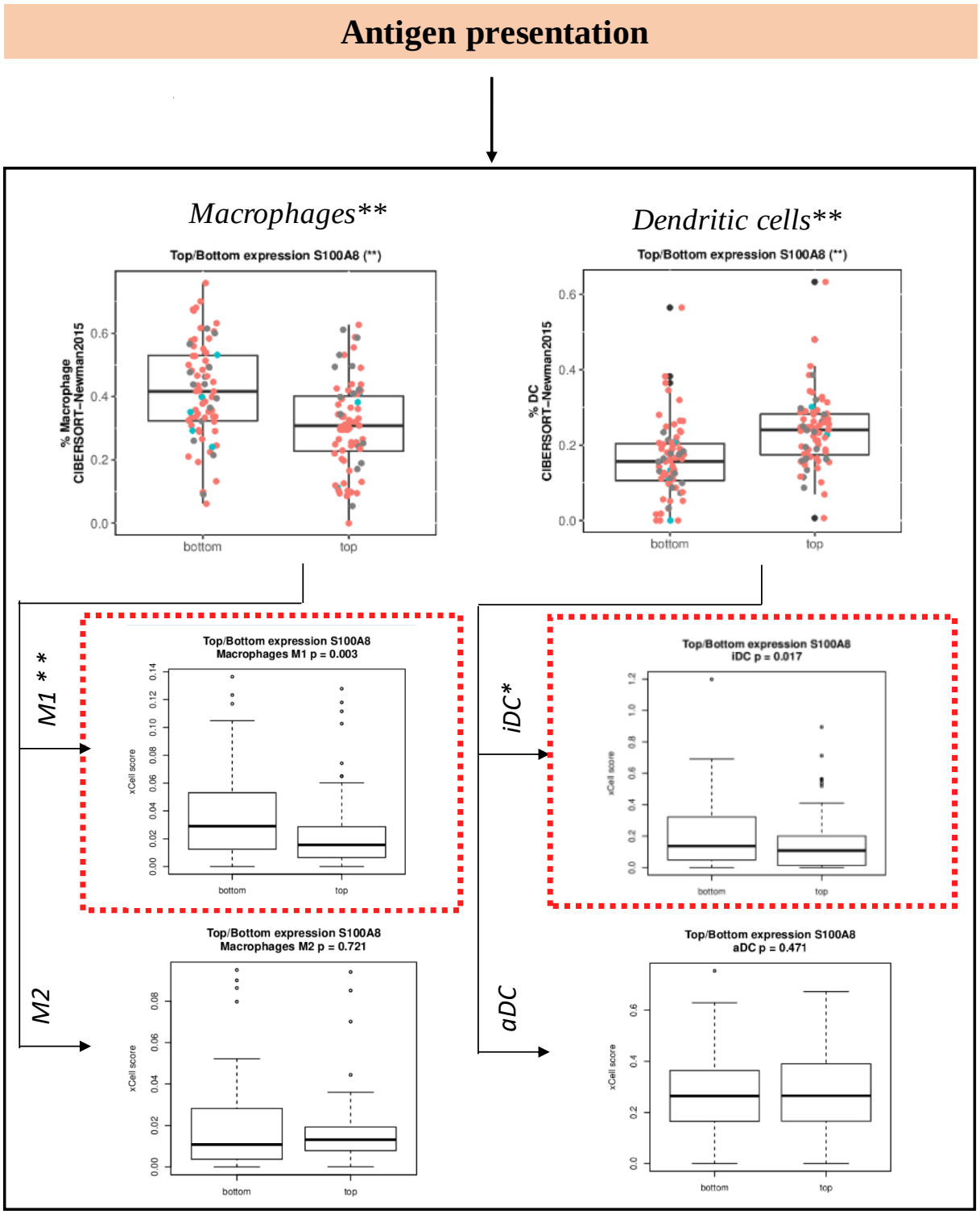
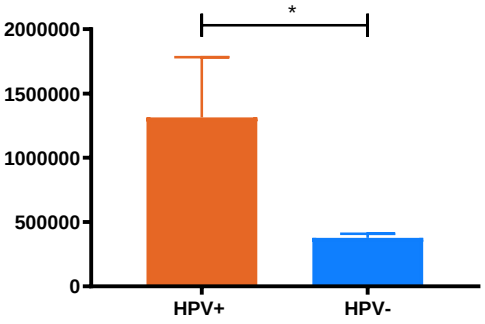

Antigen presentation
Macrophages**
Top/Bottom expression S100A8 (**)
% Macrophage CIBERSORT–Newman2015
bottom
top
Dendritic cells**
Top/Bottom expression S100A8 (**)
% DC CIBERSORT–Newman2015
bottom
top
M1 * *
Top/Bottom expression S100A8
Macrophages M1 p = 0.003
xCell score
bottom
top
iDC*
Top/Bottom expression S100A8
iDC p = 0.017
xCell score
bottom
top
M2
Top/Bottom expression S100A8
Macrophages M2 p = 0.721
xCell score
bottom
top
aDC
Top/Bottom expression S100A8
aDC p = 0.471
xCell score
bottom
top

Figure 4.

A

DISCOVERY GROUP

S100A8 LFQ INTENSTY

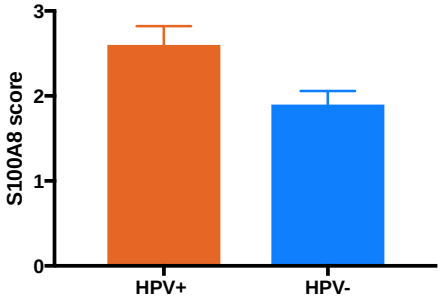


Variable	Fold change	*p* value
HPV+/ HPV-	1,8	0.004

B

VALIDATION GROUP

S100A8 TMA QUANTIFICATION



		Low	High	*p* Value*	OR	IC 95%	*p* value**
HPV	+	61.2	38.8	0.089	3.68	0.84-16.0	0.082
	-	30	70				

*Fisher Exact test
**Simple logistic regression

E

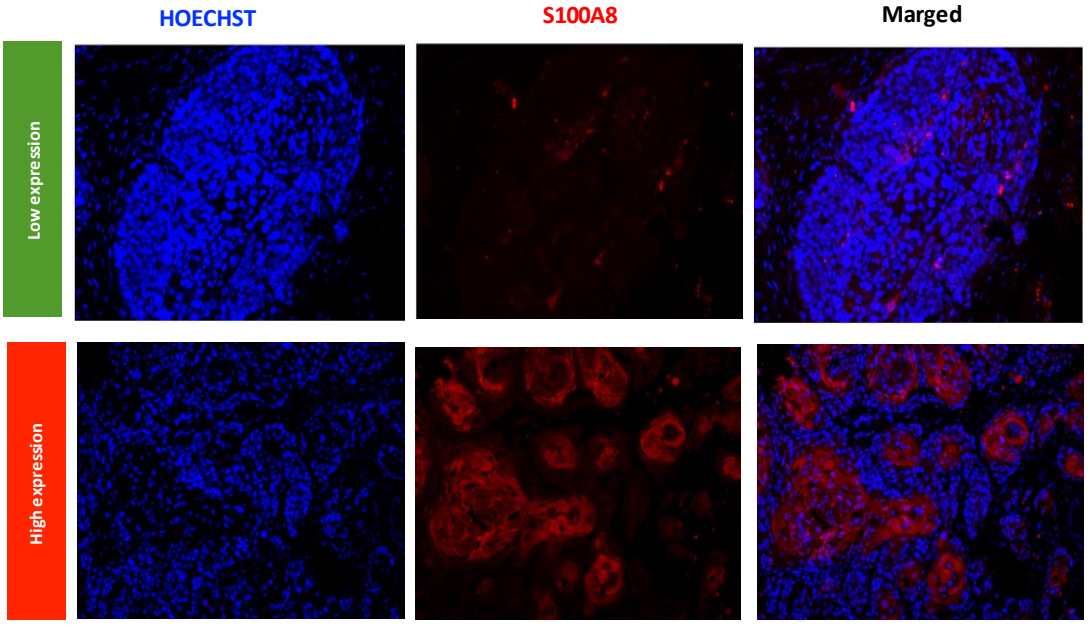


Figure 5.

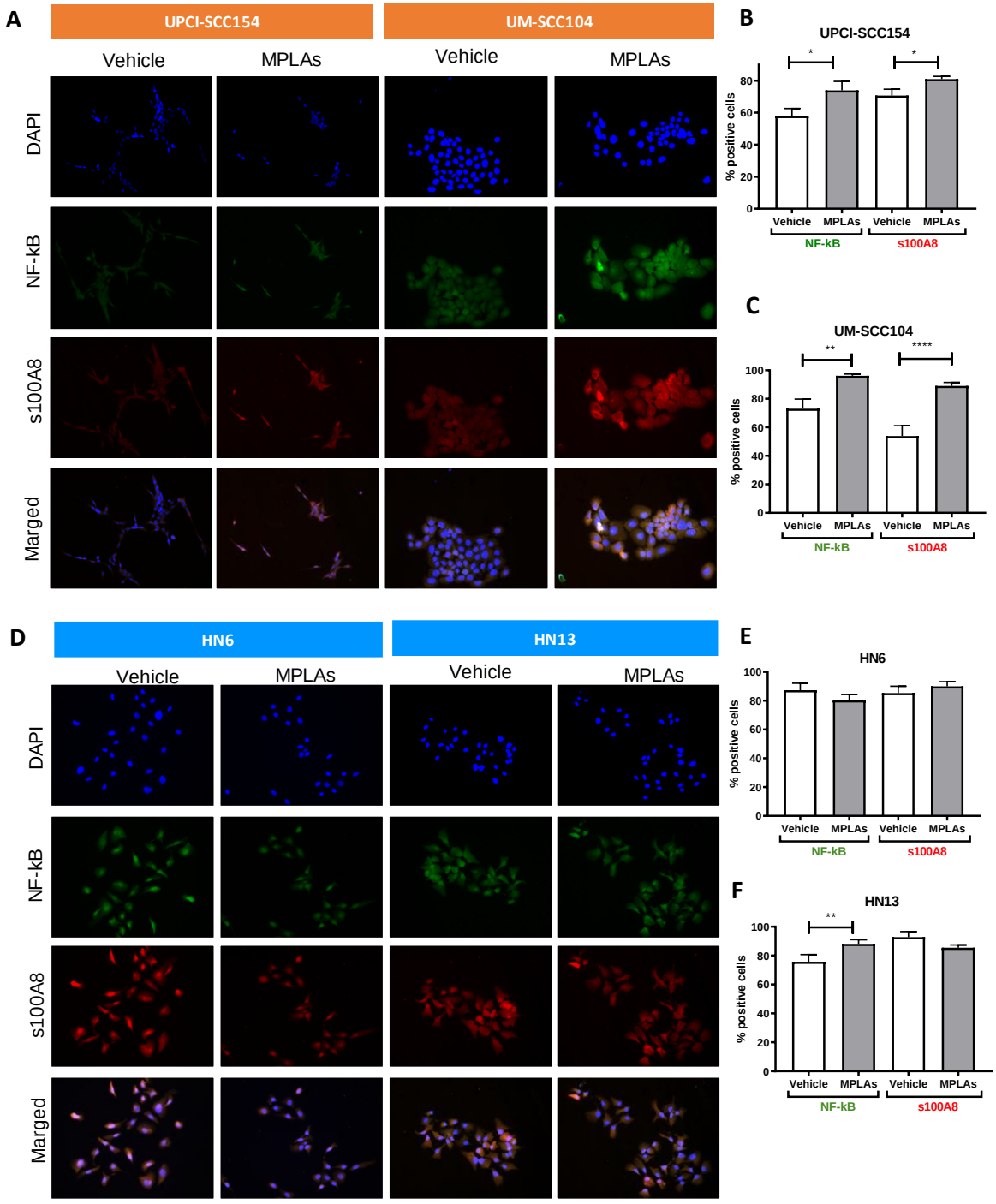

A
UPCI-SCC154
UM-SCC104
Vehicle
MPLAs
Vehicle
MPLAs
DAPI
NF-kB
s100A8
Marged
B
UPCI-SCC154
% positive cells
0
20
40
60
80
Vehicle
MPLAs
Vehicle
MPLAs
NF-kB
s100A8
C
UM-SCC104
% positive cells
0
20
40
60
80
100
Vehicle
MPLAs
Vehicle
MPLAs
NF-kB
s100A8
D
HN6
HN13
Vehicle
MPLAs
Vehicle
MPLAs
DAPI
NF-kB
s100A8
Marged
E
HN6
% positive cells
0
20
40
60
80
100
Vehicle
MPLAs
Vehicle
MPLAs
NF-kB
s100A8
F
HN13
% positive cells
0
20
40
60
80
100
Vehicle
MPLAs
Vehicle
MPLAs
NF-kB
s100A8

Figure 6.

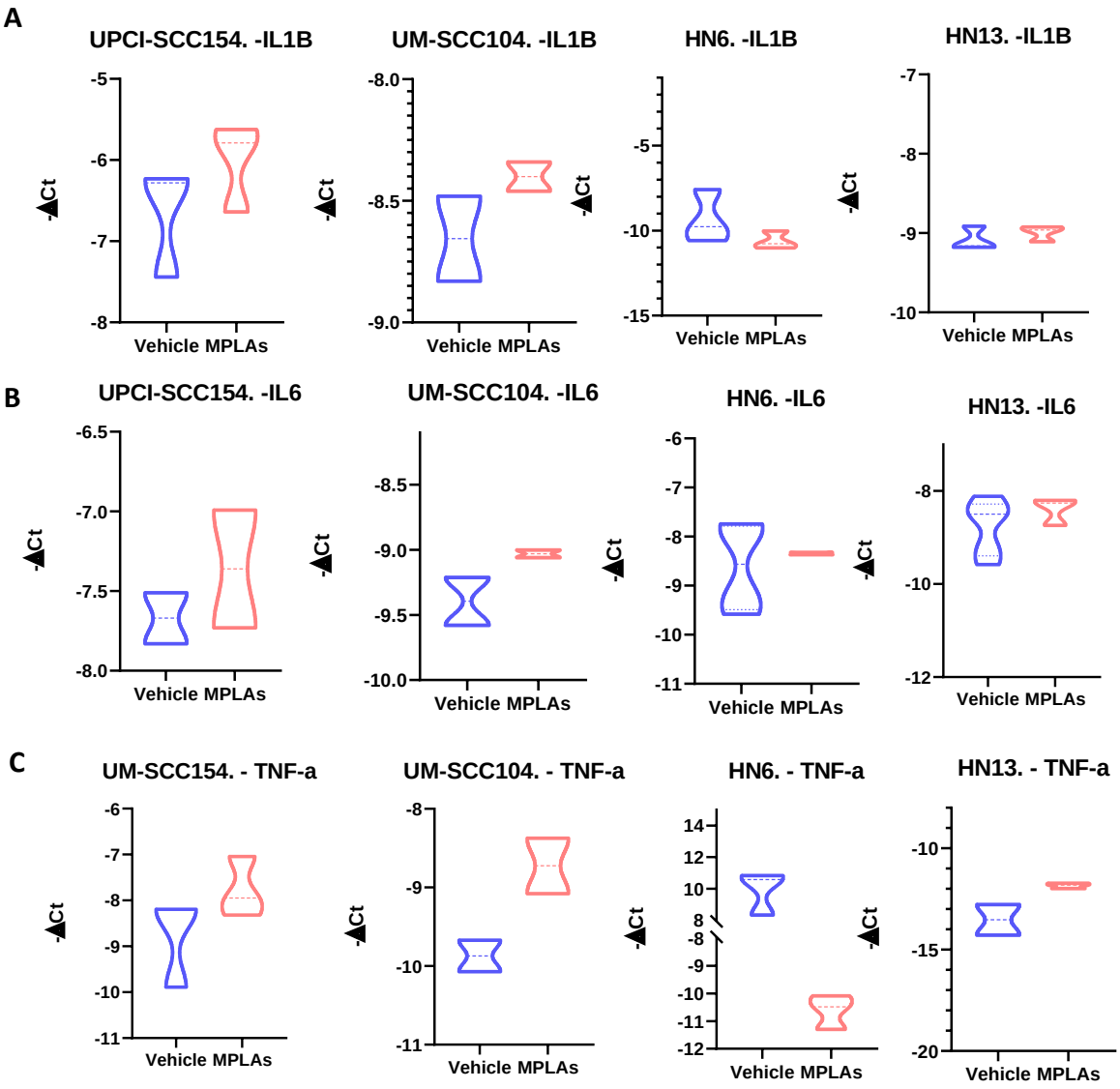
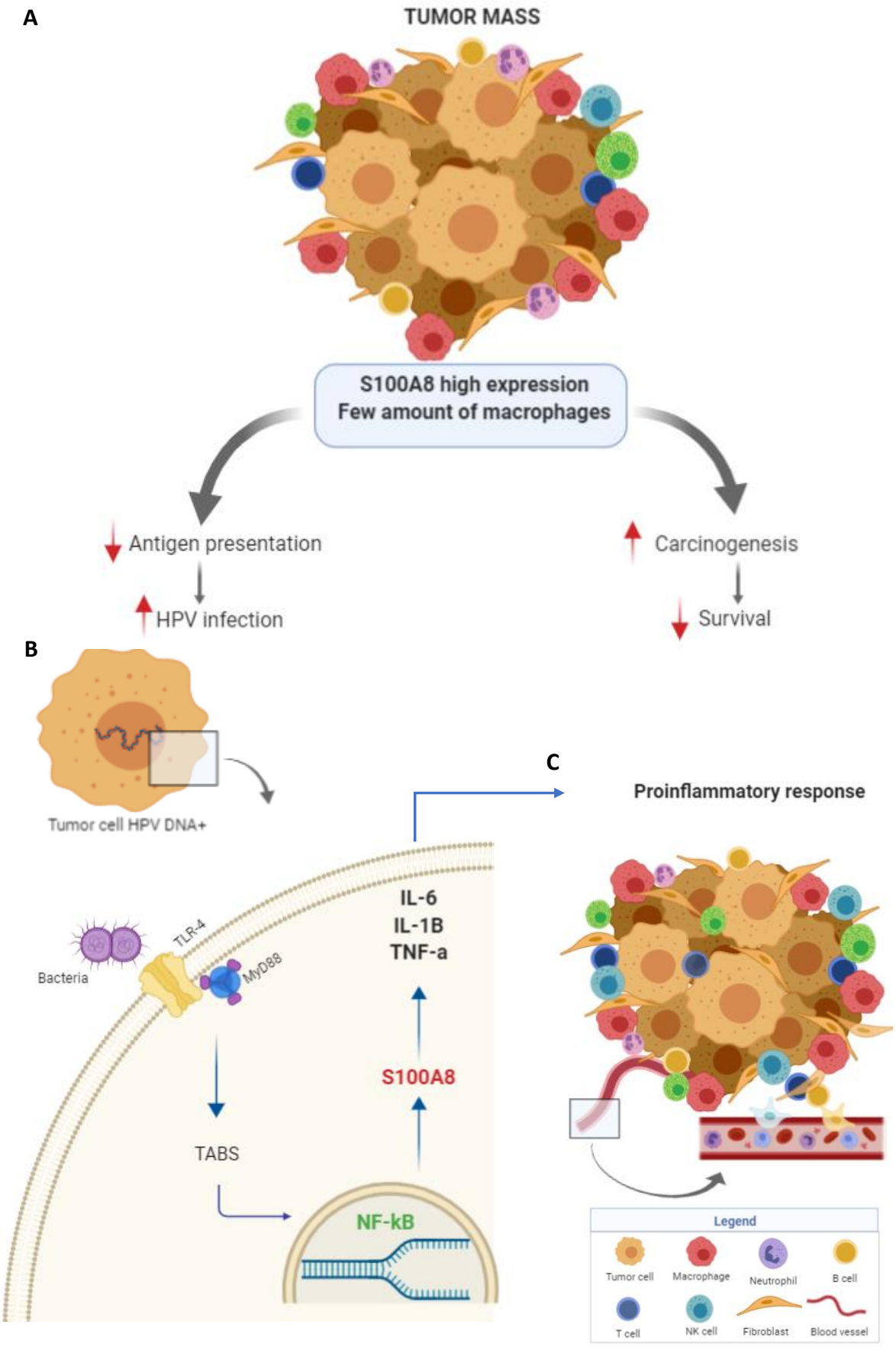

A
UPCI-SCC154. -IL1B
UM-SCC104. -IL1B
HN6. -IL1B
HN13. -IL1B
-ΔCt
Vehicle MPLAs
B
UPCI-SCC154. -IL6
UM-SCC104. -IL6
HN6. -IL6
HN13. -IL6
C
UM-SCC154. - TNF-a
UM-SCC104. - TNF-a
HN6. - TNF-a
HN13. - TNF-a

Figure 7.


A
TUMOR MASS
S100A8 high expression
Few amount of macrophages
Antigen presentation
HPV infection
Carcinogenesis
Survival
B
Tumor cell HPV DNA+
C
Proinflammatory response
IL-6
IL-1B
TNF-a
TLR-4
Bacteria
MyD88
S100A8
TABS
NF-kB
Legend
Tumor cell
Macrophage
Neutrophil
B cell
T cell
NK cell
Fibroblast
Blood vessel

SUPPLEMENTARY APPENDIX

S1. Oligonucleotide sequences used in qRT-PCR.

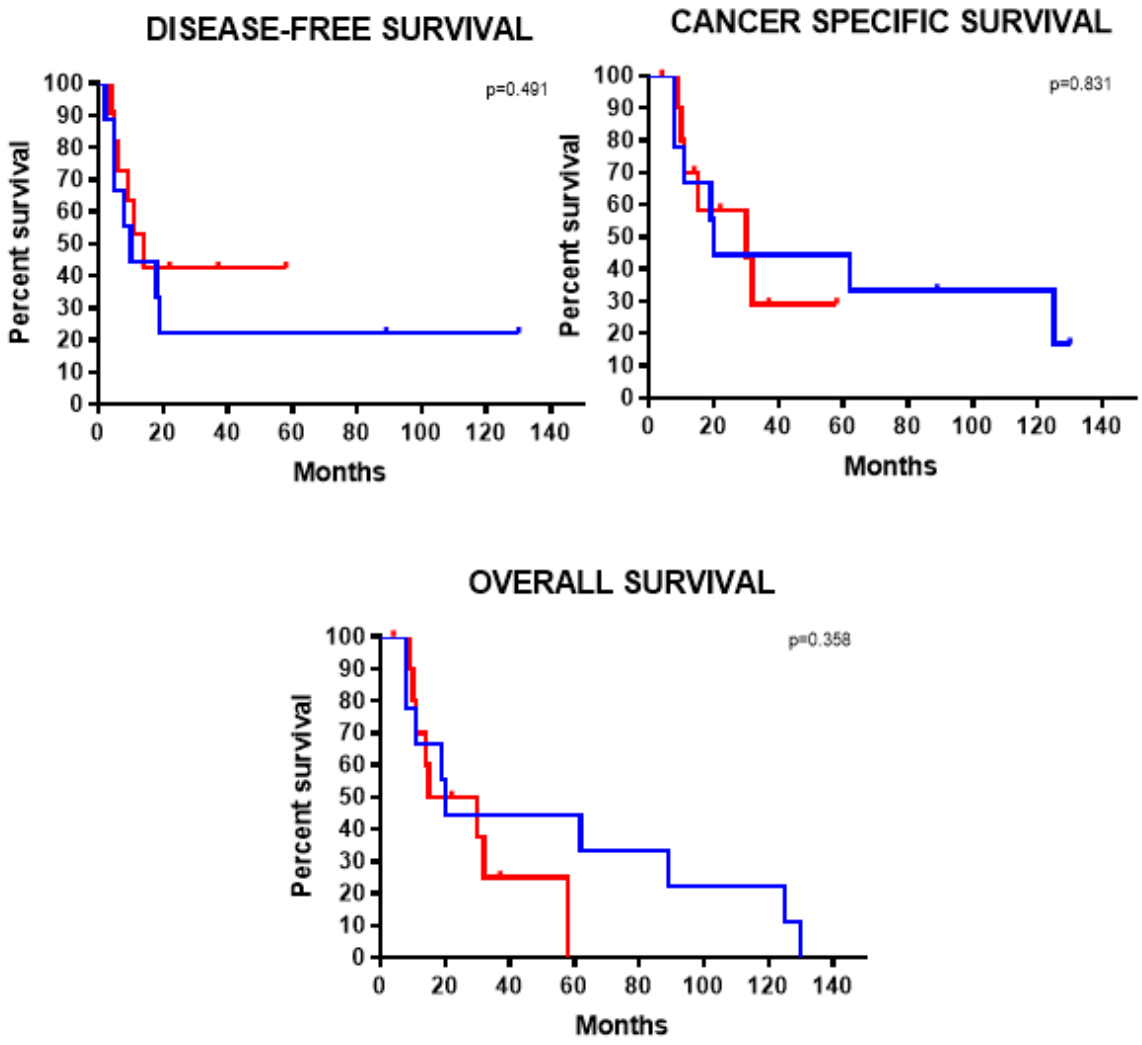
GENE	**(5’ → 3’)**	
TNF-A	GAGGCCAAGCCCTGGTATG	Forward
	CGGGCCGATTGATCTCAGC	Reverse
IL-1B	TTCGACACATGGGATAACGAGG	Forward
	TTTTTGCTGTGAGTCCCGGAG	Reverse
IL-6	ACTCACCTCTTCAGAACGAATTG	Forward
	CCATCTTTGGAAGGTTCAGGTTG	Reverse
IL-10	TCAAGGCGCATGTGAACTCC	Forward
	GATGTCAAACTCACTCATGGCT	Reverse
IRAK3	CAGCCAGTCTGAGGTTATGTTT	Forward
	TTGGGAACCAACTTTCTTCACA	Reverse
DDX58 (RIG-I)	TGCGAATCAGATCCCAGTGTA	Forward
	TGCCTGTAACTCTATACCCATGT	Reverse
MAVS	TTCTAATGCGCTCACCAATCC	Forward
	CCATGCTAGTAGGCACTTTGGA	Reverse
IRF7	GCTGGACGTGACCATCATGTA	Forward
	GGGCCGTATAGGAACGTGC	Reverse
GAPDH	ACCCACTCCTCCACCTTTGAC	Forward
	CCACCACCCTGTTGCTGTAG	Reverse

S2. Sociodemographic and clinicopathological features.

Feature	*HR-HPV positive*	*HR-HPV negative*	*p value*
	n (%)		
Age			
Mean	34,7	32,7	
Median	37	35	*0,656*
Range	20-40	20-39	
Sex			
Male	6 (66,7)	6 (54,5)	*0,670*
Female	3 (33,3)	5 (45,5)	
Tobacco consumption	5 (62,5)	10 (90,9)	*0,262*
Yes	3 (37,5)	1 (9,1)	
No			
Alcohol consumption			
Yes	3 (37,5)	10 (90,9)	*0,041*
No	5 (62,5)	1 (9,1)	
Anatomical site			
Tongue	3 (37,5)	4 (40)	*0,84*
Floor of the mouth	3 (37,5)	2 (20)	
Other	2 (25)	4 (40)	
T classification			
T1/T2	0 (0)	3 (27,3)	*0,218*
T3/T4	9 (100)	8 (72,7)	
N classification			
N0	5 (55,6)	3 (27,3)	*0,362*
N1-N3	4 (44,4)	8 (72,7)	
Histological differentiation			
I	5 (55,6)	6 (54,5)	*0,055*
II	4 (44,4)	3 (27,3)	
III	0 (0)	2 (18,2)	
Surgical margins			
Negative	6 (85,7)	7 (63,6)	*0,596*
Positive	1 (14,3)	4 (36,4)	
Treatment			
Surgery	3 (33,3)	4 (36,4)	*1*
Surgery +RT	5 (55,6)	5 (45,5)	
Surgery +RT + CTX	1 (11,1)	2 (18,2)	
Recurrence			
Yes	7 (77,8)	6 (60)	*0,628*
No	2 (22,2)	4 (40)	

Abbreviations: HR-HPV, High-risk Human Papillomavirus. RT, radiotherapy. CTX, chemotherapy.

S3. Disease-free survival, cancer specific survival and overall survival between HPV positive (blue) and HPV negative tumors (red).



S4. Differentially expressed proteins between HPV+ and HPV - OSCC samples.

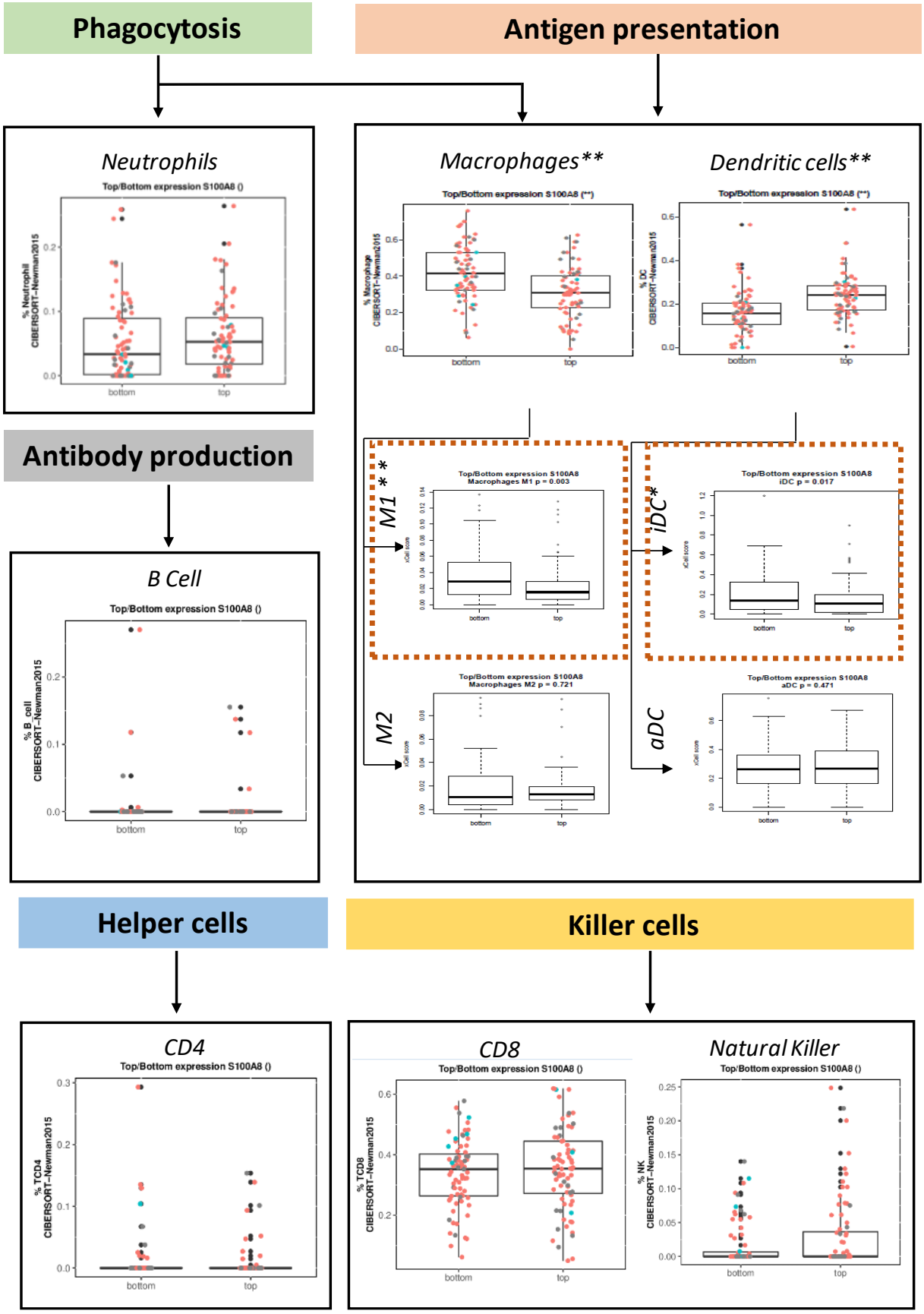
Gene	Protein	*p* value	LFQ	miRNA fold change	Pathways
Up-regulated proteins					
RACK1	Receptor of activated protein C kinase 1	0.044	0.61	-	-
CAD	CAD protein	0.019	1.99	-	-
RPL14	60S ribosomal protein L14	0.002	1.12	1.72	64
RPL29	60S ribosomal protein L29	0.048	0.68	-	-
EIF4A2	Eukaryotic initiation factor 4A-II	0.038	0.94	1.90	33
HNRNPF	Heterogeneous nuclear ribonucleoprote	0.021	0.77	-	-
SF3B3	Splicing factor 3B subunit 3	0.029	1.52	-	-
VARS	Valine—tRNA ligase	0.019	1.66	-	-
DYNC1H1	Cytoplasmic dynein 1 heavy chai1	0.001	1.49	-1.42	6
LRPPRC	Leucine-rich PPR motif-containing pro	0.042	1.02	3.11	11
CKAP4	Cytoskeleton-associated protein 4	0.038	1.50	2.00	13
COPS3	COP9 signalosome complex subunit 3	0.023	1.60	2.40	4
S100A8	Protein S100-A8	0.004	1.80	1.97	7
Down-regulated proteins					
RPL23	60S ribosomal protein L23	0.039	-0.99	-	-
RPS11	40S ribosomal protein S11	0.023	-1.85	-	-
RPS24	40S ribosomal protein S24	0.037	-0.975	-	-
EIF4A3	Eukaryotic initiation factor 4A-III	0.004	-2.03	-	32
NDRG1	NDRG1	0.001	-2.24	5.86	7
EIF4G2	Eukaryotic translation initiation factor	0.008	-1.14	3.11	17
PLP2	Proteolipid protein 2	0.012	-1.77	1.47	14
SERPINA1	Alpha-1-antitrypsin	0.009	-4.20	1.74	9
A2M	Alpha-2-macroglobulin	0.004	-4.47	1.85	2
HP1BP3	Heterochromatin protein 1-binding prot	0.042	-1.04	2.58	1
SON	SON	0.016	-1.52	-	-
PPP1R13L	RelA-associated inhibitor	0.015	-1.67	-	-
YWHAH	14-3-3 protein eta	0.017	-1.73	-	-

S5. Correlation between S100A8 expression and clinicopathological features.

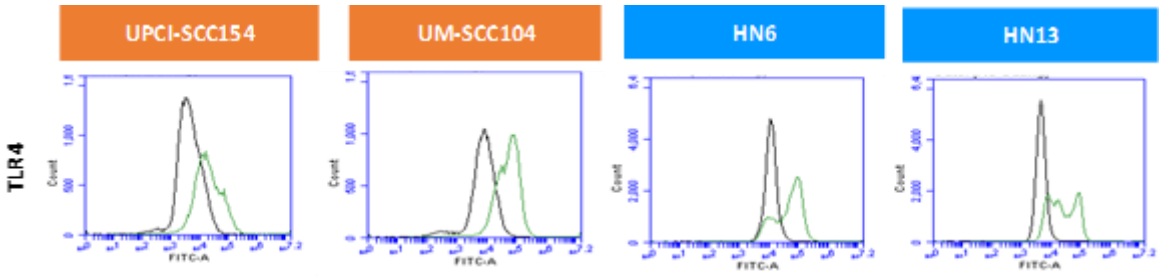
		S100A8		
Variable	***Category***	***Low***	***High***	***p***
Sex	***Male***	75.8	76.9	*0.917*
	Female	24.2	23.1	
Tobacco	***Yes***	83.9	90.5	*0.494*
	No	16.1	9.5	
Alcohol	***Yes***	64.5	66.7	*0.873*
	No	35.5	33.3	
Anatomical site	***Tongue***	43.3	50	*0.816*
	Floor m.	33.3	25	
	Other	23.3	25	
T	***T1-T2***	36.4	11.5	***0.030****
	T3-T4	63.6	88.5	
N	***N0***	0	0	*ND*
	N1-N3	100	100	
Clinical stage	**I-II**	24.2	0	***0.007****
	III-IV	75.8	100	
Histological classification	***I***	60.6	72	*0.448*
	II	30.3	16	
	III	9.1	12	
Surgical margins	***Negative***	96.3	71.4	***0.015****
	Positive	3.7	28.6	
Recurrence	***Yes***	48.5	26.9	0.092
	No	51.5	73.1	

**Statistically significant difference.*

S6. Immune cells infiltrate composition according S100A8 expression.



S7. TLR4 expression in cell lines.



2.2 Efficacy and safety of immunotherapy for head and neck cancer with focus on HPV status and PD-L1 expression: A systematic review and meta-analysis

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ABSTRACT

Background: Despite the multimodalities employed in the management of head and neck squamous cell carcinoma (HNSCC), long-term disease control in the second line is challenging, with low response rate (RR) and overall survival (OS). In this sense, novel therapeutic modalities were desperately needed and immunotherapy emerges as a promising therapeutic approach. **Objective**: To summarize the evidence to determine the effects of checkpoint inhibitors and costimulatory agonists on the RR and OS, as well as its safety and tolerability. **Methods:** Clinical trials assessing checkpoint inhibitors or costimulatory agonists as treatment for HNSCC were systematically retrieved using the databases PubMed, Cochrane, EMBASE, SCOPUS, and Web of Science. **Results:** Eleven clinical trials evaluating a total of 1,860 patients met the inclusion criteria. These studies demonstrated that the use of immunotherapy decreases the risk of death in 23%, compared to standard therapy (HR 0.77, 95% IC 0.68-0.87 $p<0.001$). Additionally, a noticeable advantage was observed for HPV positive and PD-L1-expressing tumors treated with immunotherapy. Treatment-related adverse events grade 3 or 4 were more prevalent in the patients who received standard therapy that who were treated with immunotherapy (37% vs 13%). **Conclusion:** Our findings support the use of checkpoint inhibitors and costimulatory agonists for the treatment of HNSCC, since the therapeutic agents showed safety profile and significant improvement antitumor activity restoring the immunosuppressive tumor microenvironment in HNSCC.

Keywords. Head and neck cancer; immunotherapy; HPV; PD-L1; meta-analysis.

INTRODUCTION

The global cancer statistics (GLOBOCAN) estimated for 2018 more than 905,000 new cases and 358,000 deaths due to head and neck squamous cell carcinoma (HNSCC), affecting the oropharynx, oral cavity, hypopharynx, and larynx (1). The low survival rates associated to HNSCC are related to the fact that most patients are diagnosed and treated in advanced clinical stages, mostly characterized by locoregional metastasis, extracapsular lymph node spread, vascular and/or lymphatic invasion and compromised surgical margins, with a high risk of recurrence and metastasis (2).

First-line treatment for recurrent and/or metastatic HNSCC (R/M HNSCC) patients who are not eligible to salvage surgery or radiotherapy was the EXTREME regimen, comprised of platinum-based chemotherapy, fluorouracil, and cetuximab (3). For subjects with progressive disease, second-line therapeutic options were limited to single-agent chemotherapy, such as methotrexate, docetaxel or cetuximab. Despite the multimodalities employed, a long-term disease control is challenging, with a response rate (RR) in second line of around 4% and a median overall survival (OS) of less than 10 months (4). Therefore, novel therapeutic modalities were desperately needed.

Immunotherapy emerges as a promising therapeutic approach, aiming to restore the immunosuppressive tumor microenvironment in HNSCC (5). Antitumor immune alterations in HNSCC comprise a reduction in the number and activity of natural killer cells, failure in the antigen presentation, and dysfunctional T cells that express checkpoint molecules such as Programmed cell death protein 1 (PD-1) and/or Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (6). Thereby, checkpoint inhibitors and costimulatory agonists are currently the most investigated immunomodulatory therapies, with ongoing or concluded phase I, II and III clinical trials and promising results already reported (7).

All considered, we conducted a systematic review and meta-analysis on clinical trials that evaluated the effects of checkpoint inhibitors and costimulatory agonists on the response rates and survival, as well as the safety and tolerability of such therapeutic agents as treatment options for HNSCC.

METHODS

Protocol and registration

This systematic review was reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist (8). A review protocol was submitted and approved to the Prospective Register of Systematic Reviews (PROSPERO) register (CRD42019120521).

Eligibility criteria

Inclusion criteria

The inclusion criteria were based on the PICOS questions (Population, Intervention, Comparison, Outcome, and Study Design). We included clinical trials that assessed checkpoint inhibitors or costimulatory agonists as treatment for patients with HNSCC.

Exclusion criteria

The following exclusion criteria were considered: 1) Studies on conditions other than head and neck squamous cell carcinoma; 2) Studies that did not assess checkpoint inhibitors or costimulatory agonists; 3) Studies in which survival measures, response rate, or treatment-related adverse events were not presented; 4) Pre-clinical, observational, retrospective or case report studies; 5) Studies in which the results on head and neck squamous cell carcinoma could not be individualized; 6) Studies that reported duplicated data (results that were previously published); 7) Studies written in languages other than English; 8) Reviews, letters, trial protocols, personal opinions and book chapters.

Information sources and search strategy

Individual search strategies were designed for each of the following databases: PubMed, Cochrane, EMBASE, SCOPUS, and Web of Science **(Appendix 1)**. A gray literature search was conducted on Google Scholar and ProQuest. The search strategy used for PubMed was as following: ((Head and Neck Neoplasms[MeSH Terms]) **OR** ("Head and neck cancer" OR "head and neck carcinoma" OR "HNSCC" OR "head and neck squamous cell carcinoma" OR "oral cancer" OR "oral squamous cell carcinoma" OR "oral carcinoma" OR "OSCC" OR "oropharynx cancer" OR "oropharynx squamous cell carcinoma" OR "larynx cancer" OR

"larynx squamous cell carcinoma" OR "hypopharynx cancer" OR "hypopharynx squamous cell carcinoma")) **AND** ((Immunotherapy OR "immune-therapy" OR "Immunomodulator" OR "Immunomodulation" OR "checkpoint inhibitors" OR "Immune Checkpoints" OR "Costimulatory Agonists" OR "CTLA-4 Inhibitors" OR "PD1 inhibitors" OR "PD-L1 inhibitors " OR "Anti-PD1" OR "Anti-PD-L1" OR "Anti-CTLA-4") **OR** (Pembrolizumab OR Nivolumab OR Durvalumab OR Avelumab OR Tremelimumab OR Urelumab OR Ipilimumab) **OR** (Immunotherapy[MeSH Terms])). The search included all articles published on or before January 10th, 2019, with no time restrictions nor limits. Duplicated references were removed by reference manager software (EndNote®, Thomson Reuters). Additionally, the reference lists of selected articles were hand-screened for potentially relevant studies.

Study selection

Study selection was completed in two phases. In phase 1, two authors (MMG and GAB) independently reviewed the titles and abstracts of all references and selected the studies that met the inclusion criteria. In phase 2, these studies were fully assessed by the same two authors, and the inclusion criteria were applied independently. A third author (TBO), an expert in Clinical Oncology, was consulted if disagreements were not solved by consensus between the two reviewers in both phases of study selection. The articles excluded in phase 2 are listed in **Appendix 2**.

Conference abstracts that met the selection criteria were collected, only as means to define the ongoing clinical trials. They were not included in the qualitative or quantitative analyses. Multiple publications resultant from the same trial (registered under the same NCT number) were considered a single study, for the purpose of the analyses. In these cases, the most updated (last published) article was used as source for the outcome data, while methodology and additional information were obtained from the main (primary) publication.

Data collection process and data items

One author (MMG) collected the key information from the selected studies. Another author (GAB) crosschecked the information and confirmed its accuracy. Any disagreement was resolved by discussion and mutual agreement, and other authors were involved whenever necessary. The following information was collected: study characteristics (Trial number ID, author, year, country, and study design); patients' characteristics (sample size and disease characterization); intervention and comparison; results; and main conclusion.

Risk of bias in individual studies

The risk of bias of randomized controlled trials was assessed by the Joanna Briggs Institute (JBI) Critical Appraisal Checklist for Randomized Controlled Trials (9), while non-randomized studies were evaluated by the JBI Critical Appraisal Checklist for Quasi-Experimental Studies (9). Two authors (MMG and GAB) independently scored all 10 items as "yes", "no", "unclear" or "not applicable", and assessed the quality of each included study. Disagreements were resolved by a third author (ENSG). The risk of bias for each study was considered *High* if 49% or less of the items were scored "yes", *Moderate* if 50% to 69% of the items were scored "yes", and *Low* if 70% or more of the items were scored "yes" **(Appendix 3).**

Summary measures

Any survival measures, such as OS, disease-free survival (DFS), and progression-free survival (PFS), as well as RR were considered main outcomes. Adverse effects (AE), human papillomavirus (HPV) status, programmed death-ligand 1 (PD-L1) expression, correlations between checkpoint inhibitors or costimulatory agonists response and clinical data such as tumor location and biological markers were secondary outcomes.

Synthesis of results

A meta-analysis on risk ratio and overall survival was performed with the Review Manager®5.3 software (RevMan 5.3, The Nordic Cochrane Centre, Copenhagen, Denmark). A proportion meta-analysis was developed with the appropriate data on the Microsoft Excel extension MetaXL version 5.3 (EpiGear International, Sunrise Beach, Australia). Additional graph data was constructed with the aid of Microsoft Excel software (2016).

Risk of bias across studies

The quality of evidence and grading of recommendations strength was assessed using the Grading of Recommendation, Assessment, Development and Evaluation (GRADE) instrument (10, 11). The criteria for this assessment were study design, risk of bias, inconsistency, indirectness, imprecision, and other considerations. The quality of evidence was characterized as high, moderate, low, or very low. The GRADE was assessed using tools from the following website http://gradepro.org.

RESULTS

Search results

Study selection

A total 5,800 records were identified from the databases after duplicates removal. In phase 1 of study selection, 123 articles were considered eligible for the full-text assessment. Three additional studies were retrieved from reference lists. After the exclusion criteria were applied in phase 2, a total of 91 articles were excluded and 32 studies remained. From these, 15 were complete articles and 17 abstracts. Four publications with repeated NCT numbers were identified. In the end, 11 studies were evaluated **(Figure 1)**.

Study characteristics

Most of the included studies were multicenter, developed in countries in North America, Europe and Asia, and published in the last four years (2016-2019). Seven studies were single-arm trials and four were randomized clinical trial (RCT). In one of the RCT (12), control and experimental groups were treated with different immunotherapy drugs, so they were considered single arms and analyzed accordingly. This study was not included in the meta-analysis for randomized trials. Studies were phase I (n=3), phase II (n=6), and phase III (n=2) clinical trials.

Results of individual studies

The studies evaluated the of effects of immunotherapy on the response rates and survival, as well as the safety and tolerability of such therapeutic agents as treatment options for HNSCC, the results of individual studies are shown in **Table 1**.

A total of 1,860 patients with R/M HNSCC were evaluated. Most of the studies used anti-PD-1 drugs (pembrolizumab – n=3; and nivolumab – n=2) and PD-L1 inhibitors (durvalumab – n=2; and atezolizumab – n=1). Motolimob, an anti-TLR-8, was used in two trials and monalizumab, a NKG2 inhibitor, was used in one study **(Figure 2A)**. The most common therapeutic strategy was immunotherapy alone (n=8), though other trials evaluated immunotherapy associated to chemotherapy (n=3), to therapeutic vaccine (n=1) or in combination with another immunotherapy drug (n=1) **(Figure 2B)**. The randomized clinical trials compared the effects of immunotherapy and standard therapy (EXTREME regimen or

methotrexate, docetaxel, or cetuximab as single agents). **Figure 2C** described a timeline of history of checkpoint inhibitors and costimulatory agonists.

Risk of bias within studies

The risk of bias within studies was assessed in all fifteen included studies, including both primary and subsequent publications. All nine non-randomized experimental studies were classified as of low risk of bias. Of the RCT, only two presented low risk, and four were considered of moderate risk of bias, mainly because the participants, treatment deliverer, and/or outcome assessors were not blinded **(Appendix 3)**.

Synthesis of results

Treatment with immunotherapy showed in longer overall survival and response rate than standard treatment

All randomized clinical trials demonstrated that the use of immunotherapy resulted in a longer OS. Among those patients, the risk of death was 23% lower when compared to those treated with standard therapy (HR 0.77, 95% IC 0.68-0.87 p<0.001). Low heterogeneity was found among the studies, with an I^2 of 14% (p=0.32) **(Figure 3A)**. The median OS was 8 months, yet when the study that evaluated immunotherapy plus chemotherapy were excluded (13), the median decreased to 7.65 months **(Figure 3B)**. Moreover, the estimated rate at 12 months was 36% **(Figure 3C)**. Only one study did not show OS data (14).

As expected, the comparative meta-analyzed indicated a risk ratio of 1.41 (95% CI 1.06-1.87 I^2=30%, p=0.24) favoring the immunotherapy **(Figure 4A)**. The RR was 17% (95% CI 13–21; I^2=80%, p=0.01) when all trials were analyzed **(Figure 4B)** and 14% (95% CI 0.10-0.18; I^2=71%, p=0.001) when the studies that evaluated immunotherapy plus chemotherapy were excluded (13-15) **(Figure 4C)**.

Tumors HPV positive and PD-L1 expressors are more likely to respond to immunotherapy

To understand how HPV infection and PD-L1 expression influence the response to the immunotherapy, we performed a second set of meta-analysis to evaluate the outcomes in such conditions.

First, a prevalence of 56% HPV-positive tumors (95% CI 33-77; I^2=96%, p=0.001) and 15% HPV-negative tumors (95% CI 12-18; I^2=0%, p=0.91) was found **(Figure 5A)**. Patients with HPV-positive tumors showed a better RR when compared to HPV-negative patients (risk ratio 1.6, 95% CI 1.16–2.21; I^2=0%, p=0.67) **(Figure 5B)**. In both groups, the risk of death was lower in patients treated with immunotherapy than in those treated with standard therapy (HR 0.56, 95% IC 0.42-0.7, I^2=0%, p=0.76) **(Figure 5C)**. Considering the median OS, it was higher on the HPV-positive than on the HPV-negative subgroup (10.2 months *vs* 6.3 months, respectively) **(Figure 5D)**.

Regarding PD-L1 expression, 58% of samples were expressors (95% CI 34-80; I^2=98%, p=0.001) and 22% non-expressors (95% CI 14-30; I^2=91%, p=0.001) **(Figure 6A)**. The median OS was 9.3 months among patients expressing PD-L1 and 5.6 months in patients who did not **(Figure 6B)**.

All considered, immunotherapy resulted in a higher RR and OS than the standard treatment. Additionally, a noticeable advantage was observed for HPV-positive and PD-L1-expressing tumors treated with immunotherapy.

Immunotherapy exhibited fewer treatment-related adverse events than standard treatment

AE in any grade were reported in 62% of the patients receiving immunotherapy (95% CI 59-64), with a low heterogeneity among studies (I^2=0%, p=0.85) **(Figure 7A)**. The meta-analysis demonstrated that patients who received standard therapy had more AE than those who were treated with immunotherapy (86% vs 62%) **(Figure 7B)**.

The proportion meta-analysis resulted in a 14% prevalence of grade III and IV AE (95% CI 12-15), with a low heterogeneity among studies (I^2=0%, p=0.56) **(Figure 7C)**. A lower prevalence of AE was also found in the comparative analysis: whereas only 13% of the subjects that received immunotherapy presented those effects, it was reported in 37% of the standard-treatment group **(Figure 7D)**.

Regarding treatment-related deaths, it was present in 0.1% of the immunotherapy group (95% CI 0-1; I^2=0%, p=0.85) **(Figure 7E)** and no differences were found between groups (1% vs 1%) **(Figure 7F)**.

Risk of bias across studies

The certainty of the evidence from the outcomes evaluated using the GRADE system was assessed as moderate for immunotherapy versus controls according to the response of the treatment, and high certainty for immunotherapy in HPV-positive patients versus HPV-negative patients **(Appendix 4)**. It suggests moderate and high confidence in the estimated effect from the outcomes assessed. An important limitation in this review was the few RCT studies included.

Ongoing clinical trials

Abstracts were analyzed aiming to identify in ongoing clinical trials the immunomodulators drugs, their indications, and the therapeutic strategies that have been used to treat HNSCC.

The results showed that only one new drug is being tested in two different clinical trials, ipilimumab, an anti-CTLA-4 monoclonal antibody. The first is a phase 1 trial, combining cetuximab with ipilimumab and intensity modulated radiotherapy (IMRT) on previously untreated locally advanced HNSCC. The second one is an investigator-initiated phase-1b trial using nivolumab plus ipilimumab as neo-adjuvant to surgery with or without adjuvant radiation therapy.

Another interesting finding was the new strategies used, that combined immunotherapy with other immunotherapy drugs (SD-101 + Q3W + pembrolizumab; T-VEC 1 + pembrolizumab), radiotherapy (nivolumab + SBRT; pembrolizumab + definitive-dose radiotherapy), chemotherapy (pembrolizumab + cetuximab), or chemoradiation (nivolumab concomitant with platinum-based chemoradiation; pembrolizumab prior to initiation of chemoradiation).

DISCUSSION

Head and neck cancer is a global public health problem, considering that most of the patients are only diagnosed in advanced stages, consequently leading to significant high morbidity and mortality (1). Recent advances in understanding the molecular bases of the disease associate its development, establishment, and progression with alterations in the

immune system (16). Subsequently, immunotherapy emerged as a new treatment option, able to induce, improve and/or restore the antitumor immune activity (17).

One of the mechanisms by which tumor cells evade the host immune system is through the stimulation of immune checkpoint receptors. CTLA-4 and PD-1 are members of the immunoglobulin-related receptor family, expressed by and responsible for inhibiting T cells, while PD-L1 is the PD-1 ligand, expressed in the membrane of tumor cells and also in lymphocytes or other immune cells. In this way, checkpoints inhibitor drugs target checkpoint pathways, restoring the immune system and allowing the immune-mediated elimination of tumor cells. Nivolumab and pembrolizumab are monoclonal antibodies targeting PD-1, durvalumab and atezolizumab blocks PD-L1 and tremelimumab is an anti-CTLA-4 (18). Furthermore, monalizumab is a monoclonal antibody that blocks checkpoint inhibitor pathways of T cells and natural killer cells, binding to lectin-like receptor subfamily C member 1 (NKG2A) (15).

Costimulatory agonists are another group of immunotherapy drugs that contribute to activate and improve the lymphocyte function (19). Motolimod is a toll-like receptor 8 (TLR8) agonist, that stimulates the innate and adaptive response by eradicating myeloid-derived suppressor cells. These cells are able to suppress the immune response, mainly by inhibiting the differentiation and function of T helper lymphocytes and cytotoxic cells (13).

In the current study, we systematically reviewed and meta-analyzed 11 clinical trials to verify the efficacy and safety of checkpoint inhibitors and costimulatory agonists in 1,860 patients affected by HNSCC. The results of RCT showed that immunotherapy has a clinically significant activity leading to a 23% reduction in the risk of death compared to standard therapy (single agent methotrexate, docetaxel, or cetuximab, or EXTREME regimen). Such difference may be explained by the fact that chemotherapy targets only the tumor cells, and tumor mass has intratumoral heterogeneity due to the genomic instability that promotes genetic diversity, leading to different levels of treatment sensitivity (16). On the other hand, immunotherapy leads the immune system to identify and destroy the tumor cells, and thanks to an immunological adaptation and memory, can lead to a long term effect, even after treatment completion (6). Our results are in line with recent data that stablishes immunotherapy with checkpoint inhibitors (anti-PD1 agents Nivolumab and Pembrolizumab) as standard of care second line treatment for R/M HNSCC after platinum failure, with randomized phase III trials showing significant benefit in overall survival and response rate, with a favorable toxicity

profile (20, 21). And for first line treatment, anti-PD1 Pembrolizumab, both as single agent and in combination with chemotherapy, emerged as standard options thanks to a randomized phase III trial showing better overall survival in comparison to the Extreme regimen (22). Our results, however, included also drugs besides Nivolumab and Pembrolizumab, such as other checkpoint inhibitors and costimulatory agonists, and the survival benefit demonstrated in the analysis, shows that immunotherapy has a potential role in the treatment of HNSCC.

In order to explore which patients may potentially respond better to immunotherapy, we analyzed the impact of HPV status and PD-L1 expression on the outcomes. The results demonstrated that the median OS was almost doubled in HPV-positive patients, when compared to HPV-negative patients (10.2 *vs* 6.3 months). One possible explanation for this difference is the presence of HPV-specific T-cells, type I–oriented CD4þ and CD8þ T cells, dendritic cells (DC) and DC-like macrophages in the tumor microenvironment of HPV-related tumors, and the synthesis of E6 and E7 oncoproteins, that make the tumor cells extremely detectable to the immune system (23). Also, the better prognosis observed in HPV positive population, is directed related to a better host immune response, like the presence of tumor infiltrating lymphocytes or an inflamed gene expression profile (24, 25). The initial results of phase I and phase II trials of checkpoint inhibitors in R/M HNSCC suggested a higher response rate for HPV positive in comparison to HPV negative patients (26, 27), however randomized phase III trials that evaluate endpoints in these two different populations, did not show a significant difference in RR or OS according to HPV status, with a benefit of immunotherapy in both groups (21). Our data demonstrated a higher benefit of these drugs on HPV-related cancer, but we should call attention that immunotherapy is superior to standard treatment on both populations, with the magnitude of this benefit being higher in the HPV group.

Likewise, in the PD-L1 expressing tumors, the median OS increased to 9.3, compared to 5.6 months among PD-L1-non-expressing patients, possibly because the tumor cells expressing PD-L1 evade the T-cell activity, and drugs that blocked the ligand or the receptor overpass this mechanism (28). Expression of PD-L1 is a biomarker of response to anti PD1/PD-L1 therapy in various malignancies (29), and is used in clinical decisions, as a predictive biomarker of checkpoint inhibitors efficacy, in lung cancer (30, 31), breast cancer (32), urothelial carcinoma (33), and others. For head and neck cancer, the phase I and II studies suggested an improved response rate for PD-L1 expressing tumors, especially when taking into consideration immune cells expression (PD-L1 CPS: combined positive score) (20, 26). In the phase III trials, the results were diverse, with no difference regarding PD-L1 expression on

Nivolumab efficacy on the second line Checkmate 141 trial (21), whereas a potential predictive role was observed in the Pembrolizumab trials, in second line (20) and in first line (22). Our results, analyzing a larger number of patients, that underwent different immunotherapy strategies, showed PD L1 expression as a predictive biomarker of response to treatment in this scenario.

Regarding safety, the number of deaths related to the treatment was similar to that reported as consequence of the standard therapy. Nevertheless, checkpoints inhibitors and costimulatory agonists drugs demonstrated a reduced frequency of AE in any grade (62% vs 86%) and a decrease of about 3 times the prevalence of grade III-V AE (13% vs 37%), enabling higher quality of life for the patients. Such difference might be explained by the low specificity of chemotherapy, which targets both tumor and normal cells in high mitotic activity. Going in line with our results, randomized clinical trials that evaluate the impact of immunotherapy versus standard of care treatment (chemotherapy) on patient reported outcomes and quality of life measures showed a significant benefit of checkpoint inhibitors on these outcomes (20, 34-36).

The U.S. Food and Drug Administration (FDS) has already approved numerous immunotherapy drugs for the treatment of patients affected by several cancers, including melanoma, lung cancer, kidney cancer, bladder cancer, and lymphoma. Nivolumab was in 2016 the first checkpoint inhibitor approved as treatment for R/M HNSCC in patients with disease progression during or after a platinum-based therapy. Three years later, pembrolizumab was approved for first-line treatment in patients with metastatic or unresectable recurrent head and neck squamous cell carcinoma. Immuno-oncology is a rapidly-evolving field and the data retrieved from ongoing clinical trials showed new drugs in research, as well as novels therapeutic strategies, which in the future may foster their use as first-line treatments on the early stages of the disease, to further improve survival rates.

The main strength of this systematic review and meta-analysis was evaluating OS as the primary outcome. It is unaffected by the timing of assessment, since immunotherapy may have a late clinical response, compared with chemotherapy. Furthermore, two clinical trials presented long term follow-up (21, 37). Equally important is the high quality of evidence of the included studies. All non-randomized trials were considered of low risk of bias and two RCT were classified as carrying a moderate risk of bias. Nevertheless, these trials were classified as such because the involved individuals were not blinded. It is a necessary limitation for

randomized clinical trials on immunotherapy, due to immunotherapy drugs presenting specific immunologic adverse effects. The personnel conducting the trial and the patients receiving the therapy must necessarily be informed, so that those potential adverse effects might be efficiently recognized and treated.

On the other hand, a limitation of the present study is the heterogeneity in methodology to assess HPV status and PD-L1 expression. HPV was recognized through the immunohistochemical expression of p16 antibody in most of the trials (26, 28, 35), however, some studies used polymerase chain reaction (PCR) or fluorescence in situ hybridization (FISH) techniques (13, 27, 38). HPV testing was performed only in oropharyngeal cancer in three studies (13, 20, 35), yet five other studies tested HPV in other anatomical sites, such as the oral cavity, pharynx, and larynx (12, 15, 26, 28, 38). p16 is a known marker of HPV infection in oropharyngeal cancer, however its function in other sites is not fully understood (39). Regarding PD-L1 expression, one study used a 5% cutoff (38), while the others considered 1% as the threshold (12, 28, 35, 40). Additionally, two different systems to evaluate the PD-L1 expression were used: the tumor proportion score (TPS), that evaluated only tumor cells, and the combined positive score (CPS), that included lymphocytes and macrophages. Different results were found when tumor was combined to immune cells, in contrast to the tumor score alone (20).

In conclusion, this systematic review and meta-analysis demonstrated that checkpoint inhibitors and costimulatory agonists improve OS and RR with a toxicity reduction, highlighting the ability to restore the immune system to its function of detecting and destroying tumors cells in a long-term, avoiding recurrence and metastasis. Moreover, the magnitude of this benefit is higher, but not limited, in HPV positive and PD-L1 expressers patients. Further randomized clinical trials are necessary to evaluate the effect of immunotherapy as first-line treatment and in combined therapy sets.

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CONFLICT OF INTEREST

None declared.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.
2. Haddad RI, Shin DM. Recent advances in head and neck cancer. N Engl J Med. 2008;359(11):1143-54.
3. Steinbichler TB, Lichtenecker M, Anegg M, Dejaco D, Kofler B, Schartinger VH, et al. Persistent Head and Neck Cancer Following First-Line Treatment. Cancers (Basel). 2018;10(11).
4. Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med. 2008;359(11):1116-27.
5. Mandal R, Senbabaoglu Y, Desrichard A, Havel JJ, Dalin MG, Riaz N, et al. The head and neck cancer immune landscape and its immunotherapeutic implications. JCI Insight. 2016;1(17):e89829.
6. Ferris RL. Immunology and Immunotherapy of Head and Neck Cancer. J Clin Oncol. 2015;33(29):3293-304.
7. Chan KK, Glenny AM, Weldon JC, Furness S, Worthington HV, Wakeford H. Interventions for the treatment of oral and oropharyngeal cancers: targeted therapy and immunotherapy. Cochrane Database Syst Rev. 2015(12):CD010341.
8. Moher D, Liberati A, Tetzlaff J, Altman DG, Group P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med. 2009;6(7):e1000097.
9. Tufanaru C MZ, Aromataris E, Campbell J, Hopp L. . Chapter 3: Systematic reviews of effectiveness. . Joanna Briggs Institute Reviewer's Manual. 2017.
10. Balshem H, Helfand M, Schunemann HJ, Oxman AD, Kunz R, Brozek J, et al. GRADE guidelines: 3. Rating the quality of evidence. J Clin Epidemiol. 2011;64(4):401-6.
11. Schunemann HJ, Oxman AD, Brozek J, Glasziou P, Jaeschke R, Vist GE, et al. Grading quality of evidence and strength of recommendations for diagnostic tests and strategies. BMJ. 2008;336(7653):1106-10.
12. Siu LL, Even C, Mesía R, Remenar E, Daste A, Delord JP, et al. Safety and Efficacy of Durvalumab with or Without Tremelimumab in Patients with PD-L1-Low/Negative Recurrent or Metastatic HNSCC: The Phase 2 CONDOR Randomized Clinical Trial. JAMA Oncology. 2018.
13. Ferris RL, Saba NF, Gitlitz BJ, Haddad R, Sukari A, Neupane P, et al. Effect of Adding Motolimod to Standard Combination Chemotherapy and Cetuximab Treatment of Patients With Squamous Cell Carcinoma of the Head and Neck The Active8 Randomized Clinical Trial. JAMA oncology. 2018;4(11):1583-8.
14. Chow LQM, Morishima C, Eaton KD, Baik CS, Goulart BH, Anderson LN, et al. Phase Ib Trial of the Toll-like Receptor 8 Agonist, Motolimod (VTX-2337), Combined with

Cetuximab in Patients with Recurrent or Metastatic SCCHN. Clinical cancer research : an official journal of the American Association for Cancer Research. 2017;23(10):2442-50.
15. André P, Denis C, Soulas C, Bourbon-Caillet C, Lopez J, Arnoux T, et al. Anti-NKG2A mAb Is a Checkpoint Inhibitor that Promotes Anti-tumor Immunity by Unleashing Both T and NK Cells. Cell. 2018;175(7):1731-43.e13.
16. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
17. Manca P, Raez LE, Salzberg M, Sanchez J, Hunis B, Rolfo C. The value of immunotherapy in head and neck cancer. Expert Opin Biol Ther. 2019;19(1):35-43.
18. Darvin P, Toor SM, Sasidharan Nair V, Elkord E. Immune checkpoint inhibitors: recent progress and potential biomarkers. Exp Mol Med. 2018;50(12):165.
19. Pardee AD, Wesa AK, Storkus WJ. Integrating costimulatory agonists to optimize immune-based cancer therapies. Immunotherapy. 2009;1(2):249-64.
20. Cohen EEW, Soulieres D, Le Tourneau C, Dinis J, Licitra L, Ahn MJ, et al. Pembrolizumab versus methotrexate, docetaxel, or cetuximab for recurrent or metastatic head-and-neck squamous cell carcinoma (KEYNOTE-040): a randomised, open-label, phase 3 study. Lancet. 2019;393(10167):156-67.
21. Ferris RL, Blumenschein G, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab vs investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-year long-term survival update of CheckMate 141 with analyses by tumor PD-L1 expression. Oral oncology. 2018;81:45-51.
22. Rischin D, Harrington KJ, Greil R, Soulieres D, Tahara M, Castro Gd, et al. Protocol-specified final analysis of the phase 3 KEYNOTE-048 trial of pembrolizumab (pembro) as first-line therapy for recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC). 2019;37(15_suppl):6000-.
23. Welters MJP, Ma W, Santegoets S, Goedemans R, Ehsan I, Jordanova ES, et al. Intratumoral HPV16-Specific T Cells Constitute a Type I-Oriented Tumor Microenvironment to Improve Survival in HPV16-Driven Oropharyngeal Cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2018;24(3):634-47.
24. Chakravarthy A, Henderson S, Thirdborough SM, Ottensmeier CH, Su X, Lechner M, et al. Human Papillomavirus Drives Tumor Development Throughout the Head and Neck: Improved Prognosis Is Associated With an Immune Response Largely Restricted to the Oropharynx. J Clin Oncol. 2016;34(34):4132-41.
25. Keck MK, Zuo Z, Khattri A, Stricker TP, Brown CD, Imanguli M, et al. Integrative analysis of head and neck cancer identifies two biologically distinct HPV and three non-HPV subtypes. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(4):870-81.
26. Bauml J, Seiwert TY, Pfister DG, Worden F, Liu SV, Gilbert J, et al. Pembrolizumab for Platinum- and Cetuximab-Refractory Head and Neck Cancer: Results From a Single-Arm, Phase II Study. J Clin Oncol. 2017;35(14):1542-9.
27. Zandberg DP, Algazi AP, Jimeno A, Good JS, Fayette J, Bouganim N, et al. Durvalumab for recurrent or metastatic head and neck squamous cell carcinoma: Results from a single-arm, phase II study in patients with >/=25% tumour cell PD-L1 expression who have progressed on platinum-based chemotherapy. Eur J Cancer. 2019;107:142-52.
28. Seiwert TY, Burtness B, Mehra R, Weiss J, Berger R, Eder JP, et al. Safety and clinical activity of pembrolizumab for treatment of recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): an open-label, multicentre, phase 1b trial. The Lancet Oncology. 2016;17(7):956-65.
29. Gibney GT, Weiner LM, Atkins MB. Predictive biomarkers for checkpoint inhibitor-based immunotherapy. The Lancet Oncology. 2016;17(12):e542-e51.

30. Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, et al. Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer. N Engl J Med. 2016;375(19):1823-33.
31. Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, et al. Updated Analysis of KEYNOTE-024: Pembrolizumab Versus Platinum-Based Chemotherapy for Advanced Non-Small-Cell Lung Cancer With PD-L1 Tumor Proportion Score of 50% or Greater. J Clin Oncol. 2019;37(7):537-46.
32. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. N Engl J Med. 2018;379(22):2108-21.
33. Suzman DL, Agrawal S, Ning YM, Maher VE, Fernandes LL, Karuri S, et al. FDA Approval Summary: Atezolizumab or Pembrolizumab for the Treatment of Patients with Advanced Urothelial Carcinoma Ineligible for Cisplatin-Containing Chemotherapy. Oncologist. 2019;24(4):563-9.
34. Cohen EEW, Soulieres D, Tourneau CL, Dinis J, Licitra LF, Ahn M-J, et al. Health-related quality of life (HRQoL) of pembrolizumab (pembro) vs standard of care (SOC) for recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC) in KEYNOTE-040. 2018;36(15_suppl):6013-.
35. Ferris RL, Blumenschein G, Jr., Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. N Engl J Med. 2016;375(19):1856-67.
36. Harrington KJ, Ferris RL, Blumenschein G, Jr., Colevas AD, Fayette J, Licitra L, et al. Nivolumab versus standard, single-agent therapy of investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck (CheckMate 141): health-related quality-of-life results from a randomised, phase 3 trial. The Lancet Oncology. 2017;18(8):1104-15.
37. Mehra R, Seiwert TY, Gupta S, Weiss J, Gluck I, Eder JP, et al. Efficacy and safety of pembrolizumab in recurrent/metastatic head and neck squamous cell carcinoma: pooled analyses after long-term follow-up in KEYNOTE-012. British journal of cancer. 2018;119(2):153-9.
38. Colevas AD, Bahleda R, Braiteh F, Balmanoukian A, Brana I, Chau NG, et al. Safety and clinical activity of atezolizumab in head and neck cancer: results from a phase I trial. Ann Oncol. 2018;29(11):2247-53.
39. Fakhry C, Lacchetti C, Rooper LM, Jordan RC, Rischin D, Sturgis EM, et al. Human Papillomavirus Testing in Head and Neck Carcinomas: ASCO Clinical Practice Guideline Endorsement of the College of American Pathologists Guideline. J Clin Oncol. 2018;36(31):3152-61.
40. Massarelli E, William W, Johnson F, Kies M, Ferrarotto R, Guo M, et al. Combining Immune Checkpoint Blockade and Tumor-Specific Vaccine for Patients With Incurable Human Papillomavirus 16-Related Cancer: A Phase 2 Clinical Trial. JAMA Oncol. 2019;5(1):67-73.

Table 1. Overview of descriptive characteristics of the included studies.

Figure 1. Flow chart of literature search and selection criteria.

Figure 2. Checkpoint inhibitors and costimulatory agonists used in clinical trials for head and neck cancer **(A)** and therapeutic strategies employed **(B)**. Timeline of history of immunotherapy for head and neck cancer **(C)**.

Figure 3. Forest plot of the Hazard Ratio for overall survival at 12 and 24 months comparing patients treated with immunotherapy *vs* standard therapy (control) **(A)**. Median overall survival of all trials (yellow) and individual studies (blue) **(B)**. Proportion meta-analysis graph of overall survival at 12 months **(C)**.

Figure 4. Forest plot of the Risk Ratio for response rate comparing patients treated with immunotherapy *vs* standard therapy (control) **(A)**. Proportion meta-analysis graph of response rate **(B).** The three studies that used immunotherapy combined with chemotherapy were excluded in a separate proportion meta-analysis **(C)**.

Figure 5. Proportion meta-analysis graph of HPV positive (+) **(A)** and negative (-) tumors **(B).** Forest plot of the Risk Ratio comparing HPV + vs HPV – **(C)**. Median overall survival of all trials (yellow) and individual studies (blue) comparing HPV + vs HPV –.

Figure 6. Proportion meta-analysis graph of tumors PD-L1 + **(A)** and PD-L1 - **(B).** Median overall survival of all trials (yellow) and individual studies (blue) comparing PD-L1 + vs PD-L1 -.

Figure 7. Proportion meta-analysis graphs for adverse effects any grade **(A)**, grade 3 and 4 **(B)** and treatment related deaths **(C)**. Proportion meta-analysis comparing patients treated with immunotherapy *vs* standard therapy for adverse effects any grade **(D)**, grade 3 and 4 **(E)** and treatment related deaths **(F)**

Table 1.

TRIAL NUMBER / ID	AUTHOR. YEAR / COUNTRY	STUDY DESIGN	PATIENTS	INTERVENTION	RESULTS			MAIN CONCLUSIONS
					EFICACY: RESPONSE RATE	EFICACY: SURVIVAL	SAFETY	
NCT 01848834 / KEYNOTE-012	Seiwert *et al.* 2016 / USA and Israel.	Open-label, multicenter, phase 1b trial.	60 PD-L1-positive, pretreated, R/M HNSCC: -23 HPV-positive. -37 HPV-negative.	Pembrolizumab i.v. 10 mg/kg, every 2 weeks.	**-OR**: 18% (95% CI, 8–32). According HPV status: 25% *vs* 14%. *Median* time was 8 weeks (95% CI, 7–17).	**-OS**: *Median*, 13 months (95% CI, 5 to not reached). 12 months OS, 51%. According HPV status, not reached for HPV-positive (8-not reached) *vs* 8 months (4-not reached).	**-Deaths**: 2. *Treatment-related deaths*, none. **-AE**: Any grade, 63%. Grade 3: 17%. According HPV status, any grade 70% *vs* 59%; grade 3: 22% *vs*14%.	Pembrolizumab might represent a new treatment approach for patients with HNSCC.
	Chow *et al.* 2016.	Open-label, multicenter, phase 1b trial expansion cohort.	132 R/M HNSCC, irrespective of PD-L1 or HPV status.	Pembrolizumab i.v. 200 mg once every 3 weeks.	**-OR**:18% (95% CI, 12 to 26) by central imaging vendor and 20% (95% CI, 13 to 28) by investigator review. **-OR/HPV status**: 32% vs 14%. *-Median time to response:* 2 months (2-11). **-OR/PD-L1 (TPS)**: There was no statistically significant difference. **-OR/PD-L1 (CPS)**: 22% *vs* 4%.	**-OS**: *Median* 8 months (95% CI, 6 -10). *At 6* months 59%. **-OS/HPV status**: 70% *vs* 56%. **-OS/PD-L1 expression**: *Median* OS 303 days *vs* 151 days.	**-Deaths**: 3. *Treatment-related deaths*, none. **-AE**: Any grade, 62%. Grade 3-4: 9%.	Fixed-dose pembrolizumab 200 mg administered once every 3 weeks was well tolerated and yielded a clinically meaningful OR with evidence of durable responses. The clinical benefit of pembrolizumab in biomarker-unselected patients with R/M HNSCC was similar to that seen in the initial cohort of patients who were PD-L1 positive.
	Mehra *et al.* 2018.	Multicenter, non-randomised trial	192 R/M HNSCC -60 initial cohort. -132 expansion cohort.	Pembrolizumab 10 mg/kg every 2 weeks (initial cohort) or 200 mg every 3 weeks (expansion cohort).	**-OR**: 18% (95% CI, 13–24%). **-OR/HPV status**: 24% (95% CI, 13-40%) *vs* 16% (95% CI, 10–23). **-OR/PD-L1 expression (CPS)**: 21 vs. 6%.	**-OS**: *At 12 months* 38%. *Median* was 8 months (95% CI, 6–10 months). *At 6-months* 58%, *at 12-months* 38%. **-OS/PD-L1 expression (CPS)**: *Median* (95% CI) 10 months (9–13 months) *vs* 5 months (3–8 months).	**-Deaths**: *Treatment-related deaths*, none. **-AE**: Any grade 64%, grade 3-4: 13%	Pembrolizumab exhibited durable anti-tumour activity, high survival rates, and acceptable safety in patients with heavily pre-treated advanced HNSCC.
NCT 02105636 CheckMate 141	Ferris *et al.* 2016 / USA.	Randomized, open-label, phase 3 trial.	361 R/M HNSCC: -240 nivolumab -121 standard therapy.	Nivolumab i.v. 3mg/kg, every 2 weeks *vs* standard weekly single-agent i.v. systemic therapy: methotrexate (40-60 mg/m^2 of body surface area), docetaxel (30 to 40 mg/m^2) or cetuximab (250 mg/m^2 after a loading dose of 400 mg/m^2).	**-OR**: 13.3% (95% CI, 9.3-18.3) *vs* 5.8% (95% CI, 2.4-11.6). *Median*, 2.1 months *vs* 2.0 months.	**-OS**: *Median*, 7.5 months (95% CI, 5.5-9.1) *vs* 5.1 months (95% CI, 4.0-6.0). *HR*, 0.70 (97.73% CI, 0.51-0.96). *1-year OS*, 36.0% (95% CI, 28.5-43.4) *vs* 16.6% (95% CI, 8.6-26.8). **-OS/ PD-L1 expression**: *HR*, 0.55 (95% CI, 0.36-0.83) *vs* 0.89 (95% CI, 0.54-1.45). **-OS/p16 status**: Positive tumors: *Median*, 9.1 months *vs* 4.4. *HR*, 0.56 (95% CI, 0.32-0.99). Negative tumors: *Median*, 7.5 months *vs* 5.8 months. *HR*, 0.73 (95% CI, 0.42-1.25).	**-Deaths**: 55.4% *vs* 70.2%; *Treatment-related deaths*, 2 *vs* 1. **-AE**: Any grade, were similar in both groups. Grade 3-4, 13.1% *vs* 35.1%.	Treatment with nivolumab resulted in longer overall survival than treatment with standard, single-agent therapy, regardless of tumor PD-L1 expression or p16 status.
	Gillison *et al.* 2018 / USA.	Randomized, open-label, phase 3 trial.	**First-Line Treatment.** 78 R/M HNSCC: -52 nivolumab -26 investigator's choice.	Nivolumab i.v. 3mg/kg, every 2 weeks, as first-line treatment.	**-OR (First-Line Treatment)**:19.2% *vs* 11.5%, time to response was 2.0 months in both arms. **-OR (One-Year follow-Up)**: OR did not change from the initial analysis.	**-OS (First-Line Treatment)**: *Median* (95% CI), 7.7 (3.1–13.8) *vs*. 3.3 (2.1–6.4) months. HR (95% CI), 0.56 (0.33–0.95). *12-month OS* rate 39.2% *vs* 15.4%. **-OS (One-Year follow-Up)**: *18-months OS* 21.5% *vs*. 8.3%.	**-AE (First-Line Treatment)**: Grade 3–4: 27.5% *vs* 32.0%. **-AE (One-Year follow-Up)**: AEs were consistent with the initial analysis.	The results support the use of nivolumab as first-line therapy for patients with R/M HNSCC who progressed within 6 months of platinum-based therapy in the adjuvant or primary setting.

	Ferris *et al.* 2018 / USA.	Randomized, open-label, phase 3 trial. 2-year long-term survival in the overall population, and the subgroups PD-L1 expression and HPV status.	361 R/M HNSCC: -240 nivolumab -121 standard therapy.	Nivolumab i.v. 3mg/kg, every 2 weeks *vs* standard weekly single-agent i.v. systemic therapy: methotrexate (40-60 mg/m^2 of body surface area), docetaxel (30 to 40 mg/m^2) or cetuximab (250 mg/m^2 after a loading dose of 400 mg/m^2).	**-OR (Two-Year follow-Up):** The median (range) duration of response 9.7 (2.8 to 32.8+) *vs* 4.0 (1.5+ to 11.3). **-OR (Two-Year follow-Up) / PD-L1 expression:** Nivolumab improved OR compared with Investigator's choice in PD-L1 expressors; OR was similar across treatment arms in PD-L1 non-expressors.	**-OS (Two-Year follow-Up):** *HR*=0.68 (95% CI 0.54–0.86). *Median* (95% CI) OS was 7.7 (5.7–8.8) months *vs* 5.1 (4.0–6.2) months. *24-month OS* 16.9% (95% CI 12.4%–22.0%) *vs* 6.0% (95% CI 2.7%–11.3%). **-OS (Two-Year follow-Up) / PD-L1 expression:** PD-L1 expressors, *HR*=0.55 (95% CI 0.39–0.78). OS rates with nivolumab were consistent between PD-L1 expressors and non-expressors at 18 (24.0% and 26.2%, respectively), 24 (18.5% and 20.7%), and 30 (13.7% and 11.2%) months. **-OS (Two-Year follow-Up) / HPV status:** HPV positive tumors *HR*=0.60 (95% CI 0.37–0.97), HPV-negative *HR*=0.59 (95% CI 0.38–0.92).	-Deaths: *Treatment-related deaths* 2 *vs* 1. **-AE (Two-Year follow-Up):** Grade 3–4 7.2% *vs* 15.3%.	With long-term (minimum 2-year) follow-up, nivolumab demonstrated prolonged OS benefit compared with investigator's choice and maintained a favorable safety profile in patients with R/M HNSCC post–platinum therapy. Nivolumab demonstrated OS benefit across PD-L1 expressors/non-expressors and irrespective of HPV status in this patient population.
NCT 02255097 KEYNOTE-055	Bauml *et al.* 2017 / USA.	Single-arm, multicenter, phase 2 trial.	171 R/M HNSCC.	Pembrolizumab i.v. 200 mg every 3 weeks.	**-OR:** 16% (95% CI, 11%-23%). *Median* duration of response of 8 months (2-12). **-OR/PD-L1 expression (CPS):** 18% (12% to 25%) in PD-L1–positive patients and 12% (2% to 30%) in PD-L1–negative. **-OR/HPV status:**16% *vs* 15%. *Median* (range) time to response was 2 (2 to 5) months. *Median* (range) follow-up time for responders was 9 (7 to 17) months. *Median* (range) response durations were 8 (2+ to 12+) months in all responders.	**-OS:** *Median* was 8 months (95% CI, 6 to 11 months) with similar survival observed regardless of HPV status. At 6 months was 59% in all patients, 72% in HPV-positive and 55% in HPV-negative. **-OS/PD-L1:** *At 6 months,* PD-L1– positive patients was 59% (CPS ≥ 1%) and 60% (CPS ≥ 50%); rates were 56% and 58% in patients with CPS ≤ 1% and CPS ≤ 50%, respectively.	**-Deaths:** One. *Treatment-related death:* One. **-AE:** Any grade 64%. Grade ≥ 3: 15%.	Pembrolizumab exhibited clinically meaningful antitumor activity and an acceptable safety profile in R/M HNSCC previously treated with platinum and cetuximab regardless of HPV status.. Patients whose tumors express PD-L1 may be more likely to respond to PD-1 pathway inhibition, however the therapeutic benefit of pembrolizumab is not limited to patients with PD-L1–positive tumors.
NCT 01334177	Chow et al. 2017 / USA.	Open-label, phase I, dose-finding single study.	13 R/M HNSCC: 4 cetuximab + motolimod 2.5 mg. 6 cetuximab + motolimod 3.0 mg. 3 cetuximab + motolimod 3.5 mg.	Weekly i.v dose of cetuximab (250 mg/m2) in combination with escalating doses (2.5, 3.0, or 3.5 mg/m2) of motolimod (VTX-2337) administered s.c once weekly for 3 of 4 weeks of a 28-day cycle.	**-OR:** 15%. **-Disease control rate:** 54%.		**Deaths:** None.	Motolimod can be safely administered in combination with cetuximab with an acceptable toxicity profile. Encouraging antitumor activity androbust pharmacodynamics responses were observed.
NCT 01375842	Colevas *et al.* 2018 / USA.	Cohort, phase 1 trial.	32 HNSCC: -10 without selection for PD-L1 expression -22 PD-L1 expression of ≥ 5%.	Atezolizumab i.v. every 3 weeks for 16 cycles at 15 mg/kg, 20 mg/kg or a 1200-mg fixed dose, up to 1 year, disease progression or loss of clinical benefit.	**-OR:** 22%. M*median duration* of 7.4 months (range 2.8–45.8 months).	**-OS:** *Median* 6.0 months (0.5–51.6 months). 1-, 2-, and 3-year OS rates were 36% (95% CI 19%-53%), 21% (95% CI 5%-36%), and 12% (95% CI 0%-25.0%), respectively -Responses showed no association with HPV status or PD-L1 expression level.	**-Deaths:** None. **- AE:** any grade 66%. Grade 3: 3. Grade 4: 1. Grade 5: 0.	Atezolizumab had a tolerable safety profile and encouraging activity, with responses observed regardless of HPV status and PD-L1 expression level.
NCT 01836029 Active8	Ferris *et al.* 2018 / USA.	Randomized, placebo-controlled, double-blinded, multicenter, phase 2 trial.	175 R/M HNSCC -88 motolimod -87 placebo	Motolimod s.c 3 mg/m2 on days 8 and 15 + the EXTREME regimen vs Placebo on days 8 and 15 + the EXTREME regimen in day 1	**-OR:** 38% *vs* 34%. *Median*, 84 *vs* 83.	**-OS:** *Median* was 13.5 *vs* 11.3 months. -Patients with injection site reactions had longer OS: *median* 18.7 vs 12.6; HR, 0.56; 1-sided 90% CI, 0.00-0.81. **-OS/HPV:** Motolimod-treated HPV-positive patients was 15.2 *vs* 12.6 months for placebo (HR, 0.41). -*Cox proportional hazard model* HR 0.41.	**Deaths:** Eleven patients (6%): 4 (5%) in the motolimod arm and 7 (8%) in the placebo arm. *Treatment-related death:* None. **-AE:** Any grade 25%. Notable AEs of any grade reported in 10% or more patients receiving motolimod *vs* placebo **-Serious AE:** 39% *vs* 40.	Motolimod combined with the EXTREME regimen was well tolerated but did not produce a statistically significant improvement in OS or RR *vs* placebo. Significant benefit was observed in HPV-positive patients and those with injection site reactions, suggesting that TLR8 stimulation may benefit subset- and biomarker-selected patients.

NCT 02643550	André *et al.* 2018 / USA and France.	Phase 2 clinical trial.	31 R/M HNSCC.	Monalizumab 0.4, 1, 2, 4, 10 mg/kg every 2 weeks + cetuximab 400 mg/m2 loading dose and then 250 mg/m2 weekly.	**-OR**: 31%.		**Deaths**: *Treatment-related death:* None. **AE**: 93% of the AE observed were grades 1–2.	Monalizumab is well tolerated in humans and has yielded encouraging efficacy results in clinical trials assessing its use in combination with cetuximab
NCT 02207530 HAWK	Zandberg *et al.* 2019 / USA	Single-arm, multicenter, phase 2 trial.	111 R/M HNSCC with PDL1-high expression (TPS ≥ 25%).	Durvalumab i.v. 10 mg/kg every 2 weeks.	**-OR**: 16.2% (95% CI, 9.9-24.4). *Median time* was 2.0 months (1.6-9.2). -HPV-positive 29.4% (95% CI, 15.1-47.5) *vs* 10.9% (95% CI, 4.5-21.3)..	**-OS**: *Median* 7.1 months (95% CI, 4.9-9.9). *At 12 and 18 months* was 33.6% (95% CI, 24.8-42.7) and 23.0% (95% CI, 14.3-32.9), respectively. **-OS/HPV**: 10.2 months (95% CI, 7.2-16.3) *vs* 5.0 months (95% CI, 3.4-8.4), respectively.	**AE**: Any grade 57.1%. Grade ≥ 3: 8.0% .	Durvalumab demonstrated clinically meaningful antitumour activity in patients with HNSCC with PD-L1- high expression (TPS ≥ 25%) who had progressed after first-line platinum-based therapy in the R/M setting. HPV-positive patients had a numerically higher OR and OS than HPV-negative patients.
NCT 02426892	Massarelli *et al.* 2018 / USA	Single-arm, single-center. phase 2 trial.	22 patients with incurable HPV-16–oropharyngeal cancer.	Vaccine ISA101, 100 µg/peptide in montanide adjuvant, s.c. on days 1, 22, and 50 + Nivolumab, 3mg/kg, i.v. every 2 weeks beginning day 8.	**-OR**: 36%	**-OS**: *Median* 17.5 months (95%CI, 17.5 months to inestimable). *At 6 months* 75% (95% CI, 59%-94%) and *at 12 months* 70% (95%CI, 54%-91%).		-Combining cancer vaccination with immunecheckpoint blockade to enhance efficacy of vaccine-activated T cells in the immunosuppressive tumor environment.
NCT 02252042 / KEYNOTE-040	Cohen et al. 2018/ Multicenter.	Randomized, open-label, phase 3 trial.	495 R/M HNSCC. -246 pembrolizumab -234 investigator's choice.	Pembrolizumab i.v. 200 mg every 3 weeks vs Methotrexate 40 mg/m² i.v. per week, docetaxel 75 mg/m² i.v. every 3 weeks or cetuximab 250 mg/m² i.v. per week following a loading dose of 400 mg/m².	**-OR**: 14.6% (95% CI 10.4–19.6) *vs* 10.1% (6.6–14.5). *Median* time to response 4.5 months *vs* 2.2 months (2.1- 3.5). *Median* duration of response 18.4 months (95% CI 5.8–18.4) *vs* 5.0 months (3.6–18.8). **-OR/PD-L1 expression**: OR was higher in patients whose tumors expressed PD-L1 than in those whose tumors did not, whereas the proportion in the standard-of-care group was similar regardless of PD-L1 expression.	**OS**: *Median* 8.4 months (95% CI 6.4–9.4) *vs* 6.9 months (5.9–8.0). *At 12 months* 37.0% (95% CI 31.0–43.1) *vs* 26.5% (21.2–32.2). **-OS/PD-L1**: *CPS of 1 or higher*, deaths 70% *vs* 85%, *HR* 0.74 (95% CI 0.58–0.93). *Median* OS 8.7 months (95% CI 6.9–11.4) *vs* 7.1 months (5.7–8.3). *At 12 months*, 40% (95% CI 33–47) *vs* 26% (20–33). *CPS less than 1*, deaths 84% *vs* 78%, *HR* 1.28 (95% CI 0.80–2.07). *Median* 6.3 months (3.9–8.9) *vs* 7.0 months (5.1–9.0). *TPS of 50% or higher*, deaths 64% *vs* 86%, *HR* 0.53, 95% CI 0.35–0.81. *Median* 11.6 months (95% CI 8.3–19.5) *vs* 6.6 months (4.8–9.2). *At 12 months* 47% (34–58) *vs* 25% (16–37). *TPS 50%*, 76% *vs* 82%, *HR* 0.93, 95% CI 0.73–1.17; *median* 6.5 months (95% CI 5.6–8.8) *vs* 7.1 months (5.7–8.1).	**-Deaths**: 73% *vs* 83%. *Treatment-related deaths*, 4 vs 2- **-AE**: *Any grade*, 63% *vs* 84%. *Grade 3–5*, 13% *vs* 36%.	The clinically meaningful prolongation of OS and favourable safety profile of pembrolizumab in patients with R/M HNSCC support the further evaluation of pembrolizumab as a monotherapy and as part of combination therapy in earlier stages of disease. -The benefit of pembrolizumab compared with standard-of care therapy was greater in patients with PD-L1 expression on their tumors or in the tumor microenvironment than in those without PD-L1 expression.
NCT 02319044 CONDOR	Siu, L. et al. 2018 / North America, Europe, and Asia Pacific.	Randomized, open-label, multicenter, phase 2 trial.	257 R/M HNSCC with PD-L1–low/negative (TC <25%) ARM 1: 133 Durvalumab + Tremelimumab ARM 2: 65 durvalumab monotherapy. ARM 3: 65 Tremelimumab monotherapy.	ARM 1: Durvalumab i.v. 20mg/kg every 4 weeks + tremelimumab i.v. 1mg/kg every 4 weeks for 4 cycles, followed by durvalumab 10mg/kg every 2 weeks. ARM 2: Durvalumab i.v. monotherapy, 10 mg/kg every 2 weeks. ARM 3: Tremelimumab i.v. monotherapy, 10 mg/kg every 4 weeks for 7 doses then every 12 weeks for 2 additional doses for up to 12months.	**-OR**: 7.8% (95% CI, 3.78%-13.79%) in ARM1, 9.2% (95% CI, 3.46%-19.02%) in ARM2 and 1.6% (95% CI, 0.04%-8.53%) ARM3. -*Median time to response* (range) was 2.0 (2-6) months in ARM1, 4.1 (2-6) months in ARM2, and 1.8 months ARM3. **–OR/PD-L1**: TC<1% was 7.4% in ARM1, 8.8% in ARM2. TC<10% was 6.8% in ARM1, 8.9% in ARM2. **-OR/HPV**: HPV-positive tumors 5.4%(95%CI,0.66%-18.19%)in ARM1, 16.7% (95%CI, 3.58%-41.42%) in ARM2.	**-OS**: *Median* (95% CI) 7.6 (4.9-10.6) months in ARM1, 6.0 (4.0-11.3) months in ARM2, and 5.5 (3.9-7.0) months in ARM3. *Risk of death*: combination vs durvalumab monotherapy HR, 0.99 (95%CI, 0.69-1.43) or tremelimumab monotherapy HR, 0.72 (95%CI, 0.51-1.03). *At 12 months* was 37% in ARM1, 36% in ARM2, and 24% in ARM3.	**-Deaths**: One treatment-related death. - **AE**: Any grade 57.9% in ARM 1, 63.1% in ARM2, and 55.4% ARM 3. Grade 3-4: 15.8% ARM1, 12.3% ARM2 and 16.9% ARM3.	Durvalumab monotherapy showed a manageable toxicity profile and clinical benefit for patients with R/M HNSCC and low or no PD-L1 TC expression; durvalumab + tremelimumab demonstrated similar efficacy to durvalumab monotherapy. -The findings do not appear to support the hypothesis that tremelimumab combinedwith durvalumab exerts a synergistic therapeutic effect in this populationwith lowor no expression ofPD-L1.

Abbreviations: AE, Adverse effects; CI, confidence interval; CPS, combined positive score; HNSCC, Head and Neck Squamous Cell Carcinoma; HPV, human papillomavirus; HR, hazard ratio; i.v. intravenous; OR, Overall response; OS, Overall survival; PD-L1, Programmed death-ligand 1; R/M recurrent/metastatic; s.c., subcutaneous; TPS, tumor proportion.

In results, always are showing the data in the following order: intervention *vs* control; according HPV status, positive *vs* negative; according PD-L1 expression, positive *vs* negative.

Figure 1.

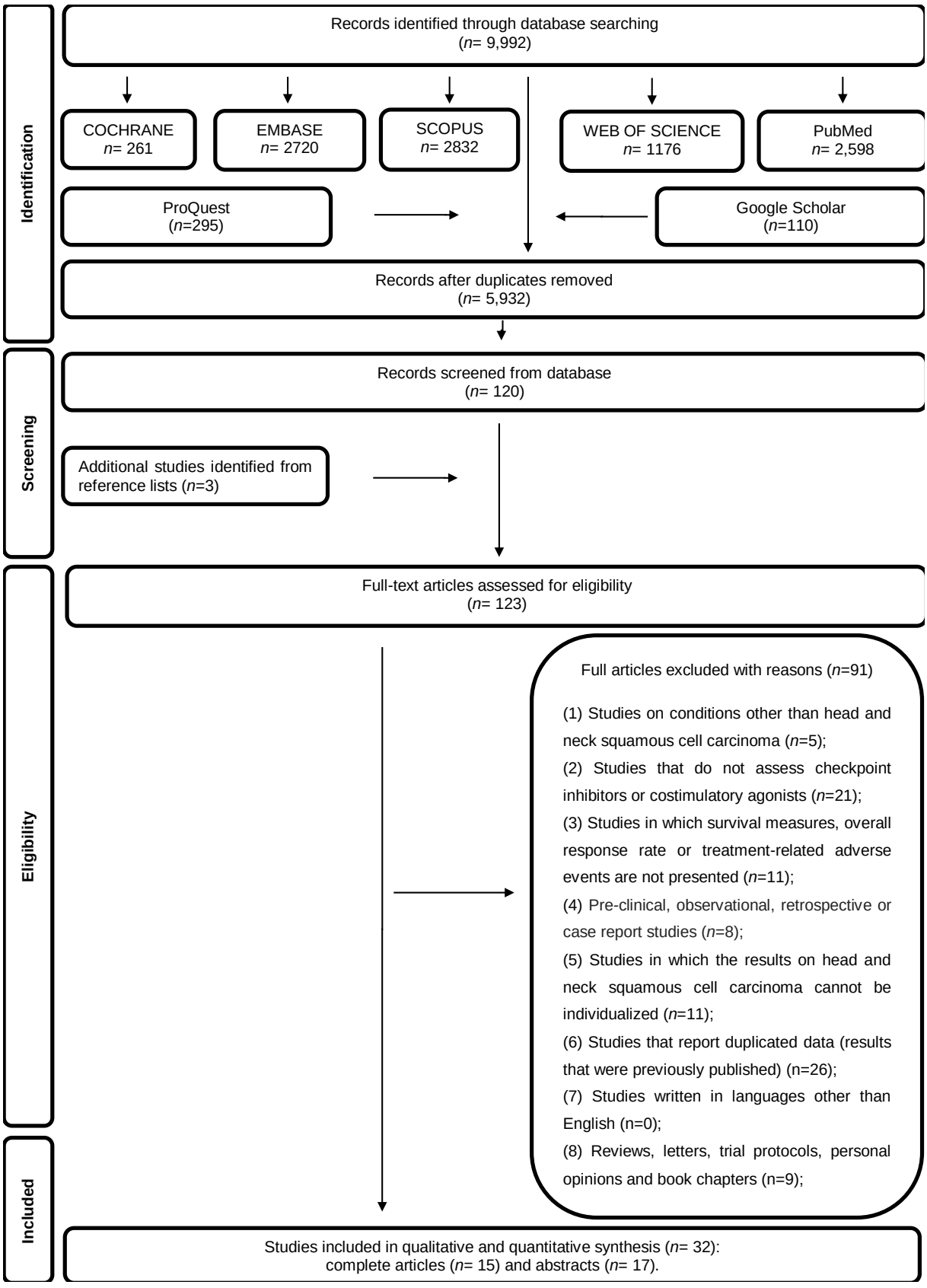
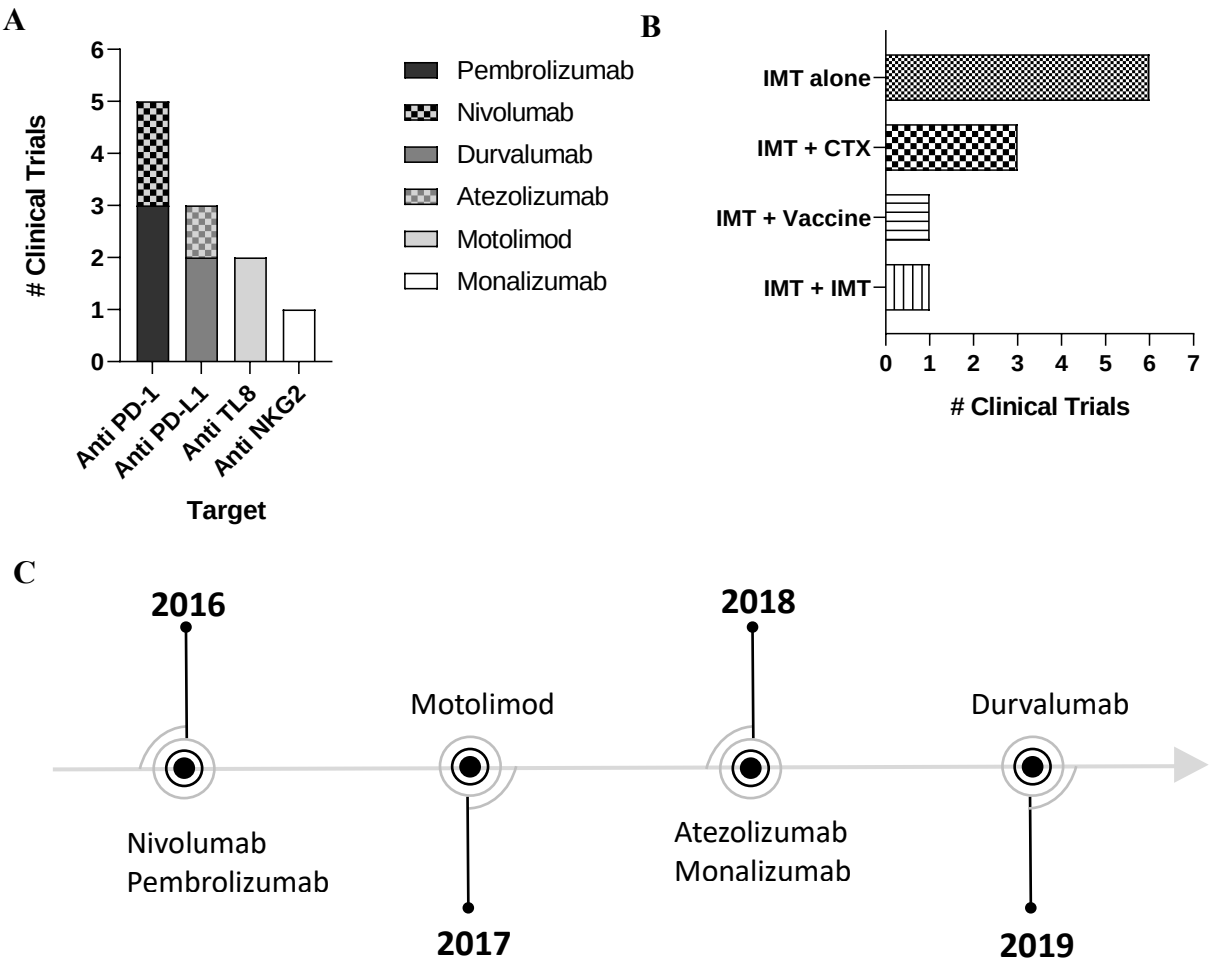

Identification
Records identified through database searching
(n= 9,992)
COCHRANE
n= 261
EMBASE
n= 2720
SCOPUS
n= 2832
WEB OF SCIENCE
n= 1176
PubMed
n= 2,598
ProQuest
(n=295)
Google Scholar
(n=110)
Records after duplicates removed
(n= 5,932)
Screening
Records screened from database
(n= 120)
Additional studies identified from reference lists (n=3)
Eligibility
Full-text articles assessed for eligibility
(n= 123)
Full articles excluded with reasons (n=91)
(1) Studies on conditions other than head and neck squamous cell carcinoma (n=5);
(2) Studies that do not assess checkpoint inhibitors or costimulatory agonists (n=21);
(3) Studies in which survival measures, overall response rate or treatment-related adverse events are not presented (n=11);
(4) Pre-clinical, observational, retrospective or case report studies (n=8);
(5) Studies in which the results on head and neck squamous cell carcinoma cannot be individualized (n=11);
(6) Studies that report duplicated data (results that were previously published) (n=26);
(7) Studies written in languages other than English (n=0);
(8) Reviews, letters, trial protocols, personal opinions and book chapters (n=9);
Included
Studies included in qualitative and quantitative synthesis (n= 32):
complete articles (n= 15) and abstracts (n= 17).

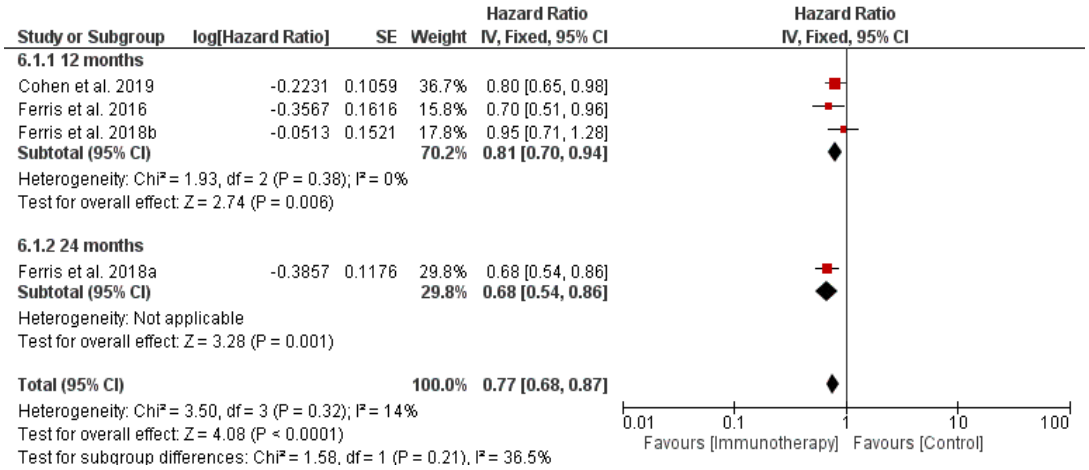
Figure 2.



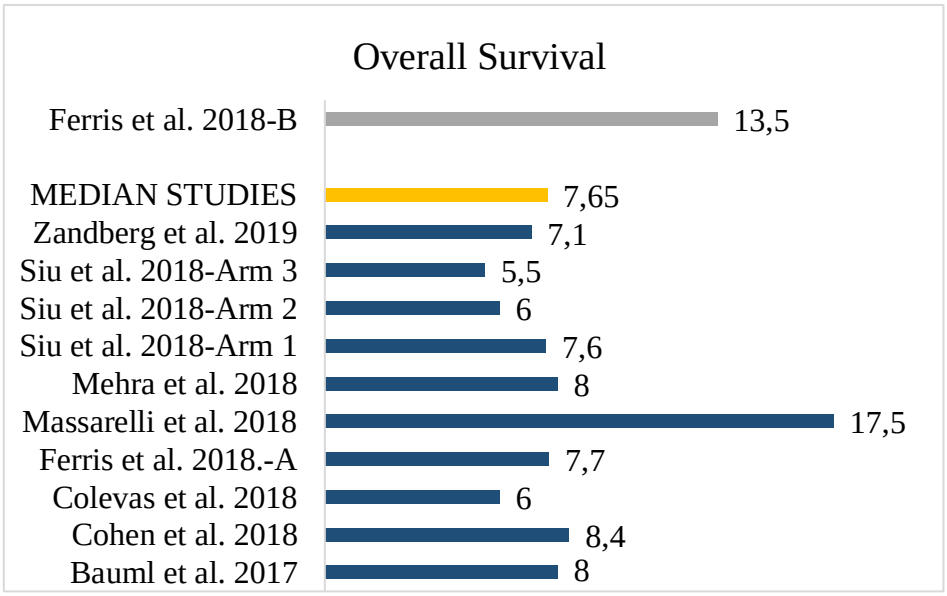
Abbreviations: CTX, chemotherapy; IMT, immunotherapy; PD-L1, Programmed death-ligand 1; PD-1, Programmed death 1; TLR-8, Toll-like receptor 8.

Figure 3.

A


Study or Subgroup log[Hazard Ratio] SE Weight Hazard Ratio IV, Fixed, 95% CI
Hazard Ratio IV, Fixed, 95% CI
6.1.1 12 months
Cohen et al. 2019 -0.2231 0.1059 36.7% 0.80 [0.65, 0.98]
Ferris et al. 2016 -0.3567 0.1616 15.8% 0.70 [0.51, 0.96]
Ferris et al. 2018b -0.0513 0.1521 17.8% 0.95 [0.71, 1.28]
Subtotal (95% CI) 70.2% 0.81 [0.70, 0.94]
Heterogeneity: Chi² = 1.93, df = 2 (P = 0.38); I² = 0%
Test for overall effect: Z = 2.74 (P = 0.006)
6.1.2 24 months
Ferris et al. 2018a -0.3857 0.1176 29.8% 0.68 [0.54, 0.86]
Subtotal (95% CI) 29.8% 0.68 [0.54, 0.86]
Heterogeneity: Not applicable
Test for overall effect: Z = 3.28 (P = 0.001)
Total (95% CI) 100.0% 0.77 [0.68, 0.87]
Heterogeneity: Chi² = 3.50, df = 3 (P = 0.32); I² = 14%
Test for overall effect: Z = 4.08 (P < 0.0001)
Test for subgroup differences: Chi² = 1.58, df = 1 (P = 0.21), I² = 36.5%
0.01 0.1 1 10 100
Favours [Immunotherapy] Favours [Control]

B


Overall Survival
Ferris et al. 2018-B 13,5
MEDIAN STUDIES 7,65
Zandberg et al. 2019 7,1
Siu et al. 2018-Arm 3 5,5
Siu et al. 2018-Arm 2 6
Siu et al. 2018-Arm 1 7,6
Mehra et al. 2018 8
Massarelli et al. 2018 17,5
Ferris et al. 2018.-A 7,7
Colevas et al. 2018 6
Cohen et al. 2018 8,4
Bauml et al. 2017 8

C

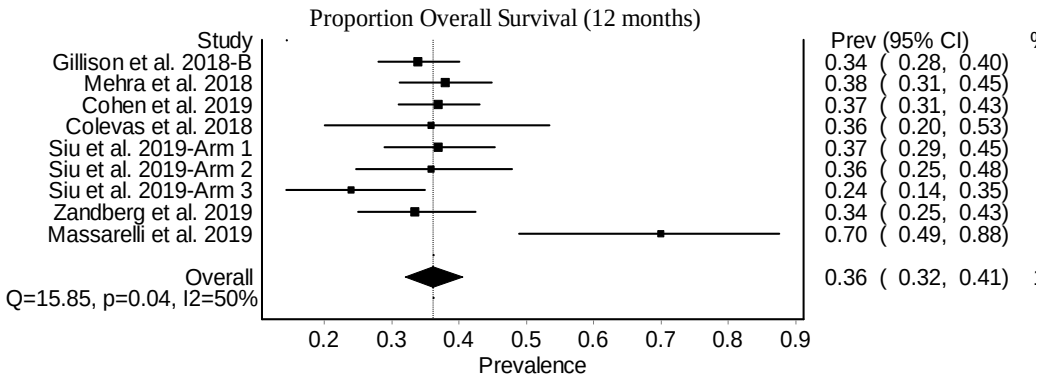
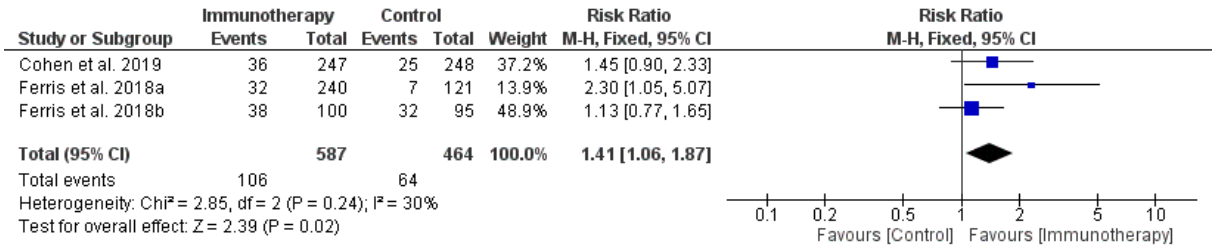
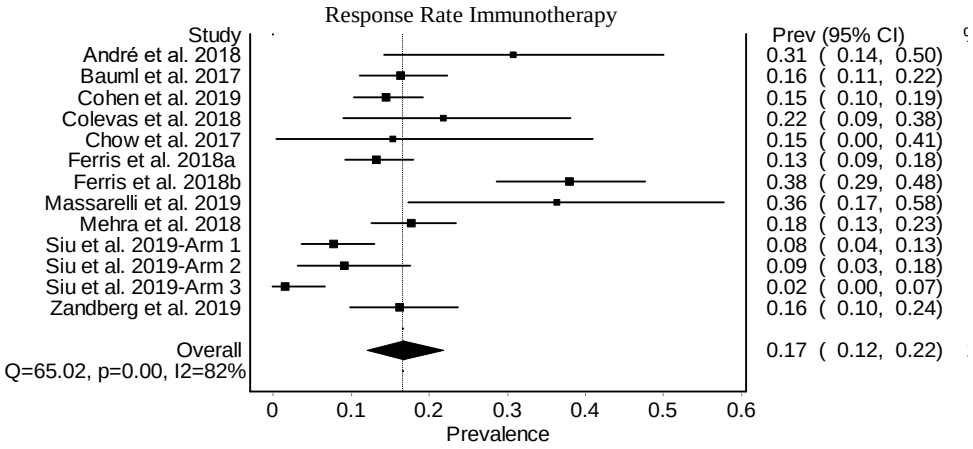

Proportion Overall Survival (12 months)
Study
Prev (95% CI)
Gillison et al. 2018-B 0.34 (0.28, 0.40)
Mehra et al. 2018 0.38 (0.31, 0.45)
Cohen et al. 2019 0.37 (0.31, 0.43)
Colevas et al. 2018 0.36 (0.20, 0.53)
Siu et al. 2019-Arm 1 0.37 (0.29, 0.45)
Siu et al. 2019-Arm 2 0.36 (0.25, 0.48)
Siu et al. 2019-Arm 3 0.24 (0.14, 0.35)
Zandberg et al. 2019 0.34 (0.25, 0.43)
Massarelli et al. 2019 0.70 (0.49, 0.88)
Overall 0.36 (0.32, 0.41)
Q=15.85, p=0.04, I2=50%
0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Prevalence

Figure 4.

A


Immunotherapy
Control
Risk Ratio
Risk Ratio
Study or Subgroup Events Total Events Total Weight M-H, Fixed, 95% CI
M-H, Fixed, 95% CI
Cohen et al. 2019 36 247 25 248 37.2% 1.45 [0.90, 2.33]
Ferris et al. 2018a 32 240 7 121 13.9% 2.30 [1.05, 5.07]
Ferris et al. 2018b 38 100 32 95 48.9% 1.13 [0.77, 1.65]
Total (95% CI) 587 464 100.0% 1.41 [1.06, 1.87]
Total events 106 64
Heterogeneity: Chi² = 2.85, df = 2 (P = 0.24); I² = 30%
Test for overall effect: Z = 2.39 (P = 0.02)
0.1 0.2 0.5 1 2 5 10
Favours [Control] Favours [Immunotherapy]

B


Response Rate Immunotherapy
Study
Prev (95% CI)
André et al. 2018 0.31 (0.14, 0.50)
Bauml et al. 2017 0.16 (0.11, 0.22)
Cohen et al. 2019 0.15 (0.10, 0.19)
Colevas et al. 2018 0.22 (0.09, 0.38)
Chow et al. 2017 0.15 (0.00, 0.41)
Ferris et al. 2018a 0.13 (0.09, 0.18)
Ferris et al. 2018b 0.38 (0.29, 0.48)
Massarelli et al. 2019 0.36 (0.17, 0.58)
Mehra et al. 2018 0.18 (0.13, 0.23)
Siu et al. 2019-Arm 1 0.08 (0.04, 0.13)
Siu et al. 2019-Arm 2 0.09 (0.03, 0.18)
Siu et al. 2019-Arm 3 0.02 (0.00, 0.07)
Zandberg et al. 2019 0.16 (0.10, 0.24)
Overall 0.17 (0.12, 0.22)
Q=65.02, p=0.00, I2=82%
0 0.1 0.2 0.3 0.4 0.5 0.6
Prevalence

C

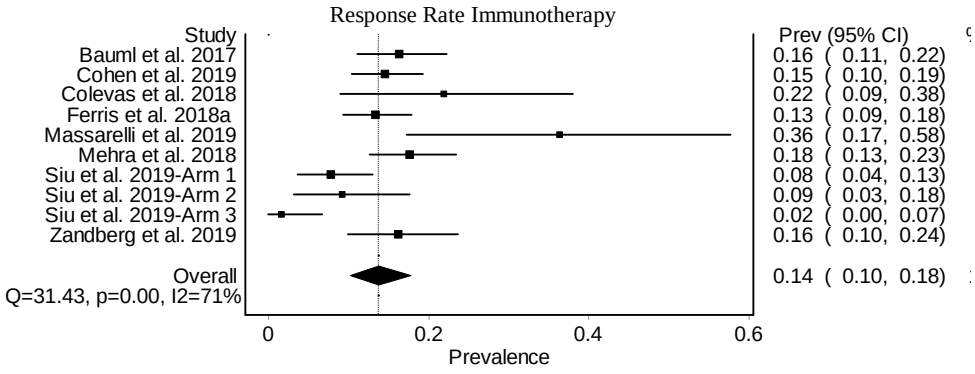
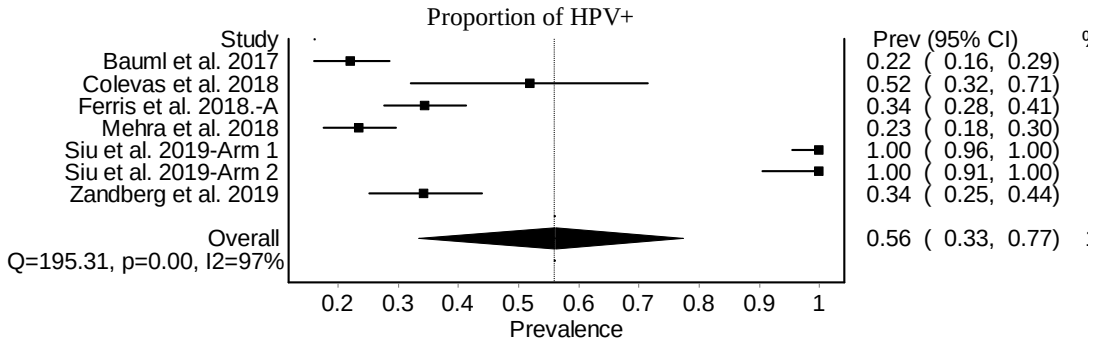
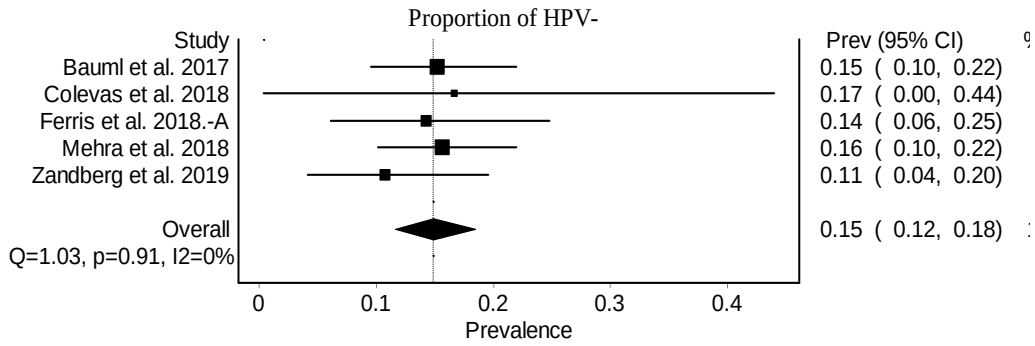

Response Rate Immunotherapy
Study
Prev (95% CI)
Bauml et al. 2017 0.16 (0.11, 0.22)
Cohen et al. 2019 0.15 (0.10, 0.19)
Colevas et al. 2018 0.22 (0.09, 0.38)
Ferris et al. 2018a 0.13 (0.09, 0.18)
Massarelli et al. 2019 0.36 (0.17, 0.58)
Mehra et al. 2018 0.18 (0.13, 0.23)
Siu et al. 2019-Arm 1 0.08 (0.04, 0.13)
Siu et al. 2019-Arm 2 0.09 (0.03, 0.18)
Siu et al. 2019-Arm 3 0.02 (0.00, 0.07)
Zandberg et al. 2019 0.16 (0.10, 0.24)
Overall 0.14 (0.10, 0.18)
Q=31.43, p=0.00, I2=71%
0 0.2 0.4 0.6
Prevalence

Figure 5.

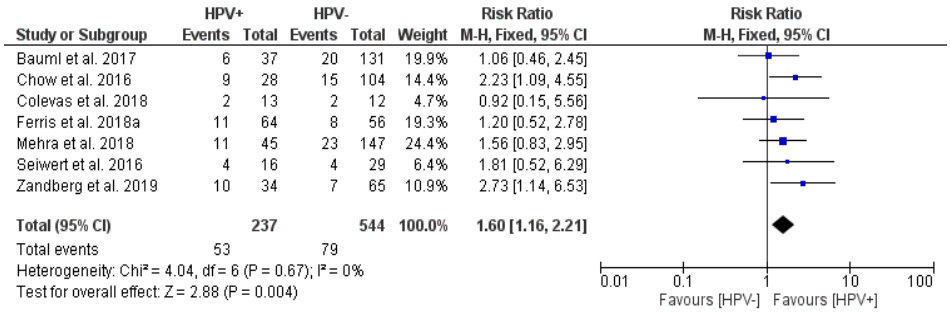
A



B



C



Study or Subgroup	HPV+ Events	HPV+ Total	HPV- Events	HPV- Total	Weight	Risk Ratio M-H, Fixed, 95% CI
Bauml et al. 2017	6	37	20	131	19.9%	1.06 [0.46, 2.45]
Chow et al. 2016	9	28	15	104	14.4%	2.23 [1.09, 4.55]
Colevas et al. 2018	2	13	2	12	4.7%	0.92 [0.15, 5.56]
Ferris et al. 2018a	11	64	8	56	19.3%	1.20 [0.52, 2.78]
Mehra et al. 2018	11	45	23	147	24.4%	1.56 [0.83, 2.95]
Seiwert et al. 2016	4	16	4	29	6.4%	1.81 [0.52, 6.29]
Zandberg et al. 2019	10	34	7	65	10.9%	2.73 [1.14, 6.53]
Total (95% CI)		**237**		**544**	**100.0%**	**1.60 [1.16, 2.21]**
Total events	53		79			

Heterogeneity: Chi² = 4.04, df = 6 (P = 0.67); I² = 0%
Test for overall effect: Z = 2.88 (P = 0.004)

D

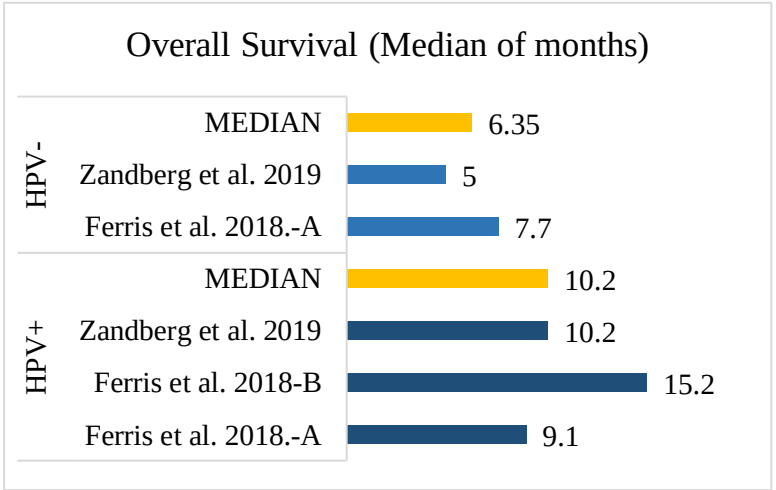
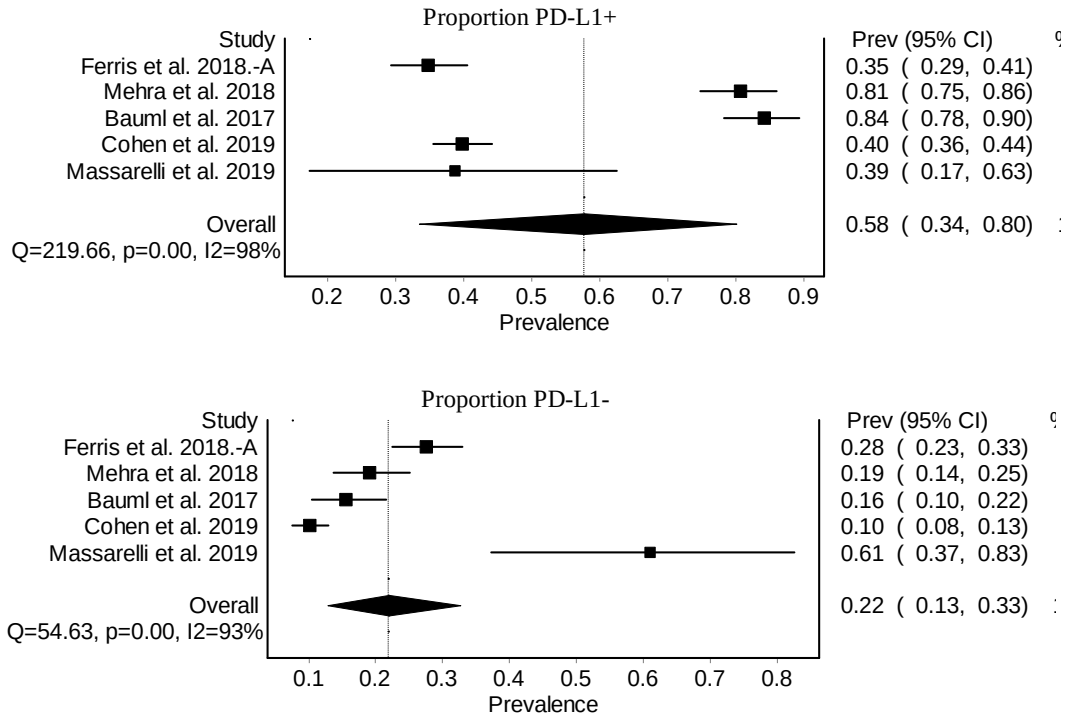


Figure 6.

A


Proportion PD-L1+
Study
Ferris et al. 2018.-A
Mehra et al. 2018
Bauml et al. 2017
Cohen et al. 2019
Massarelli et al. 2019
Overall
Q=219.66, p=0.00, I2=98%
Prev (95% CI)
0.35 (0.29, 0.41)
0.81 (0.75, 0.86)
0.84 (0.78, 0.90)
0.40 (0.36, 0.44)
0.39 (0.17, 0.63)
0.58 (0.34, 0.80)
0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Prevalence
Proportion PD-L1-
Study
Ferris et al. 2018.-A
Mehra et al. 2018
Bauml et al. 2017
Cohen et al. 2019
Massarelli et al. 2019
Overall
Q=54.63, p=0.00, I2=93%
Prev (95% CI)
0.28 (0.23, 0.33)
0.19 (0.14, 0.25)
0.16 (0.10, 0.22)
0.10 (0.08, 0.13)
0.61 (0.37, 0.83)
0.22 (0.13, 0.33)
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
Prevalence

B

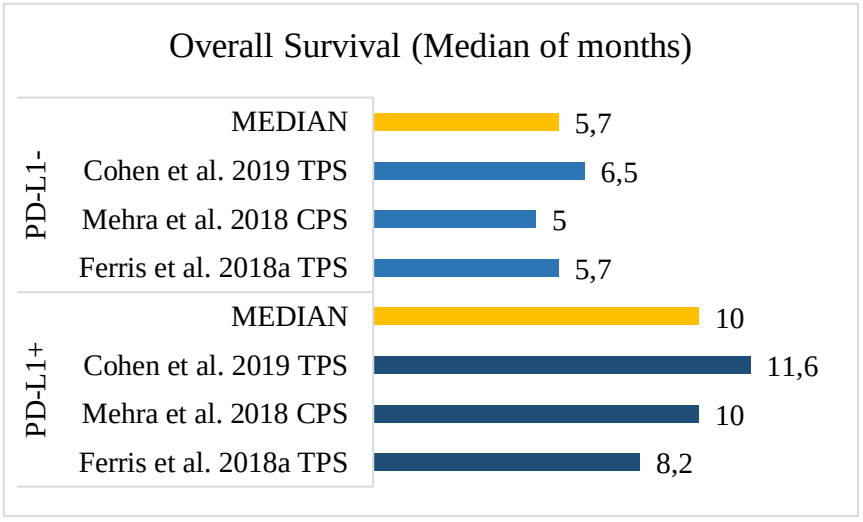
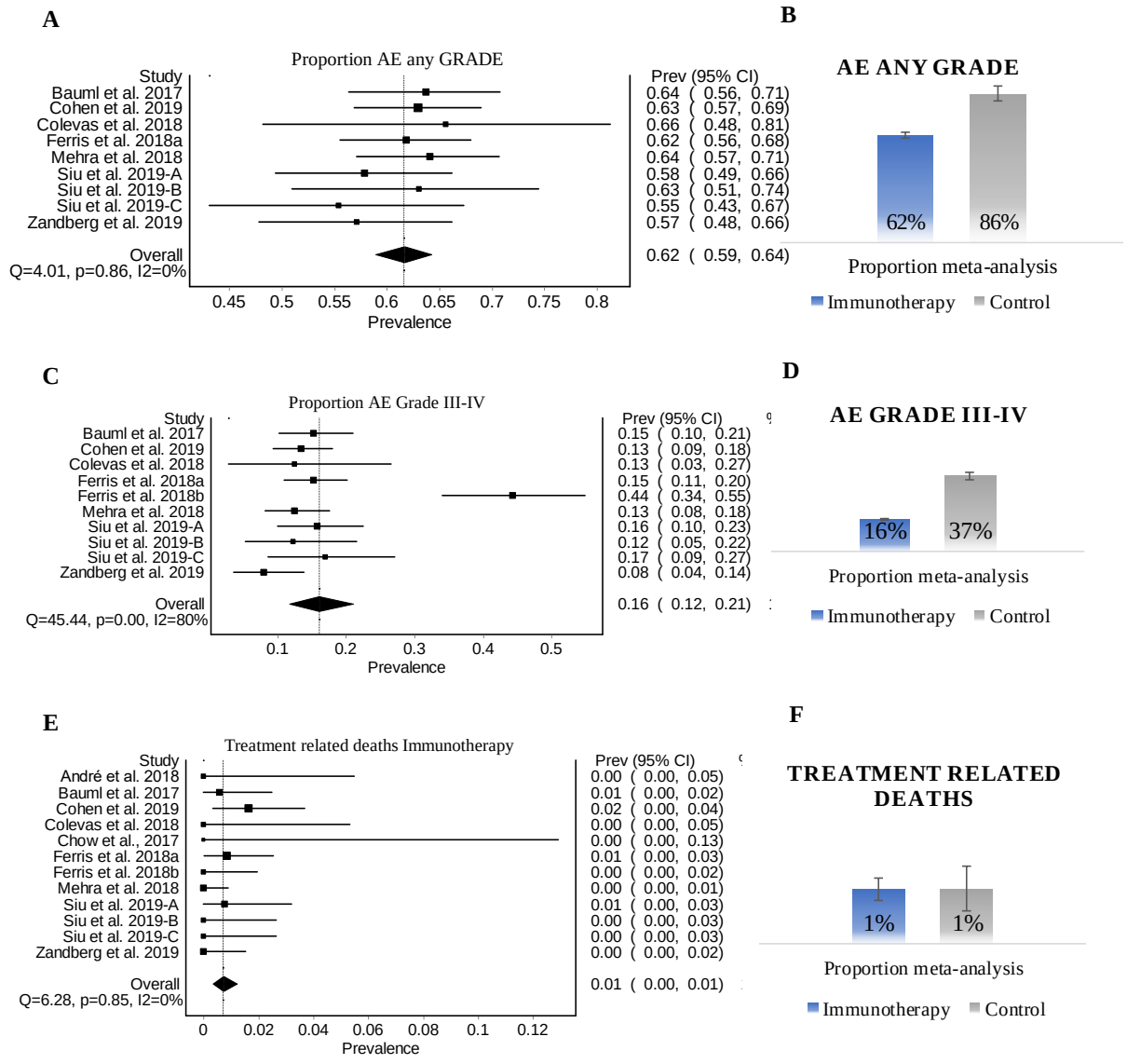

Overall Survival (Median of months)
PD-L1-
MEDIAN 5,7
Cohen et al. 2019 TPS 6,5
Mehra et al. 2018 CPS 5
Ferris et al. 2018a TPS 5,7
PD-L1+
MEDIAN 10
Cohen et al. 2019 TPS 11,6
Mehra et al. 2018 CPS 10
Ferris et al. 2018a TPS 8,2

Figure 7.



Abbreviations: AE, adverse effects.

Appendix 1 - Database search strategy (January 10th 2019).

Database	Search
PubMed	("Head and neck cancer" OR "head and neck carcinoma" OR "HNSCC" OR "head and neck squamous cell carcinoma" OR "oral cancer" OR "oral squamous cell carcinoma" OR "oral carcinoma" OR "OSCC" OR "oropharynx cancer" OR "oropharynx squamous cell carcinoma" OR "larynx cancer" OR "larynx squamous cell carcinoma" OR "hypopharynx cancer" OR "hypopharynx squamous cell carcinoma") **OR** (Head and Neck Neoplasms[MeSH Terms]) **AND** (Immunotherapy OR "immuno-therapy" OR "Immunomodulator" OR "Immunomodulation" OR "checkpoint inhibitors" OR "Immune Checkpoints" OR "Costimulatory Agonists" OR "CTLA-4 Inhibitors" OR "PD1 inhibitors" OR "PD-L1 inhibitors" OR "IDO inhibitors" OR "Anti-PD1" OR "Anti-PD-L1" OR "Anti-CTLA-4") **OR** (Pembrolizumab OR Nivolumab OR Durvalumab OR Avelumab OR Tremelimumab OR Urelumab OR Ipilimumab) **OR** (Immunotherapy[MeSH Terms]).
Cochrane	("Head and neck cancer" OR "head and neck carcinoma" OR "HNSCC" OR "head and neck squamous cell carcinoma" OR "oral cancer" OR "oral squamous cell carcinoma" OR "oral carcinoma" OR "OSCC" OR "oropharynx cancer" OR "oropharynx squamous cell carcinoma" OR "larynx cancer" OR "larynx squamous cell carcinoma" OR "hypopharynx cancer" OR "hypopharynx squamous cell carcinoma") **OR** (MeSH descriptor: [Head and Neck Neoplasms]) **AND** (Immunotherapy OR "immuno-therapy" OR "Immunomodulator" OR "checkpoint inhibitors" OR "Immune Checkpoints" OR "Costimulatory Agonists" OR "CTLA-4 Inhibitors" OR "PD1 inhibitors" OR "PD-L1 inhibitors" OR "Anti-PD1" OR "Anti-PD-L1" OR "Anti-CTLA-4") **OR** (Pembrolizumab OR Nivolumab OR Durvalumab OR Avelumab OR Tremelimumab OR Urelumab OR Ipilimumab) **OR** (MeSH descriptor: [Immunotherapy]).
EMBASE	(('head':ti,ab,kw AND 'neck cancer':ti,ab,kw OR 'head':ti,ab,kw) AND 'neck carcinoma':ti,ab,kw OR 'hnscc':ti,ab,kw OR 'head':ti,ab,kw) AND 'neck squamous cell carcinoma':ti,ab,kw OR 'oral cancer':ti,ab,kw OR 'oral squamous cell carcinoma':ti,ab,kw OR 'oral carcinoma':ti,ab,kw OR 'oscc':ti,ab,kw OR 'oropharynx cancer':ti,ab,kw OR 'oropharynx squamous cell carcinoma':ti,ab,kw OR 'larynx cancer':ti,ab,kw OR 'larynx squamous cell carcinoma':ti,ab,kw OR 'hypopharynx cancer':ti,ab,kw OR 'hypopharynx squamous cell carcinoma':ti,ab,kw) **AND** ('immunotherapy':ti,ab,kw OR 'immuno-therapy':ti,ab,kw OR 'immunomodulator':ti,ab,kw OR 'immunomodulation':ti,ab,kw OR 'checkpoint inhibitors':ti,ab,kw OR 'immune checkpoints':ti,ab,kw OR 'costimulatory agonists':ti,ab,kw OR 'ctla-4 inhibitors':ti,ab,kw OR 'pd1 inhibitors':ti,ab,kw OR 'pd-l1 inhibitors':ti,ab,kw OR 'anti-pd1':ti,ab,kw OR 'anti-pd-l1':ti,ab,kw OR 'anti-ctla-4':ti,ab,kw) **OR** ('pembrolizumab':ti,ab,kw OR 'nivolumab':ti,ab,kw OR 'durvalumab':ti,ab,kw OR 'avelumab':ti,ab,kw OR 'tremelimumab':ti,ab,kw OR 'urelumab':ti,ab,kw OR 'ipilimumab':ti,ab,kw).
SCOPUS	(TITLE-ABS-KEY ("Head and neck cancer" OR "head and neck carcinoma" OR "HNSCC" OR "head and neck squamous cell carcinoma" OR "oral cancer" OR "oral squamous cell carcinoma" OR "oral carcinoma" OR "OSCC" OR "oropharynx cancer" OR "oropharynx

	squamous cell carcinoma" OR "larynx cancer" OR "larynx squamous cell carcinoma" OR "hypopharynx cancer" OR "hypopharynx squamous cell carcinoma")) **AND** (TITLE-ABS-KEY ((immunotherapy OR "immuno-therapy" OR "Immunomodulator" OR "Immunomodulation" OR "checkpoint inhibitors" OR "Immune Checkpoints" OR "Costimulatory Agonists" OR "CTLA-4 Inhibitors" OR "PD1 inhibitors" OR "PD-L1 inhibitors" OR "Anti-PD1" OR "Anti-PD-L1" OR "Anti-CTLA-4")) **OR** (TITLE-ABS-KEY (pembrolizumab OR nivolumab OR durvalumab OR avelumab OR tremelimumab OR urelumab OR ipilimumab)))
Web of Science	ALL FIELDS: (("Head and neck cancer" OR "head and neck carcinoma" OR "HNSCC" OR "head and neck squamous cell carcinoma" OR "oral cancer" OR "oral squamous cell carcinoma" OR "oral carcinoma" OR "OSCC" OR oropharynx cancer OR oropharynx squamous cell carcinoma OR larynx cancer OR larynx squamous cell carcinoma OR hypopharynx cancer OR hypopharynx squamous cell carcinoma)) **AND** (Immunotherapy OR "immuno-therapy" OR "Immunomodulator" OR "Immunomodulation" OR "checkpoint inhibitors" OR "Immune Checkpoints" OR "Costimulatory Agonists" OR "CTLA-4 Inhibitors" OR "PD1 inhibitors" OR "PD-L1 inhibitors" OR "Anti-PD1" OR "Anti-PD-L1" OR "Anti-CTLA-4") **OR** (Pembrolizumab OR Nivolumab OR Durvalumab OR Avelumab OR Tremelimumab OR Urelumab OR Ipilimumab).
Google Scholar	IN THE TITLE OF THE ARTICLE: Immunotherapy **AND** "head and neck cancer"
Proquest	Immunotherapy **AND** "head and neck cancer"

Appendix 2 - Articles excluded and the reasons for exclusion (n=91).

Reasons for exclusion*	Author, year
1	Callahan et al., 2017; Chen et al., 2018; Dredge et al., 2018; Juric et al., 2017; Taylor et al., 2016.
2	Adkins et al., 2018; Bentzen et al., 2018; Bertino et al., 2016; Chindavijak et al., 2018; Colnot et al., 2002; Colnot et al., 2001; Colnot et al., 2000; Colnot et al., 2003; Colnot et al., 2001; Freeman et al., 2011; Hadden et al., 2003; Michaluart et al., 2008; Miyazaki et al., 2011; Reuschenbach et al., 2016; Rodríguez et al., 2010; Schuler et al., 2014; Vlahovic et al., 2018; Yang et al., 2015; Yoshitake et al., 2015; Yuta et al., 2011; Zandberg et al., 2015.
3	Barrera et al., 2001; Barrera et al., 2000; Bell et al., 2017; Chow et al., 2016; Harrington et al., 2016; Kiyota et al., 2016; Leidner et al., 2017; Seiwert et al., 2015; Shayan et al., 2018; Uppaluri et al., 2017; Wise-Draper et al., 2018.
5	Bartkowiak et al., 2015.
6	Chung et al., 2018; Khagi et al., 2017; Levy et al., 2016; Margolin et al., 2018; Mitchell et al., 2018; Park et al., 2018; Powderly et al., 2015; Sanborn et al., 2016; Segal et al., 2017; Segal et al., 2016; Yap et al., 2016.
7	Bauml et al., 2016; Chow et al., 2015; Chow et al., 2014; Chow et al., 2014; Cohen et al., 2016; Cohen et al., 2017; Ferris et al., 2018; Ferris et al., 2018; Ferris et al., 2017; Ferris et al., 2018; Gangadhar et al., 2015; Gillison et al., 2016; Gillison et al., 2017; Glisson et al., 2017; Haddad et al., 2017; Haddad et al., 2016; Hasegawa et al., 2016; Kasper et al., 2017; Kiyota et al., 2017; Mehra et al., 2016; Gillison1 et al., 2017; Soulieres et al., 2018; Tahara et al., 2016; Tahara et al., 2018; Zandberg et al., 2017; Zandberg et al., 2018.
9	Bonomo et al., 2017; Brahmer et al., 2016; Karabajakian et al., 2018; Larkins et al., 2017; Machiels et al., 2017; Russel et al., 2016; Seymour et al., 2015; Yen et al., 2015; Yu et al., 2018.
10	Chen et al., 2017; Das et al., 2015; Granados et al., 2018; Kao et al., 2017; Lin et al., 2018; Saada-Bouzid et al., 2017; Saada-Bouzid et al., 2016.

*Legend: (1) Studies on conditions other than head and neck squamous cell carcinoma; (2) Studies that do not assess checkpoint inhibitors or costimulatory agonists; (3) Studies in which survival measures, overall response rate or treatment-related adverse events are not presented; (4) Pre-clinical, observational, retrospective or case report studies; (5) Studies in which the results on head and neck squamous cell carcinoma cannot be individualized; (6) Studies that report duplicated data (results that were previously published); (7) Studies written in languages other than English; (8) Reviews, letters, trial protocols, personal opinions and book chapters.

References of excluded articles

1. Adkins D, Ley J, Atiq O, Rigden C, Trinkaus K, Wildes TM, et al. Multicenter phase 2 trial of cis/carboplatin, nab-paclitaxel, and cetuximab (CACTUX) as first-line therapy for recurrent/metastatic head and neck squamous cell carcinoma. International Journal of Radiation Oncology Biology Physics. 2018;100(5):1311-2.
2. Barrera J, Verastegui E, Meneses A, Zinser J, De la Garza J, Hadden J. Clinical and pathological bio-responses induced with a cytokine mixture (IRX-2) in patients with oral cavity squamous cell carcinoma. Clinical and Applied Immunology Reviews. 2001;1(3-4):181-5.

3. Barrera JL, Verastegui E, Meneses A, Zinser J, de la Garza J, Hadden JW. Combination immunotherapy of squamous cell carcinoma of the head and neck: a phase 2 trial. Archives of otolaryngology--head & neck surgery. 2000;126(3):345-51.
4. Bartkowiak T, Singh S, Yang G, Galvan G, Allison J, Sastry J, et al. Unique potential of 4-1BB agonist antibody to promote durable regression of HPV+ tumors when combined with an E6/E7 peptide vaccine (TUM2P.1016). Journal of Immunology. 2015;194(1).
5. Bauml J, Seiwert TY, Pfister DG, Worden FP, Liu SV, Gilbert J, et al. Preliminary results from KEYNOTE-055: Pembrolizumab after platinum and cetuximab failure in head and neck squamous cell carcinoma (HNSCC). Journal of Clinical Oncology. 2016;34.
6. Bell BR, Leidner R, Duhen R, Koguchi Y, Weinberg A. A phase IB neoadjuvant trial of anti-OX-40 (medi6469) prior to definitive surgical resection in patients with head and neck squamous cell carcinoma. Head and Neck. 2017;39:E46-E7.
7. Bentzen JKD, Kristensen CA, Overgaard M, Rytter C, Jensen K, Hansen HS. a non Platinum regimen for the Treatment of recurrent or Metastatic Squamous Cell Carcinoma of the Head and neck region. results From an Extended Phase II Study With Paclitaxel and Capecitabine. Frontiers in oncology. 2018;8.
8. Bertino EM, McMichael EL, Mo X, Trikha P, Davis M, Paul B, et al. A Phase I Trial to Evaluate Antibody-Dependent Cellular Cytotoxicity of Cetuximab and Lenalidomide in Advanced Colorectal and Head and Neck Cancer. Molecular cancer therapeutics. 2016;15(9):2244-50.
9. Bonomo P, Desideri I, Loi M, Mangoni M, Sottili M, Greto D, et al. Anti-PD-L1 durvalumab combined with cetuximab and radiotherapy in locally advanced squamous cell carcinoma of the head and neck: A phase II study. Journal of Clinical Oncology. 2017;35(15).
10. Brahmer J, Rasco D, Chen M, Masteller E, Qazi I, Rogers S, et al. A phase 1a/1b study of FPA008 in combination with nivolumab in patients with selected advanced cancers. Cancer immunology research Conference: CRI-CIMT-EATI-AACR inaugural international cancer immunotherapy conference: translating science into survival United states Conference start: 20150916 Conference end: 20150919. 2016;4(1 Supplement) (no pagination).
11. Callahan MK, Odunsi K, Sznol M, Nemunaitis JJ, Ott PA, Dillon PM, et al. Phase 1 study to evaluate the safety and tolerability of MEDI4736 (durvalumab, DUR) + tremelimumab (TRE) in patients with advanced solid tumors. Journal of clinical oncology Conference: 2017 annual meeting of the american society of clinical oncology, ASCO United states. 2017;35(15 Supplement 1) (no pagination).
12. Chen CL, Pan QZ, Weng DS, Xie CM, Zhao JJ, Chen MS, et al. Safety and activity of PD-1 blockade-activated DC-CIK cells in patients with advanced solid tumors. Oncoimmunology. 2018;7(4).
13. Chen WC, Chu PY, Lee YT, Lu WB, Liu CY, Chang PM, et al. Pembrolizumab for recurrent/metastatic head and neck squamous cell carcinoma in an Asian population. Medicine. 2017;96(52):e9519.
14. Chindavijak S, Har-Noy M, Lausoontornsiri W. Effect of therapeutic vaccine on CTLA4 and tumor debulking response in recurrent and metastatic HNSCC. Journal of Clinical Oncology. 2018;36(5).
15. Chow L, Mehra R, Haddad R, Gupta S, Weiss J, Berger R, et al. Antitumor activity of the Anti-PD-1 antibody pembrolizumab in subgroups of patients with recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC): Exploratory analyses from KEYNOTE-012. European Journal of Cancer. 2015;51:S579.
16. Chow LQM, Eaton KD, Baik C, Goulart B, Morishima C, Disis ML, et al. Phase 1b trial of TLR8 Agonist VTX-2337 in combination with cetuximab in patients with recurrent or metastatic squamous cell carcinomas of the head and neck (SCCHN) immunology and

immunotherapy. International journal of radiation oncology biology physics. 2014;88(2):503-4.
17. Chow LQM, Eaton KD, Baik C, Goulart B, Morishima C, Disis ML, et al. Phase 1b Trial of TLR8 Agonist VTX-2337 in Combination With Cetuximab in Patients With Recurrent or Metastatic Squamous Cell Carcinomas of the Head and Neck (SCCHN): Immunology and Immunotherapy. International Journal of Radiation Oncology • Biology • Physics. 2014;88(2):503-4.
18. Chow LQM, Mehra R, Haddad RI, Mahipal A, Weiss J, Berger R, et al. Biomarkers and response to pembrolizumab (pembro) in recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC). Journal of Clinical Oncology. 2016;34.
19. Chung V, Kos FJ, Hardwick N, Yuan Y, Chao J, Li D, et al. Evaluation of safety and efficacy of p53MVA vaccine combined with pembrolizumab in patients with advanced solid cancers. Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico. 2018.
20. Cohen E, Saba NF, Gitlitz B, Haddad R, Sukari A, Neupane P, et al. Active8: a randomized, double-blind, placebo-controlled study of chemotherapy plus cetuximab in combination with motolimod immunotherapy in patients with recurrent or metastatic squamous cell carcinoma of the head and neck. Annals of oncology Conference: 41st european society for medical oncology congress, ESMO 2016 Denmark Conference start: 20161007 Conference end: 20161011. 2016;27(no pagination).
21. Cohen EE, Harrington KJ, Le Tourneau C, Dinis J, Licitra L, Ahn MJ, et al. PR Pembrolizumab (pembro) vs standard of care (SOC) for recurrent or metastatic head and neck squamous cell carcinoma (R/M HNSCC): phase 3 KEYNOTE-040 trial. Annals of oncology Conference: 42nd ESMO congress, ESMO 2017 Spain. 2017;28(Supplement 5):v628.
22. Colnot DR, Ossenkoppele GJ, Roos JC, Quak JJ, de Bree R, Borjesson PK, et al. Reinfusion of unprocessed, granulocyte colony-stimulating factor-stimulated whole blood allows dose escalation of 186Relabeled chimeric monoclonal antibody U36 radioimmunotherapy in a phase I dose escalation study. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002;8(11):3401-6.
23. Colnot DR, Quak JJ, Roos JC, De Bree R, Wilhelm AJ. Radioimmunotherapy in patients with head and neck squamous cell carcinoma: Initial experience. Head and Neck. 2001;23(7):559-65.
24. Colnot DR, Quak JJ, Roos JC, van Lingen A, Wilhelm AJ, van Kamp GJ, et al. Phase I therapy study of Re-186-labeled chimeric monoclonal antibody U36 in patients with squamous cell carcinoma of the head and neck. Journal of Nuclear Medicine. 2000;41(12):1999-2010.
25. Colnot DR, Roos JC, de Bree R, Wilhelm AJ, Kummer JA, Hanft G, et al. Safety, biodistribution, pharmacokinetics, and immunogenicity of Tc-99m-labeled humanized monoclonal antibody BIWA 4 (bivatuzumab) in patients with squamous cell carcinoma of the head and neck. Cancer Immunology Immunotherapy. 2003;52(9):576-82.
26. Colnot DR, Wilhelm AJ, Cloos J, Roos JC, de Bree R, Quak JJ, et al. Evaluation of limited blood sampling in a preceding 99mTc-labeled diagnostic study to predict the pharmacokinetics and myelotoxicity of 186Re-cMAb U36 radioimmunotherapy. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 2001;42(9):1364-7.
27. Das LC, Haraf D, Brisson R, McHugh M, Cheng J, Heath K, et al. Evaluation of patients with progressive metastatic head and neck cancer treated with PD-1 inhibition with Pembrolizumab and radiation therapy: Assessment of local and systemic effects. European Journal of Cancer. 2015;51:S105.
28. Dredge K, Brennan TV, Hammond E, Lickliter JD, Lin L, Bampton D, et al. A Phase i study of the novel immunomodulatory agent PG545 (pixatimod) in subjects with advanced solid tumours. British journal of cancer. 2018;118(8):1035-41.

29. Ferris RL, Blumenschein GR, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Two-year update from checkmate 141: outcomes with nivolumab (NIVO) vs investigator's choice (IC) in recurrent or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN) in the overall population and PD-l1 subgroups. International journal of radiation oncology biology physics Conference: 2018 multidisciplinary head and neck cancer symposium United states. 2018;100(5):1317.
30. Ferris RL, Blumenschein GR, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab (Nivo) vs investigator's choice (IC) in recurrent or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN): 2-yr outcomes in the overall population and PD-L1 subgroups of CheckMate 141. Cancer research Conference: 2018 annual meeting of the american association for cancer research, AACR 2018 United states. 2018;78(13 Supplement 1) (no pagination).
31. Ferris RL, Clump DA, Ohr J, Gooding W, Kim S, Karlovits BJ, et al. Phase I trial of cetuximab, intensity modulated radiotherapy (IMRT), and ipilimumab in previously untreated, locally advanced head and neck squamous cell carcinoma (PULA HNSCC). Annals of Oncology. 2017;28:v379.
32. Ferris RL, Gillison ML, Harris J, Colevas AD, Mell LK, Kong C. Safety evaluation of nivolumab (Nivo) concomitant with cetuximab-radiotherapy for intermediate (IR) and high-risk (HR) local-regionally advanced head and neck squamous cell carcinoma (HNSCC): RTOG 3504. Journal of Clinical Oncology. 2018;36(15).
33. Freeman SM, Franco JL, Kenady DE, Baltzer L, Roth Z, Brandwein HJ, et al. A phase 1 safety study of an IRX-2 regimen in patients with squamous cell carcinoma of the head and neck. American journal of clinical oncology. 2011;34(2):173-8.
34. Gangadhar TC, Hamid O, Smith DC, Bauer TM, Wasser JS, Luke JJ, et al. Preliminary results from a Phase I/II study of epacadostat (incb024360) in combination with pembrolizumab in patients with selected advanced cancers. Journal for immunotherapy of cancer. 2015;3.
35. Gillison ML, Blumenschein G, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab (nivo) vs investigator's choice (IC) for recurrent or metastatic (R/M) head and neck squamous cell carcinoma (HNSCC): checkMate-141. Cancer research Conference: 107th annual meeting of the american association for cancer research, AACR 2016 United states Conference start: 20160416 Conference end: 20160420. 2016;76(14 Supplement) (no pagination).
36. Gillison ML, Blumenschein G, Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab versus investigator's choice (IC) for platinum-refractory recurrent or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN; CHECKMATE 141): outcomes in first-line R/M patients and updated safety and efficacy. Asia-pacific journal of clinical oncology Conference: 44th annual scientific meeting of the clinical oncology society of australia, COSA 2017 Australia. 2017;13(Supplement 4):144.
37. Glisson B, Massarelli E, William WN, Johnson FM, Kies MS, Ferrarotto R, et al. Nivolumab and ISA 101 HPV vaccine in incurable HPV-161 cancer. Annals of oncology Conference: 42nd ESMO congress, ESMO 2017 Spain. 2017;28(Supplement 5):v403-v4.
38. Granados ALO, Caro NL, Pozo JFM, Vargas FA, Navarro MF, Cabrera CD. Survival with nivolumab therapy in recurrent/advanced squamous cell head and neck carcinoma. A single center experience. Journal of Clinical Oncology. 2018;36(15).
39. Haddad R, Ferris RL, Blumenschein G, Fayette J, Guigay J, Colevas AD, et al. Treatment beyond progression with nivolumab in patients with recurrent or metastatic squamous cell carcinoma of the head and neck in the phase 3 Checkmate 141 study. Cancer research Conference: american association for cancer research annual meeting 2017 United states. 2017;77(13 Supplement 1) (no pagination).

40. Haddad R, Seiwert T, Pfister DG, Worden F, Liu SV, Gilbert J, et al. Pembrolizumab after progression on platinum and cetuximab in head and neck squamous cell carcinoma (HNSCC): results from KEYNOTE-055. Annals of oncology Conference: 41st european society for medical oncology congress, ESMO 2016 Denmark Conference start: 20161007 Conference end: 20161011. 2016;27(no pagination).
41. Hadden J, Verastegui E, Barrera JL, Kurman M, Meneses A, Zinser JW, et al. A trial of IRX-2 in patients with squamous cell carcinomas of the head and neck. International immunopharmacology. 2003;3(8):1073-81.
42. Harrington K, Ferris RL, Shaw J, Taylor F, Derosa M, Turner-Bowker D, et al. PR Patient-reported outcomes (PROs) in recurrent or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN) treated with nivolumab (nivo) or investigator's choice (IC): checkMate 141. Annals of oncology Conference: 41st european society for medical oncology congress, ESMO 2016 Denmark Conference start: 20161007 Conference end: 20161011. 2016;27(no pagination).
43. Hasegawa Y, Kiyota N, Takahashi S, Yokota T, Yen CJ, Iwae S, et al. Efficacy and safety of nivolumab for recurrent or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN) in Asia: checkMate 141 subgroup analysis. Annals of oncology Conference: ESMO asia congress 2016 Singapore. 2016;27(Supplement 9):ix112-ix3.
44. Juric D, Faucette S, Wang L, Niu H, Kannan K, Stumpo K, et al. A phase 1b study to evaluate TAK-659 in combination with nivolumab in patients (pts) with advanced solid tumors. Journal of Clinical Oncology. 2017;35(15).
45. Kao HF, Huang HC, Liao BC, Hong RL. Pembrolizumab and afatinib for recurrent or metastatic head and neck squamous cell carcinoma. Journal for immunotherapy of cancer. 2017;5.
46. Karabajakian A, Reverdy T, Gau M, Fayette J. Nivolumab in recurrent/metastatic head and neck cancers. Future oncology (London, England). 2018;14(7):603-9.
47. Kasper S, Haddad R, Ferris RL, Blumenschein G, Fayette J, Guigay J, et al. Treatment beyond progression with nivolumab (nivo) in patients with recurrent or metastatic (R/M) Squamous Cell Carcinoma of the Head and Neck (SCCHN) in the phase 3 CheckMate 141 study. Oncology research and treatment Conference: jahrestagung der deutschen, osterreichischen und schweizerischen gesellschaften fur hamatologie und medizinische onkologie 2017 Germany. 2017;40(Supplement 3):250-1.
48. Khagi Y, Goodman AM, Daniels GA, Patel SP, Sacco AG, Randall JM, et al. Hypermutated circulating tumor DNA: Correlation with response to checkpoint inhibitor–based immunotherapy. Clinical Cancer Research. 2017;23(19):5729-36.
49. Kiyota N, Harrington K, Ferris RL, Shaw JW, Taylor F, Derosa M, et al. Patient-reported outcomes (PROs) in recurrent or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN) treated with nivolumab (Nivo) or Investigator's Choice (IC): checkMate 141. Annals of oncology Conference: ESMO asia congress 2016 Singapore. 2016;27(Supplement 9):ix113.
50. Kiyota N, Hasegawa Y, Takahashi S, Yokota T, Yen CJ, Iwae S, et al. A randomized, open-label, Phase III clinical trial of nivolumab vs. therapy of investigator's choice in recurrent squamous cell carcinoma of the head and neck: A subanalysis of Asian patients versus the global population in checkmate 141. Oral oncology. 2017;73:138-46.
51. Larkins E, Blumenthal GM, Yuan W, He K, Sridhara R, Subramaniam S, et al. FDA Approval Summary: Pembrolizumab for the Treatment of Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma with Disease Progression on or After Platinum-Containing Chemotherapy. The oncologist. 2017;22(7):873-8.
52. Leidner R, Crittenden M, Gough M, Fox BA, Weinberg AD, Dierks EJ, et al. A potential solution to immunosuppression of the tumour microenvironment: Results of a phase 1 clinical

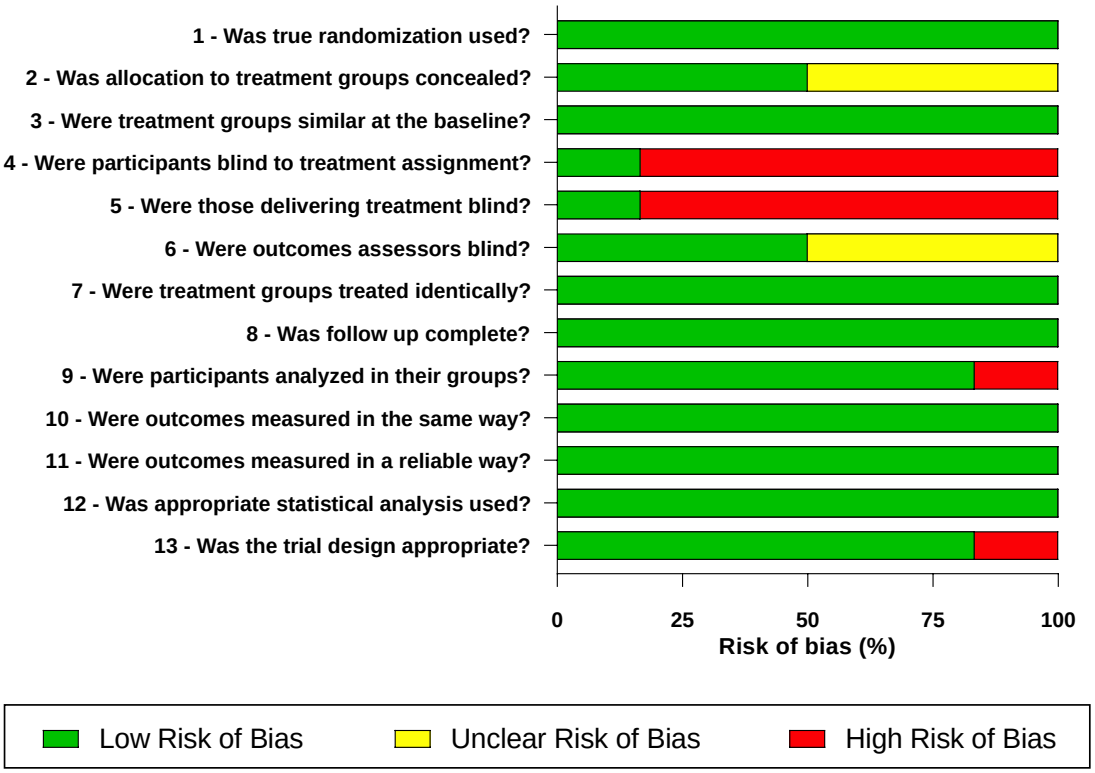
trial of anti-OX-40 in head and neck squamous cell carcinoma. International journal of oral and maxillofacial surgery. 2017;46:126.
53. Levy A, Massard C, Soria JC, Deutsch E. Concurrent irradiation with the anti-programmed cell death ligand-1 immune checkpoint blocker durvalumab: Single centre subset analysis from a phase 1/2 trial. European journal of cancer (Oxford, England : 1990). 2016;68:156-62.
54. Lin YC, Uen WC, Hao SP, Hsiao CY, Lai HC. Triple combination treatment of cetuximab, chemotherapy, and anti-PD1 check-point inhibitor for recurrent and/or metastatic head and neck squamous cell carcinoma: A single institute experience. Journal of Clinical Oncology. 2018;36(15).
55. Machiels JP, Licitra L, Rischin D, Waldron J, Burtness B, Gregoire V, et al. KEYNOTE-412: pembrolizumab (pembro) in combination with chemoradiation versus chemoradiation alone in locally advanced head and neck squamous cell carcinoma (LA-HNSCC). Journal of clinical oncology. 2017;35(15 Suppl 1).
56. Margolin K, Morishima C, Velcheti V, Miller JS, Lee SM, Silk AW, et al. Phase I Trial of ALT-803, A Novel Recombinant IL15 Complex, in Patients with Advanced Solid Tumors. Clinical cancer research : an official journal of the American Association for Cancer Research. 2018;24(22):5552-61.
57. Mehra R, Seiwert TY, Mahipal A, Weiss J, Berger R, Eder JP, et al. Efficacy and safety of pembrolizumab in recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC): Pooled analyses after long-term follow-up in KEYNOTE-012. Journal of Clinical Oncology. 2016;34.
58. Michaluart P, Abdallah KA, Lima FD, Smith R, Moyses RA, Coelho V, et al. Phase I trial of DNA-hsp65 immunotherapy for advanced squamous cell carcinoma of the head and neck. Cancer gene therapy. 2008;15(10):676-84.
59. Mitchell TC, Hamid O, Smith DC, Bauer TM, Wasser JS, Olszanski AJ, et al. Epacadostat plus pembrolizumab in patients with advanced solid tumors: Phase I results from a multicenter, open-label phase I/II trial (ECHO-202/KEYNOTE-037). Journal of Clinical Oncology. 2018;36(32):3223-30.
60. Miyazaki A, Kobayashi J, Torigoe T, Hirohashi Y, Yamamoto T, Yamaguchi A, et al. Phase I clinical trial of survivin-derived peptide vaccine therapy for patients with advanced or recurrent oral cancer. Cancer science. 2011;102(2):324-9.
61. ML Gillison1 KH, Rl Ferris3, J Guigay4, G Blumenschein, Jr.5, J Fayette6, Ad Colevas7, N Kiyota8, Jw Shaw9, M Monga9, M Lynch9, J Kopit9, F Taylor10, M DeRosa10, L Morrissey10, K Cocks10, Nf Saba. Nivolumab versus investigator's choice therapy among patients with human papillomavirus (hpv)-associated squamous cell carcinoma of the head and neck (scchn): updated results from checkmate 141. 2017.
62. Park S, Sacco AG, Cohen EEW, Daniels GA. Safety and efficacy of checkpoint inhibition (CI) in cancer patients (pts) with concurrent human immunodeficiency virus (HIV) infection. Journal of Clinical Oncology. 2018;36(5).
63. Powderly J, Cote G, Flaherty K, Szmulewitz RZ, Ribas A, Weber J, et al. Interim results of an ongoing Phase I, dose escalation study of MGA271 (Fc-optimized humanized anti-B7-H3 monoclonal antibody) in patients with refractory B7-H3-expressing neoplasms or neoplasms whose vasculature expresses B7-H3. Journal for immunotherapy of cancer. 2015;3.
64. Reuschenbach M, Pauligk C, Karbach J, Rafiyan MR, Kloor M, Prigge ES, et al. A phase 1/2a study to test the safety and immunogenicity of a p16INK4a peptide vaccine in patients with advanced human papillomavirus-associated cancers. Cancer. 2016;122(9):1425-33.

65. Rodríguez MO, Rivero TC, Bahi RDC, Muchuli CR, Bilbao MA, Vinageras EN, et al. Nimotuzumab plus radiotherapy for unresectable squamous-cell carcinoma of the head and neck. Cancer Biology and Therapy. 2010;9(5):343-9.
66. Russel J, Von Einem J, Keilholz U, Dietz A, Grunewald V, Maschmeyer G, et al. RAPTOR, the global phase II, III clinical development program of the PD-L1 inhibitor Durvalumab (MEDI4736) in 2nd-line recurrent/metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN)-A German update on the HAWK, EAGLE and CONDOR trials. Oncology research and treatment Conference: jahrestagung der deutschen, osterreichischen und schweizerischen gesellschaften fur hamatologie und medizinische onkologie 2016 Germany Conference start: 20161014 Conference end: 20161018. 2016;39:173-4.
67. Saada-Bouzid E, Defaucheux C, Karabajakian A, Coloma VP, Servois V, Paoletti X, et al. Hyperprogression during anti-PD-1/PD-L1 therapy in patients with recurrent and/or metastatic head and neck squamous cell carcinoma. Annals of oncology : official journal of the European Society for Medical Oncology. 2017;28(7):1605-11.
68. Saada-Bouzid E, Defaucheux C, Karabajakian A, Coloma VP, Servois V, Paoletti X, et al. Tumor's flare-up and patterns of recurrence in patients (pts) with recurrent and/or metastatic (R/M) head and neck squamous cell carcinoma (HNSCC) treated with anti-PD1/PDL1 inhibitors. Journal of Clinical Oncology. 2016;34.
69. Sanborn RE, Pishvain MJ, Callahan MK, Rizvi N, Kluger H, Yellin M, et al. Phase I results from the combination of an immune-activating anti-CD27 antibody (varlilumab) in combination with PD-1 blockade (nivolumab): activation across multiple immune pathways without untoward immune-related adverse events. Cancer research Conference: 107th annual meeting of the american association for cancer research, AACR 2016 United states Conference start: 20160416 Conference end: 20160420. 2016;76(14 Supplement) (no pagination).
70. Schuler PJ, Harasymczuk M, Visus C, Deleo A, Trivedi S, Lei Y, et al. Phase I dendritic cell p53 peptide vaccine for head and neck cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2014;20(9):2433-44.
71. Segal NH, Logan TF, Hodi FS, McDermott D, Melero I, Hamid O, et al. Results from an integrated safety analysis of urelumab, an agonist anti-CD137 monoclonal antibody. Clinical Cancer Research. 2017;23(8):1929-36.
72. Segal NH, Sharma M, Le DT, Ott PA, Ferris RL, Zelenetz AD, et al. Assessing the potential for enhanced antibody-dependent cellmediated cytotoxicity (ADCC) by combining the CD137 antibody urelumab with rituximab or cetuximab in patients with refractory lymphoma or select advanced solid tumors. Journal for immunotherapy of cancer Conference: 31st annual meeting and associated programs of the society for immunotherapy of cancer, SITC 2016 United states Conference start: 20161109 Conference end: 20161113. 2016;4(no pagination).
73. Seiwert TY, Burtness B, Weiss J, Eder JP, Yearley J, Murphy E, et al. Inflamed-phenotype gene expression signatures to predict benefit from the anti-PD-1 antibody pembrolizumab in PD-L1+ head and neck cancer patients. Journal of Clinical Oncology. 2015;33(15).
74. Seymour L, Tinker A, Hirte H, Wagtmann N, Dodion P. Phase I and dose ranging, phase II studies with IPH2201, a humanized monoclonal antibody targeting HLA-E receptor CD94/NKG2A. Annals of oncology. 2015;26:ii3.
75. Shayan G, Kansy BA, Gibson SP, Srivastava RM, Bryan JK, Bauman JE, et al. Phase Ib study of immune biomarker modulation with neoadjuvant cetuximab and TLR8 stimulation in head and neck cancer to overcome suppressive myeloid signals. Clinical Cancer Research. 2018;24(1):62-72.

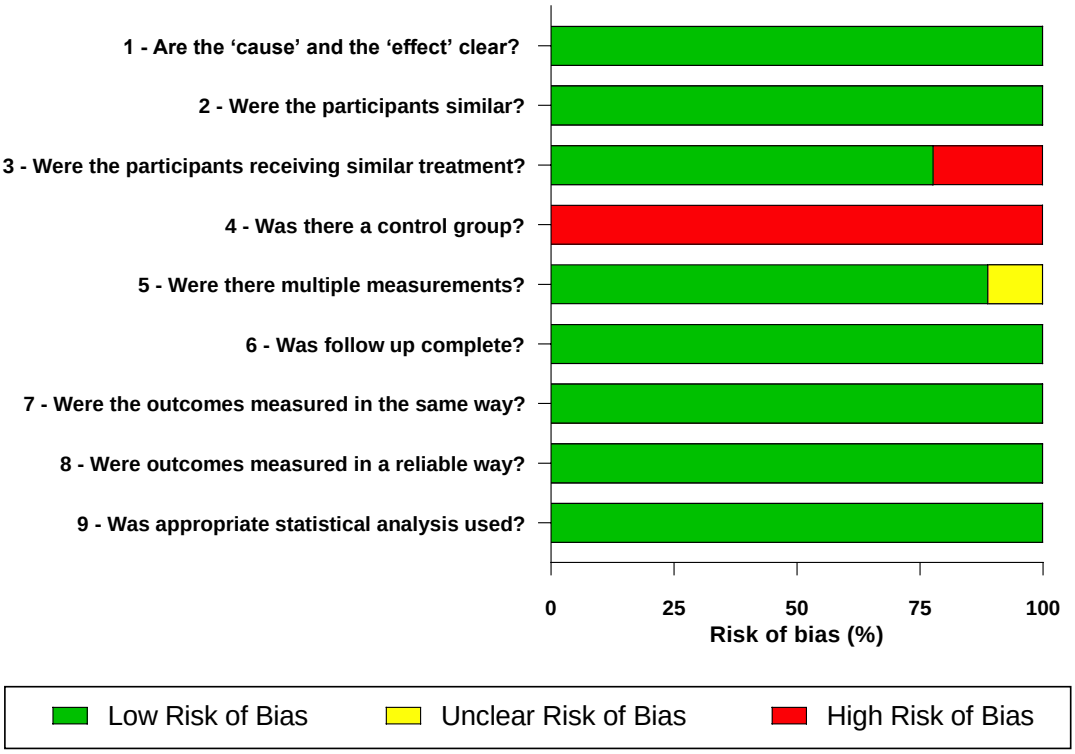
76. Soulieres D, Cohen E, Tourneau CL, Dinis J, Licitra L, Ahn MJ, et al. Updated survival results of the KEYNOTE-040 study of pembrolizumab vs standard-ofcare chemotherapy for recurrent or metastatic head and neck squamous cell carcinoma. Cancer research. 2018;78(13).
77. Tahara M, Muro K, Hasegawa Y, Chung HC, Lin CC, Keam B, et al. Efficacy and safety of pembrolizumab (MK-3475) in recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC): Subset analyses for Asia-pacific patients in KEYNOTE-012 (KN-012) B2 cohort. Annals of Oncology. 2016;27:ix112.
78. Tahara M, Muro K, Hasegawa Y, Chung HC, Lin CC, Keam B, et al. Pembrolizumab in Asia-Pacific patients with advanced head and neck squamous cell carcinoma: Analyses from KEYNOTE-012. Cancer science. 2018;109(3):771-6.
79. Taylor M, Dutcus CE, Schmidt E, Bagulho T, Li D, Shumaker R, et al. A phase 1b trial of lenvatinib (LEN) plus pembrolizumab (PEM) in patients with selected solid tumors. Annals of Oncology. 2016;27.
80. Uppaluri R, Zolkind P, Lin T, Nussenbaum B, R SJ, Rich J, et al. Neoadjuvant pembrolizumab in surgically resectable, HPV negative, locally advanced head and neck squamous cell carcinoma (HNSCC). Journal of Clinical Oncology. 2017;35(15).
81. Vlahovic G, Meadows KL, Hatch AJ, Jia J, Nixon AB, Uronis HE, et al. A Phase I Trial of the IGF-1R Antibody Ganitumab (AMG 479) in Combination with Everolimus (RAD001) and Panitumumab in Patients with Advanced Cancer. The oncologist. 2018;23(7):782-90.
82. Wise-Draper TM, Old MO, Worden FP, O'Brien PE, Cohen EEW, Dunlap N. Phase II multi-site investigation of neoadjuvant pembrolizumab and adjuvant concurrent radiation and pembrolizumab with or without cisplatin in resected head and neck squamous cell carcinoma. Journal of Clinical Oncology. 2018;36(15).
83. Yang Z, Aggarwal C, Cohen R, Morrow M, Bauml J, Weinstein G, et al. Immunotherapy with INO-3112 (HPV16 and HPV18 plasmids + IL-12 DNA) in human papillomavirus (HPV) associated head and neck squamous cell carcinoma (HNSCCa). Annals of Oncology. 2015;26:viii1.
84. Yap TA, Krebs MG, Postel-Vinay S, Bang YJ, El-Khoueiry A, Abida W, et al. Phase I modular study of AZD6738, a novel oral, potent and selective ataxia telangiectasia Rad3-related (ATR) inhibitor in combination (combo) with carboplatin, olaparib or durvalumab in patients (pts) with advanced cancers. European journal of cancer Conference: 28th EORTC-NCI-AACR symposium on molecular targets and cancer therapeutics Germany. 2016;69:S2.
85. Yen CJ, Klochikhin A, Cohen E, Vermorken J, Harrington K, Tahara M, et al. Phase 3 trial of pembrolizumab as a first-line treatment in subjects with recurrent/metastatic head and neck squamous cell carcinoma: KEYNOTE-048. Annals of Oncology. 2015;26:101-.
86. Yoshitake Y, Fukuma D, Yuno A, Hirayama M, Nakayama H, Tanaka T, et al. Phase II clinical trial of multiple peptide vaccination for advanced head and neck cancer patients revealed induction of immune responses and improved OS. Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(2):312-21.
87. Yu Y, Lee NY. JAVELIN Head and Neck 100: a Phase III trial of avelumab and chemoradiation for locally advanced head and neck cancer. Future oncology (London, England). 2018.
88. Yuta A, Shiku H. A clinical trial of combined immunotherapy with MAGE-A4 peptide and cultured T lymphocyte for patients with head and neck squamous cell carcinoma. Japanese Journal of Head and Neck Cancer. 2011;37(3):366-9.
89. Zandberg D, Algazi A, Jimeno A, Good JS, Fayette J, Bouganim N, et al. Durvalumab for recurrent/metastatic (R/M) head and neck squamous cell carcinoma (HNSCC): Preliminary results from a single-arm, phase 2 study. Annals of Oncology. 2017;28:v372.

90. Zandberg DP, Algazi A, Jimeno A, Good J, Fayette J, Bouganim N, et al. Durvalumab for recurrent/metastatic (R/M) head and neck squamous cell carcinoma (HNSCC): Preliminary results from a single-arm, phase 2 study. Oncology research and treatment. 2018;41:13.
91. Zandberg DP, Rollins S, Goloubeva O, Morales RE, Tan M, Taylor R, et al. A phase I dose escalation trial of MAGE-A3- and HPV16-specific peptide immunomodulatory vaccines in patients with recurrent/metastatic (RM) squamous cell carcinoma of the head and neck (SCCHN). Cancer Immunology, Immunotherapy. 2015;64(3):367-79.

Appendix 3A - Risk of bias summary, assessed with Joanna Briggs Institute Critical Appraisal Checklist for Randomized Controlled Trials: author's judgments for each included study.



Appendix 3B - Risk of bias summary, assessed with Joanna Briggs Institute Critical Appraisal Checklist for Quasi-Experimental Studies (non-randomized experimental studies): author's judgments for each included study.



Appendix 4. Grading of Recommendation, Assessment, Development, and Evaluation (GRADE) instrument.

Certainty assessment							# of patients		Effect	Certainty	Importance
# of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	[intervention]	[comparison]	Fixed (95% CI)		
Immunotherapy vs. controls											
3	RCT	not serious[a]	serious [b]	not serious	not serious[c]	none	106/587	64/464	**RR 1.41** (1.06 to 1.87)	⊕⊕⊕◯ MODERATE	IMPORTANT
Immunotherapy (HPV+ vs. HPV-)											
7	RCT No RCT	not serious[a]	not serious	not serious	not serious[c]	none	HPV+ 53/237	HPV- 79/544	**RR 1.60** (1.16 to 2.21)	⊕⊕⊕⊕ HIGH	IMPORTANT

CI: Confidence interval; **RR:** Risk ratio

a. Most studies were graded as of low risk of bias

b. I^2 shows moderate heterogeneity

c. Risk relative shows that there was statistical significance between intervention and control

GRADE Working Group grades of evidence
High Certainty: We are very confident that the true effect lies close to that of the estimate of the effect.
Moderate Certainty: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low Certainty: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect.
Very low Certainty: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect.

3 DISCUSSÃO

Apesar do grande avanço na melhoria das estratégias terapêuticas para o câncer de cabeça e pescoço, as taxas de sobrevida se mantem em aproximadamente 50% em 5 anos (Vermorken et al. 2008). Estudos que avaliam os diferentes níveis de sensibilidade ao tratamento propõem o conceito de o câncer ser uma doença dinâmica, onde no seu desenvolvimento e progressão torna-se heterogêneo. Tem sido reconhecido dois tipos de heterogeneidade: a intertumoral e a intratumoral. Pacientes com tumores classificados na mesma entidade clinicopatológica, podem apresentar diferentes variações genéticas e fatores de risco, isto faz referência a heterogeneidade intertumoral. Em relação à heterogeneidade intratumoral, refere-se as diferenças entre as células tumorais de um mesmo paciente, devido à instabilidade genômica que promove a diversidade genética. Neste cenário, há a necessidade de uma melhor compreensão dos fatores etiológicos e promotores, alterações genéticas e epigenéticas, características moleculares e perfil imunológico destas neoplasias, que permita o desenvolvimento de estratégias terapêuticas seguras, eficazes e duráveis (Dagogo-Jack and Shaw 2018; Stanta and Bonin 2018; Lawson et al. 2018).

A abordagem proteômica permite analisar a função celular mediante o estudo das estruturas, funções, interações e mudanças das proteínas expressas nas células (Petricoin and Liotta 2003). É uma valiosa ferramenta para a identificação de novas proteínas alvo, descoberta de novos biomarcadores de doenças para aplicações clínicas e de diagnóstico e exploração de mecanismos de ação e toxicologia de produtos farmacêuticos (Hu et al. 2007). Na aplicação da proteômica no estudo do câncer, duas tecnologias são usadas: eletroforese bidimensional em gel de poliacrilamida (2D-PAGE) e espectrometria de massa (MS) (Reymond and Schlegel 2007), sendo a MS o principal método usado devido a sua alta sensibilidade e precisão, que permitem em um único experimento identificar um grande número de proteínas (Hu et al. 2007).

O espectrômetro de massa é composto por três elementos principais, uma fonte de íons, um analisador de massa (mede a relação massa-carga (m/z) e um detector (registra o número de íons de cada valor de m/z) (Aebersold and Mann 2003); onde se realiza o processo de análise proteômica em cinco estádios. O primeiro consiste em diversos fraccionamentos dos tecidos ou fluidos a ser analisados para obter a separação das proteínas presentes. Posteriormente é realizada a degradação enzimática das proteínas em peptídeos, que geralmente são tripsinas. No terceiro estádio é utilizada a cromatografia líquida de alto desempenho para a separação dos peptídeos em capilares finos, seguido por nebulização em pequenas gotas por

uma fonte iónica de eletropulverização. Após a evaporação, múltiplos peptídeos protonados entram no espectrômetro de massa. No quarto estádio um espectro de massa dos peptídeos eluem a cada certo tempo. Por último, os peptídeos mais intensos são fragmentados, seguido por uma série de espectrometria de massa para obter a sequência de cada um; informação que é comparada com um banco de dados de sequencias de proteínas para a posterior identificação (van der Merwe et al. 2007).

Em amostras teciduais heterogêneas a microdissecção a laser (LMD – do inglês *Laser Microdissection*) pode ser empregada juntamente com a MS para o isolamento de uma população celular especifica. A LMD consiste em um emissor de feixes de raio laser e um microscópio ótico para visualizar as imagens e orientar a dissecção por disparos sucessivos de feixes de laser de alta precisão apenas da área de interesse (Domazet et al. 2008).

A espectrometria de massa tem sido aplicada na pesquisa de biomarcadores oncológicos e no ano de 2006 foram aprovados 14 biomarcadores pela *US Food and Drug Administration* (FDA) para o diagnóstico de câncer de peritônio, mama, urotelial, de tiroide metastásico, hepatocelular, próstata, pulmão, pâncreas e ovário (Polanski and Anderson 2007). No CCE oral potenciais biomarcadores pesquisados através da MS tem sido relatados: Isoforma Rab-2A (RAB2A), peroxiredoxina-1(PRDX1) (Dey et al. 2015) cofilin-1 (Polachini et al. 2012) e fator eucariótico de enlongação delta 1 (EEF1D) (Flores et al. 2016); embora a especificidade e sensibilidade desses biomarcadores necessitam ser validados em estudos futuros.

Nos últimos anos o HPV foi reconhecido como um fator etiológico e de prognóstico num subgrupo de CCE de cabeça e pescoço (Gillison et al. 2000). Apesar da baixa prevalência deste vírus no CCE oral, existe uma população de pacientes jovens, com tumores HR-HPV ADN positivos (do inglês *High Risk-HPV-DNA*) (Kaminagakura et al. 2012), e em alguns casos sendo identificado o vírus transcriptalmente ativo (Gillison et al. 2000). Com o objetivo de explorar o papel do vírus nestes tumores, primeiramente, nós realizamos um estudo proteômico baseado em espectrometria de massa em amostras de CCE oral, que identificou 26 proteínas diferencialmente expressas entre HR-HPV DNA positivos e negativos. Entre essas proteínas, a S100A8 foi superexpresa nos casos de HR-HPV positivos, e adicionalmente apresentou a correlação mais relevante com a sobrevida.

A família de proteínas S100 é composta por 21 membros que tem principalmente atividade na regulação dos níveis de cálcio intra e extracelular, apresentando, portanto, função

na modulação da reposta celular. Alterações em várias proteínas desta família, principalmente sobreexpressão, tem sido reportada no câncer de mama, pulmão, próstata, cabeça e pescoço, fígado, colorectal, cérebro, gástrico, bexiga, pâncreas, rim, tireóide, timo e no osteosarcoma, linfoma e melanoma. Tendo em visa o anteriormente descrito, *clinical trials* em andamento estão avaliando a resposta à inibição de S100B e S100A9 no câncer de próstata e melanoma, respectivamente (Bresnick, Weber, and Zimmer 2015; Cancemi et al. 2018; Chen et al. 2014).

Em relação à proteína S100A8, esta tem uma importante função nos processos inflamatórios, na resposta imune, e na regulação da apoptose. A ligação de S100A8 com o *Toll like receptor 4* -TLR4 e o *Receptor for advanced glycation endproducts* - RAGE, ativa a via de sinalização *Mitogen Activated Protein Kinases* - MAP-quinase e o F*actor nuclear kappa B* - NF-kB, resulta na amplificação da cascata pró-inflamatória. A atividade antimicrobiana contra bactérias e fungos é realizada através da quelação de Zn^{2+}. Adicionalmente, a apoptose é regulada através da interferência de mitocôndrias e lisossomos via espécies reativas de oxigênio e BNIP3. Por este motivo, alterações na proteína S100A8 podem resultar em amplificações da resposta inflamatória em doenças autoimunes, bem como no desenvolvimento e disseminação de tumores malignos (Wang, Song, et al. 2018; Ryckman et al. 2003; Wang, Liu, et al. 2018).

Ainda no que diz respeito à participação da proteína S100A8 em câncer, prévios estudos demostraram a sua superexpressão nas neoplasia malignas de pulmão, e o correlacionaram com proliferação celular, tumorigênese e metástase (Huang et al. 2018). Esta relação também foi reportada no carcinoma anaplásico da tireóide, por meio da interação S100A8/RAGE e ativação das vias de sinalização p38, ERK1/2 e JNK (Reeb et al. 2015). Em acréscimo ao exposto, no câncer de mama, estabilizou-se a interação da via de sinalização S100A8, RAGE e NF-κB, induzindo transição epitelial-mesenquimal e subsequentes metástases locoregionais e distantes (Yin et al. 2013).

No presente trabalho, ensaios *in vitro* demostraram que a ativação do TLR4 aumenta significativamente os níveis de S100A8, NF-κB, e a resposta pró-inflamatória em linhagens celulares HPV positivas, mas não na sua contraparte negativa. A proteína NF-κB desempenha um papel fundamental na resposta celular a estímulos externos, e está altamente expressa em células cancerígenas participando da inibição à apoptose e amplificação da resposta inflamatória (Karin 2009).

A nova geração de *hallmark of cancer* introduz quatro novas capacidades biológicas adquiridas pelas células cancerígenas durante o desenvolvimento e progressão dos tumores. De importância para o presente trabalho cabe citar a capacidade das células neoplásicas para promover a inflamação. Entre os mecanismos pelos quais a inflamação contribui na progressão do tumor, destacam-se a sínteses de fatores de crescimento pró-proliferativos, fatores de crescimento que inibem a apoptose, e fatores pro-angiogênicos, que ativam a transição epitélio mesenquima e consequentemente invasão e metástase. Complementarmente, estudos recentes demostraram que a inflamação também tem um papel importante na carcinogênese, ao liberar espécies reativas de oxigênio que são altamente mutagênicas, acelerando as alterações genéticas de células pré-cancerígenas. Tendo em vista o exposto, o S100A8 pode ser um participante crucial no início e progressão do CCE oral relacionado ao HPV, através de NF-κB e aumentado a resposta pró-inflamatória (Hanahan and Weinberg 2011; Grivennikov, Greten, and Karin 2010).

No intuito de avaliar o papel do HPV na resposta à imunoterapia, nos propusemos realizar uma revisão à literatura e meta-análise para avaliar a eficácia e segurança da imunoterapia no câncer de cabeça e pescoço, enfatizando o status do HPV. A revisão sistemática e meta análise encontram-se no topo da pirâmide da escala de evidência de estudos científicos para toma de decisões terapêuticas. A revisão sistemática tem como finalidade integrar de forma organizada vários estudos independentes, mas com o mesmo objetivo de pesquisa, na qual pode ser utilizada a meta-análise como técnica estatística para sintetizar todos os resultados em uma medida única (Dawson, Pihlstrom, and Blanchette 2016). Em síntese nossos resultados mostraram que a imunoterapia aumentou significativamente as taxas de resposta e sobrevida levando a uma redução de 23% no risco de morte em comparação com a terapia padrão (metotrexato de agente único, docetaxel ou cetuximabe ou regime EXTREME). Essa diferença pode ser explicada pelo fato da heterogeneidade intratumoral e a presença de *cancer steam cells* resistentes à quimiorradiação e com capacidade de autorenovação (Abdullah and Chow 2013; Dagogo-Jack and Shaw 2018). Por outro lado, a imunoterapia leva ao sistema imunológico a identificar e destruir as células tumorais, resultando em uma memória imunológica com efeito a longo prazo, mesmo após a conclusão do tratamento (Ferris 2015). Em adição, maior benefício foi observado nos tumores HPV positivos. Uma possível explicação para essa diferença é a presença de células T específicas para HPV, células T CD4þ e CD8þ orientadas a tipo I, células dendríticas (DC) e macrófagos semelhantes a DC no microambiente

tumoral de tumores relacionados ao HPV e a síntese das oncoproteínas E6 e E7, que tornam as células tumorais extremamente detectáveis pelo sistema imunológico (Welters et al. 2018).

4 CONCLUSÃO

✓ O perfil proteômico dos CCE HR-HPV ADN positivos apresenta diferenças dos HR-HPV-DNA negativos, e permitiu a identificação de algumas proteínas com mecanismos biológicos associados à carcinogênese oral, podendo auxiliar no entendimento do papel do vírus transcricionalmente inativo nestes tumores.

✓ Alta expressão de S100A8 é um marcador prognóstico independente para um menor tempo de sobrevidas livre de doença, câncer especifica e geral, independente do status do HPV. Além disso, S100A8 foi associado a tumores localmente avançados, estádio clinico III-IV e margens cirúrgicas comprometidas, sugerindo a sua participação na progressão tumoral.

✓ A expressão da proteína S100A8 foi significativamente maior nos tumores HR-HPV ADN positivos em comparação com aos negativos, no qual essa alta expressão foi validada por imunofluorescência. Esta diferença indica possíveis modificações na regulação dos processos inflamatórios e na resposta imune nos tumores de cavidade oral HPV ADN positivos.

✓ A ativação da via das proteínas S100A8 e NF-κB leva a uma reposta pró inflamatória só nos tumores HPV positivos, sugerindo que a presença do vírus pode levar a uma modificação no microambiente tumoral com uma presumível influência na carcinogênese oral.

✓ A revisão da literatura e meta-análise demostraram que a imunoterapia melhora as taxas de resposta e sobrevida com redução nas toxicidades nos pacientes com câncer de cabeça e pescoço recorrente e/ou metastáticos, em comparação com os atuais protocolos de tratamento. Estes achados demostram a capacidade do tratamento em restaurar a função do sistema imunológico, levando à detecção e destruição das células tumorais a longo prazo, diminuindo as recorrências e metástases.

✓ A meta-análise avaliando a resposta da imunoterapia segundo o status do HPV, aumentou o número de evidência cientifica de que pacientes HPV positivos podem se beneficiar ainda mais com o uso da imunoterapia, suportando a teoria que o melhor prognóstico nos pacientes com tumores associados ao HPV pode estar ligado a uma melhor resposta imune do hospedeiro.

REFERÊNCIAS

Abdullah, L. N., and E. K. Chow. 2013. 'Mechanisms of chemoresistance in cancer stem cells', *Clin Transl Med*, 2: 3.

Aebersold, R., and M. Mann. 2003. 'Mass spectrometry-based proteomics', *Nature*, 422: 198-207.

Bray, F., J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal. 2018. 'Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries', *CA Cancer J Clin*, 68: 394-424.

Bresnick, A. R., D. J. Weber, and D. B. Zimmer. 2015. 'S100 proteins in cancer', *Nat Rev Cancer*, 15: 96-109.

Cancemi, P., M. Buttacavoli, G. Di Cara, N. N. Albanese, S. Bivona, I. Pucci-Minafra, and S. Feo. 2018. 'A multiomics analysis of S100 protein family in breast cancer', *Oncotarget*, 9: 29064-81.

Chen, H., C. Xu, Q. Jin, and Z. Liu. 2014. 'S100 protein family in human cancer', *Am J Cancer Res*, 4: 89-115.

Dagogo-Jack, I., and A. T. Shaw. 2018. 'Tumour heterogeneity and resistance to cancer therapies', *Nat Rev Clin Oncol*, 15: 81-94.

Dawson, D. V., B. L. Pihlstrom, and D. R. Blanchette. 2016. 'Understanding and evaluating meta-analysis', *J Am Dent Assoc*, 147: 264-70.

de Villiers, E. M., C. Fauquet, T. R. Broker, H. U. Bernard, and H. zur Hausen. 2004. 'Classification of papillomaviruses', *Virology*, 324: 17-27.

Deschuymer, S., H. Mehanna, and S. Nuyts. 2018. 'Toxicity Reduction in the Treatment of HPV Positive Oropharyngeal Cancer: Emerging Combined Modality Approaches', *Front Oncol*, 8: 439.

Dey, K. K., I. Pal, R. Bharti, G. Dey, B. N. Kumar, S. Rajput, A. Parekh, S. Parida, P. Halder, I. Kulavi, and M. Mandal. 2015. 'Identification of RAB2A and PRDX1 as the potential biomarkers for oral squamous cell carcinoma using mass spectrometry-based comparative proteomic approach', *Tumour Biol*, 36: 9829-37.

Domazet, B., G. T. Maclennan, A. Lopez-Beltran, R. Montironi, and L. Cheng. 2008. 'Laser capture microdissection in the genomic and proteomic era: targeting the genetic basis of cancer', *Int J Clin Exp Pathol*, 1: 475-88.

Duensing, S., L. Y. Lee, A. Duensing, J. Basile, S. Piboonniyom, S. Gonzalez, C. P. Crum, and K. Munger. 2000. 'The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle', *Proc Natl Acad Sci U S A*, 97: 10002-7.

Egawa, N., K. Egawa, H. Griffin, and J. Doorbar. 2015. 'Human Papillomaviruses; Epithelial Tropisms, and the Development of Neoplasia', *Viruses*, 7: 3863-90.

EI-Naggar, Adel K., John K.C. Chan, Jennifer R. Grandis, Takashi Takata, and Pieter J. Slootweg. 2017. 'WHO Classification of Head and Neck Tumours', *lnternational Agency for Research on Cancer*.

Ferris, R. L. 2015. 'Immunology and Immunotherapy of Head and Neck Cancer', *J Clin Oncol*, 33: 3293-304.

Ferris, R. L., N. F. Saba, B. J. Gitlitz, R. Haddad, A. Sukari, P. Neupane, J. C. Morris, K. Misiukiewicz, J. E. Bauman, M. Fenton, A. Jimeno, D. R. Adkins, C. J. Schneider, A. G. Sacco, K. Shirai, D. W. Bowles, M. Gibson, T. Nwizu, R. Gottardo, K. L. Manjarrez, G. N. Dietsch, J. K. Bryan, R. M. Hershberg, and E. E. W. Cohen. 2018. 'Effect of Adding Motolimod to Standard Combination Chemotherapy and Cetuximab Treatment

of Patients With Squamous Cell Carcinoma of the Head and Neck: The Active8 Randomized Clinical Trial', *JAMA Oncol*, 4: 1583-88.

Flores, I. L., R. Kawahara, M. C. Miguel, D. C. Granato, R. R. Domingues, C. C. Macedo, C. M. Carnielli, S. Yokoo, P. C. Rodrigues, B. V. Monteiro, C. E. Oliveira, C. R. Salmon, F. H. Nociti, Jr., M. A. Lopes, A. Santos-Silva, F. V. Winck, R. D. Coletta, and A. F. Paes Leme. 2016. 'EEF1D modulates proliferation and epithelial-mesenchymal transition in oral squamous cell carcinoma', *Clin Sci (Lond)*, 130: 785-99.

Gillison, M. L., W. M. Koch, R. B. Capone, M. Spafford, W. H. Westra, L. Wu, M. L. Zahurak, R. W. Daniel, M. Viglione, D. E. Symer, K. V. Shah, and D. Sidransky. 2000. 'Evidence for a causal association between human papillomavirus and a subset of head and neck cancers', *J Natl Cancer Inst*, 92: 709-20.

Gillison, M. L., A. M. Trotti, J. Harris, A. Eisbruch, P. M. Harari, D. J. Adelstein, E. M. Sturgis, B. Burtness, J. A. Ridge, J. Ringash, J. Galvin, M. Yao, S. A. Koyfman, D. M. Blakaj, M. A. Razaq, A. D. Colevas, J. J. Beitler, C. U. Jones, N. E. Dunlap, S. A. Seaward, S. Spencer, T. J. Galloway, J. Phan, J. J. Dignam, and Q. T. Le. 2019. 'Radiotherapy plus cetuximab or cisplatin in human papillomavirus-positive oropharyngeal cancer (NRG Oncology RTOG 1016): a randomised, multicentre, non-inferiority trial', *Lancet*, 393: 40-50.

Grivennikov, S. I., F. R. Greten, and M. Karin. 2010. 'Immunity, inflammation, and cancer', *Cell*, 140: 883-99.

Hanahan, D., and R. A. Weinberg. 2011. 'Hallmarks of cancer: the next generation', *Cell*, 144: 646-74.

Hashibe, M., P. Brennan, S. Benhamou, X. Castellsague, C. Chen, M. P. Curado, L. Dal Maso, A. W. Daudt, E. Fabianova, L. Fernandez, V. Wunsch-Filho, S. Franceschi, R. B. Hayes, R. Herrero, S. Koifman, C. La Vecchia, P. Lazarus, F. Levi, D. Mates, E. Matos, A. Menezes, J. Muscat, J. Eluf-Neto, A. F. Olshan, P. Rudnai, S. M. Schwartz, E. Smith, E. M. Sturgis, N. Szeszenia-Dabrowska, R. Talamini, Q. Wei, D. M. Winn, D. Zaridze, W. Zatonski, Z. F. Zhang, J. Berthiller, and P. Boffetta. 2007. 'Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium', *J Natl Cancer Inst*, 99: 777-89.

Hashibe, M., P. Brennan, S. C. Chuang, S. Boccia, X. Castellsague, C. Chen, M. P. Curado, L. Dal Maso, A. W. Daudt, E. Fabianova, L. Fernandez, V. Wunsch-Filho, S. Franceschi, R. B. Hayes, R. Herrero, K. Kelsey, S. Koifman, C. La Vecchia, P. Lazarus, F. Levi, J. J. Lence, D. Mates, E. Matos, A. Menezes, M. D. McClean, J. Muscat, J. Eluf-Neto, A. F. Olshan, M. Purdue, P. Rudnai, S. M. Schwartz, E. Smith, E. M. Sturgis, N. Szeszenia-Dabrowska, R. Talamini, Q. Wei, D. M. Winn, O. Shangina, A. Pilarska, Z. F. Zhang, G. Ferro, J. Berthiller, and P. Boffetta. 2009. 'Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium', *Cancer Epidemiol Biomarkers Prev*, 18: 541-50.

Hu, S., T. Yu, Y. Xie, Y. Yang, Y. Li, X. Zhou, S. Tsung, R. R. Loo, J. R. Loo, and D. T. Wong. 2007. 'Discovery of oral fluid biomarkers for human oral cancer by mass spectrometry', *Cancer Genomics Proteomics*, 4: 55-64.

Huang, H., Q. Huang, T. Tang, L. Gu, J. Du, Z. Li, X. Lu, and X. Zhou. 2018. 'Clinical significance of calcium-binding protein S100A8 and S100A9 expression in non-small cell lung cancer', *Thorac Cancer*, 9: 800-04.

INCA. 2017. 'Estimativa 2018, Incidência de Câncer no Brasil', *INCA*.

Kaminagakura, E., L. L. Villa, M. A. Andreoli, J. S. Sobrinho, J. G. Vartanian, F. A. Soares, I. N. Nishimoto, R. Rocha, and L. P. Kowalski. 2012. 'High-risk human papillomavirus in oral squamous cell carcinoma of young patients', *Int J Cancer*, 130: 1726-32.

Karin, M. 2009. 'NF-kappaB as a critical link between inflammation and cancer', *Cold Spring Harb Perspect Biol*, 1: a000141.

Kowalski, L. P. 2002. 'Results of salvage treatment of the neck in patients with oral cancer', *Arch Otolaryngol Head Neck Surg*, 128: 58-62.

Kowalski LP, Nishimoto IN. 2000. 'Epidemiologia do câncer de boca. In: Parise Jr. O. Câncer de boca: aspectos básicos e terapêuticos', *Sarvier*.

Lawson, D. A., K. Kessenbrock, R. T. Davis, N. Pervolarakis, and Z. Werb. 2018. 'Tumour heterogeneity and metastasis at single-cell resolution', *Nat Cell Biol*, 20: 1349-60.

Mehanna, H., M. Robinson, A. Hartley, A. Kong, B. Foran, T. Fulton-Lieuw, M. Dalby, P. Mistry, M. Sen, L. O'Toole, H. Al Booz, K. Dyker, R. Moleron, S. Whitaker, S. Brennan, A. Cook, M. Griffin, E. Aynsley, M. Rolles, E. De Winton, A. Chan, D. Srinivasan, I. Nixon, J. Grumett, C. R. Leemans, J. Buter, J. Henderson, K. Harrington, C. McConkey, A. Gray, J. Dunn, and ESCALaTE H. P. V. Trial Group De. 2019. 'Radiotherapy plus cisplatin or cetuximab in low-risk human papillomavirus-positive oropharyngeal cancer (De-ESCALaTE HPV): an open-label randomised controlled phase 3 trial', *Lancet*, 393: 51-60.

Petricoin, E. F., and L. A. Liotta. 2003. 'Clinical applications of proteomics', *J Nutr*, 133: 2476S-84S.

Polachini, G. M., L. M. Sobral, A. M. Mercante, A. F. Paes-Leme, F. C. Xavier, T. Henrique, D. M. Guimaraes, A. Vidotto, E. E. Fukuyama, J. F. Gois-Filho, P. M. Cury, O. A. Curioni, P. Michaluart, Jr., A. M. Silva, V. Wunsch-Filho, F. D. Nunes, A. M. Leopoldino, and E. H. Tajara. 2012. 'Proteomic approaches identify members of cofilin pathway involved in oral tumorigenesis', *PLoS One*, 7: e50517.

Polanski, M., and N. L. Anderson. 2007. 'A list of candidate cancer biomarkers for targeted proteomics', *Biomark Insights*, 1: 1-48.

Reeb, A. N., W. Li, W. Sewell, L. A. Marlow, H. W. Tun, R. C. Smallridge, J. A. Copland, K. Spradling, R. Chernock, and R. Y. Lin. 2015. 'S100A8 is a novel therapeutic target for anaplastic thyroid carcinoma', *J Clin Endocrinol Metab*, 100: E232-42.

Reymond, M. A., and W. Schlegel. 2007. 'Proteomics in cancer', *Adv Clin Chem*, 44: 103-42.

Ryckman, C., K. Vandal, P. Rouleau, M. Talbot, and P. A. Tessier. 2003. 'Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion', *J Immunol*, 170: 3233-42.

Stanta, G., and S. Bonin. 2018. 'Overview on Clinical Relevance of Intra-Tumor Heterogeneity', *Front Med (Lausanne)*, 5: 85.

Stewart BW, Wild CP. . 2014. 'World Cancer Report 2014 ', *Lyon: IARC Press*.

Vairaktaris, E., S. Spyridonidou, V. Papakosta, A. Vylliotis, A. Lazaris, D. Perrea, C. Yapijakis, and E. Patsouris. 2008. 'The hamster model of sequential oral oncogenesis', *Oral Oncol*, 44: 315-24.

van der Merwe, D. E., K. Oikonomopoulou, J. Marshall, and E. P. Diamandis. 2007. 'Mass spectrometry: uncovering the cancer proteome for diagnostics', *Adv Cancer Res*, 96: 23-50.

Vermorken, J. B., R. Mesia, F. Rivera, E. Remenar, A. Kawecki, S. Rottey, J. Erfan, D. Zabolotnyy, H. R. Kienzer, D. Cupissol, F. Peyrade, M. Benasso, I. Vynnychenko, D. De Raucourt, C. Bokemeyer, A. Schueler, N. Amellal, and R. Hitt. 2008. 'Platinum-based chemotherapy plus cetuximab in head and neck cancer', *N Engl J Med*, 359: 1116-27.

Wang, D., G. Liu, B. Wu, L. Chen, L. Zeng, and Y. Pan. 2018. 'Clinical Significance of Elevated S100A8 Expression in Breast Cancer Patients', *Front Oncol*, 8: 496.

Wang, S., R. Song, Z. Wang, Z. Jing, S. Wang, and J. Ma. 2018. 'S100A8/A9 in Inflammation', *Front Immunol*, 9: 1298.

Welters, M. J. P., W. Ma, Sjam Santegoets, R. Goedemans, I. Ehsan, E. S. Jordanova, V. J. van Ham, V. van Unen, F. Koning, S. I. van Egmond, P. Charoentong, Z. Trajanoski, L. A. van der Velden, and S. H. van der Burg. 2018. 'Intratumoral HPV16-Specific T Cells Constitute a Type I-Oriented Tumor Microenvironment to Improve Survival in HPV16-Driven Oropharyngeal Cancer', *Clin Cancer Res*, 24: 634-47.

Yin, C., H. Li, B. Zhang, Y. Liu, G. Lu, S. Lu, L. Sun, Y. Qi, X. Li, and W. Chen. 2013. 'RAGE-binding S100A8/A9 promotes the migration and invasion of human breast cancer cells through actin polymerization and epithelial-mesenchymal transition', *Breast Cancer Res Treat*, 142: 297-309.

Zandberg, D. P., A. P. Algazi, A. Jimeno, J. S. Good, J. Fayette, N. Bouganim, N. E. Ready, P. M. Clement, C. Even, R. W. Jang, S. Wong, U. Keilholz, J. Gilbert, M. Fenton, I. Brana, S. Henry, E. Remenar, Z. Papai, L. L. Siu, A. Jarkowski, J. M. Armstrong, K. Asubonteng, J. Fan, G. Melillo, and R. Mesia. 2019. 'Durvalumab for recurrent or metastatic head and neck squamous cell carcinoma: Results from a single-arm, phase II study in patients with >/=25% tumour cell PD-L1 expression who have progressed on platinum-based chemotherapy', *Eur J Cancer*, 107: 142-52.

ANEXO 1 - Certificado do Comitê de Ética em Pesquisa

A.C.Camargo Cancer Center

Comitê de Ética em Pesquisa - CEP

APROVAÇÃO

Os membros do Comitê de Ética em Pesquisa em Seres Humanos da Fundação Antonio Prudente – A.C. Camargo Cancer Center, em sua última reunião de **09/08/2016,** após analisarem as respostas aos questionamentos realizados em reunião de 24/05/2016, **aprovaram** a realização do projeto nº **2199/16** intitulado: "**ANÁLISE PROTEÔMICA POR ESPECTROMETRIA DE MASSAS DO CARCINOMA DE CÉLULAS ESCAMOSAS ORAL EM PACIENTES JOVENS.**".

Pesquisador responsável: Dr. Luiz Paulo Kowalski.

Informações a respeito do andamento do referido projeto deverão ser encaminhadas ao CEP dentro de 06 meses em relatório (modelo CEP).

São Paulo, 17 de Agosto de 2016.

Atenciosamente,

Dra. Sandra Caires Serrano
2ª Vice-Coordenadora do Comitê de Ética em Pesquisa

1/1

A.C.Camargo Cancer Center
Centro Integrado de Diagnóstico, Tratamento, Ensino e Pesquisa

COMITÊ DE ÉTICA
EM PESQUISA - CEP

São Paulo, 13 de setembro de 2017.

Ao
Dr. Luiz Paulo Kowalski

Ref.: Projeto de Pesquisa nº. 2199/16B
"ANÁLISE FUNCIONAL DE PROTEÍNAS RELACIONADAS AO VIRUS DE PAPILOMA HUMANO NO CARCINOMA DE CÉLULAS ESCAMOSAS ORAL."

Os membros do Comitê de Ética em Pesquisa em Seres Humanos da Fundação Antonio Prudente – A.C. Camargo Cancer Center, em sua última reunião de **05/09/2017**, **tomaram conhecimento e aprovaram** o seguinte documento:

- Solicitação de dispensa da submissão da documentação obrigatória e análise ética do projeto acima mencionado por se tratar de um projeto afiliado ao temático intitulado: "ANÁLISE PROTEÔMICA POR ESPECTROMETRIA DE MASSAS DO CARCINOMA DE CÉLULAS ESCAMOSAS ORAL EM PACIENTES JOVENS" registrado neste CEP sob nº 2199/16. O projeto afiliado em referência será um projeto Departamental.

Atenciosamente,

Dra. Sandra Caires Serrano
2ª. Vice-Coordenadora do Comitê de Ética em Pesquisa

A.C.Camargo Cancer Center
Centro Integrado de Diagnóstico, Tratamento, Ensino e Pesquisa

COMITÊ DE ÉTICA EM PESQUISA - CEP

São Paulo, 27 de agosto de 2019.

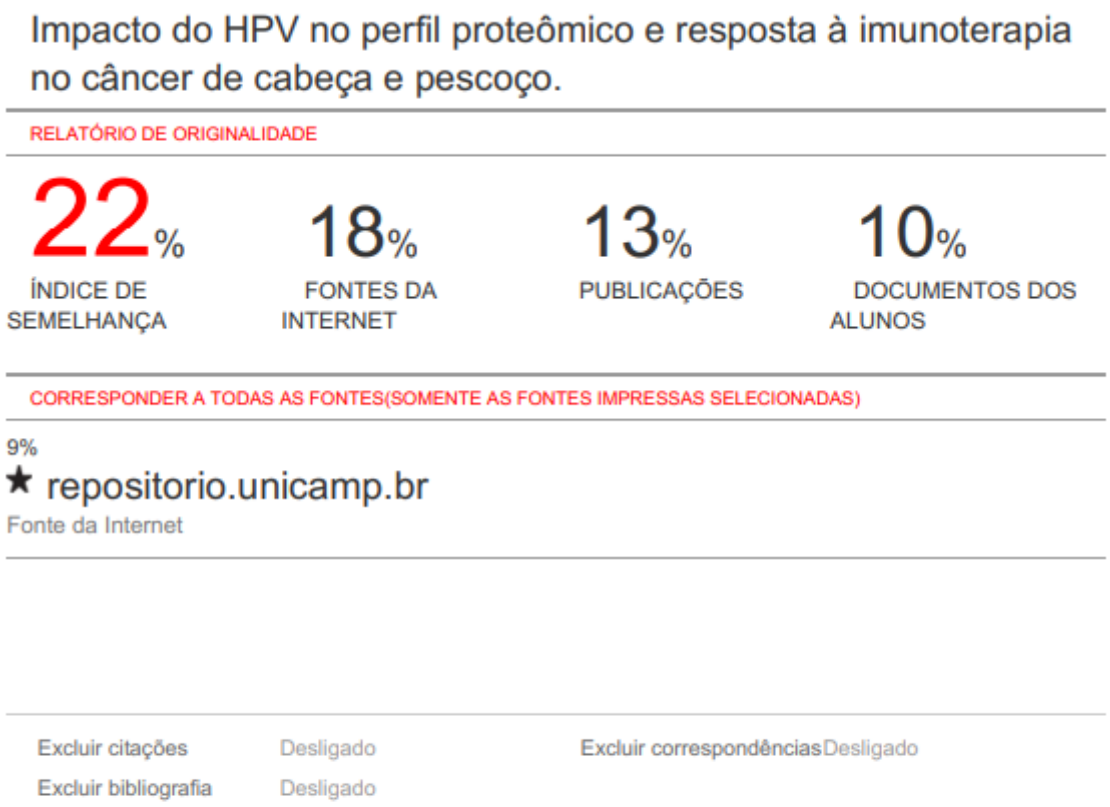
DECLARAÇÃO

Declaro para os devidos fins que a **Sra. Marisol Miranda Galvis,** é co-pesquisadora do Projeto: **"ANÁLISE PROTEÔMICA POR ESPECTROMETRIA DE MASSAS DO CARCINOMA DE CÉLULAS ESCAMOSAS ORAL EM PACIENTES JOVENS."**, aprovado neste CEP sob o Registro nº **2199/16**, em reunião de **09/08/2016**, tendo como pesquisador responsável: **Prof. Dr. Luiz Paulo Kowalski.**

Atenciosamente,

Luciana Facure Moredo
1ª. Vice-Coordenadora do Comitê de Ética em Pesquisa

ANEXO 2 – Verificação de originalidade e prevenção de plágio



Impacto do HPV no perfil proteômico e resposta à imunoterapia no câncer de cabeça e pescoço.

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ANEXO 3 – Comprovante de submissão de artigo

Dear Dr. Miranda,

Thank you for submitting your manuscript "NEW INSIGHTS IN THE IMPACT OF HR-HPV-DNA IN ORAL CANCER: PROTEOMIC APPROACH REVEALS A NOVEL ROLE FOR S100A8" to Cancer Cell. It has been forwarded to the editors.

The manuscript number for your submission is CANCER-CELL-D-19-00974. Please reference this number in any further correspondence about the paper. If I can be of assistance, please let me know.

Sincerely,

Alex Dvorkin

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