UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

RAÍSA SALES DE SÁ

CARACTERIZAÇÃO DO PERFIL IMUNE SOLÚVEL E CORRELAÇÃO CLÍNICO-PATOLÓGICA E IMUNOEXPRESSÃO DE PD-L1 EM CARCINOMA ESPINOCELULAR ORAL

CHARACTERIZATION OF THE SOLUBLE IMMUNE PROFILE AND CLINICALPATHOLOGICAL CORRELATION WITH PD-L1 IMMUNOEXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA

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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.

Thes is presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Estomatopatologia, in Patology area.

Orientador: Prof. Dr. Luiz Paulo Kowalski

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA RAÍSA SALES DE SÁ E ORIENTADA PELO PROF. DR. LUIZ PAULO KOWALSKI

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PROF. DR. LUIZ PAULO KOWALSKI

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RESUMO

Estima-se cerca de 350 mil novos casos e 170 mil mortes ao ano por câncer de cavidade oral no mundo. As opções de tratamento para este tipo de câncer oral foram modificadas nos últimos 5 anos, principalmente pelo avanço das técnicas cirúrgicas e das terapias adjuvantes. Contudo, a taxa de sobrevivência a 5 anos não apresentou melhoras significativas nas últimas décadas. Novas terapias surgiram como uma opção promissora, como a imunoterapia. Contudo, não são todos os pacientes que se beneficiam desse tratamento. Desta forma, este trabalho teve como objetivo caracterizar do perfil imune celular através do perfil solúvel sistêmico através de plataforma Iluminex e pela análise imunohistoquimica de PD-L1 correlacionando com os dados clinicopatologicos. Dividimos esse trabalho em duas etapas, na primeira realizamos a caracterização do perfil de imunoexpressão de PD-L1 em pacientes com Carcinoma espinocelular oral (CECO), através de um estudo retrospectivo. Observamos a presença de 4 perfis através da imuno-histoquímica, que revelou as células tumorais expressaram PD-L1 em padrões irregulares ou difusos. O padrão irregular previu uma sobrevida pior. Além disso, os padrões de expressão no microambiente imunológico do tumor mostraram que a maioria dos casos expressava PD-L1 tanto nas células tumorais quanto nas células imunes, enquanto os não-expressores de PD-L1 tinham a menor sobrevida geral. Na segunda parte desse trabalho, realizamos um estudo prospectivo, onde incluímos 23 pacientes com a coleta de sangue (plasma) e blocos de parafina. Com o plasma, realizamos a caracterização do perfil imune solúvel, com a análise de 32 analitos (citocinas, quimiocinas e fatores de crescimento) e correlacionamos com características clínico-patológicas, como estadiamento clínico, pT, tabagismo, metástase linfonodal. Sendo possível observar que em pacientes com CECO tem um perfil imune ativo e que é capaz de predizer a progressão tumoral. Em seguida, analisamos através da imunohistoquímica a imunoexpressão de PD-L1 desses pacientes e dividimos conforme o estudo anterior, notamos que havia 2 padrões de pacientes, um que representava um perfil mais ativo e propenso a ser respondedor a imunoterapia e um outro perfil imune inativo. Dividimos esses dois perfis de expressão e correlacionamos com o perfil imune solúvel, onde foi possível notar presença de diferenças significativas nos níveis de citocinas, quimiocinas e fatores de crescimento entre os grupos dois perfis de expressão de PD-L1 destacando a importância de considerar a resposta imunológica

do paciente ao planejar estratégias terapêuticas personalizadas. Por fim, a identificação de marcadores imunológicos, como as citocinas, quimiocinas e fatores de crescimento analisadas, pode ajudar a prever a resposta ao tratamento e estratificar os pacientes, permitindo uma abordagem mais direcionada e eficaz. A compreensão desses perfis imunológicos pode ajudar a otimizar a terapia e melhorar os resultados clínicos em pacientes com diferentes perfis de resposta imunológica.

Palavras-chaves: Carcinoma espinocelular oral, PD-L1, Bloqueadores de Checkpoint imunológico.

ABSTRACT

Oral cavity cancer accounts for approximately 350,000 new cases and 170,000 deaths annually worldwide. Despite advances in surgical techniques and adjuvant therapies over the past five years, the 5-year survival rate has not significantly improved. Immunotherapy has emerged as a promising treatment, but its effectiveness is not universal. This study aimed to characterize the immune profile in patients with oral squamous cell carcinoma (OSCC) through systemic soluble immune markers using the Luminex platform and PD-L1 immunohistochemistry, correlating these findings with clinicopathological data. In the first phase, a retrospective analysis of PD-L1 immunoexpression was performed in OSCC patients. Four distinct patterns were identified through immunohistochemistry, with tumor cells showing irregular or diffuse PD-L1 expression. Irregular patterns were associated with poorer survival, and cases expressing PD-L1 in both tumor and immune cells had better outcomes, whereas non-expressors exhibited the worst overall survival. The second phase involved a prospective study of 23 patients, analyzing plasma for 32 immune analytes (cytokines, chemokines, and growth factors) and correlating these with clinicopathological features. Patients with an active immune profile were more likely to show tumor progression. PD-L1 immunoexpression divided patients into two groups: those with an active immune profile responsive to immunotherapy and those with an inactive immune profile. Significant differences in cytokine, chemokine, and growth factor levels were observed between the two groups, emphasizing the need to consider immune response when developing personalized therapies. The identification of immune markers, such as cytokines, chemokines, and growth factors, can aid in predicting treatment response and patient stratification, enabling more targeted and effective approaches. Understanding these immune profiles may optimize treatment strategies and improve clinical outcomes in OSCC patients.

Keywords: Oral squamous cell carcinoma, PD-L1, Blockade, PD-1-PD-L1.

SÚMARIO

1. INTRODUÇÃO

Câncer oral

O câncer oral é considerado um grave problema de saúde mundial. Estima-se o surgimento de cerca de 350 mil novos casos e 170 mil mortes no mundo, das quais 77% ocorrem em países em desenvolvimento (Bray *et al*, 2018). No Brasil, o câncer de boca é o quinto tumor mais incidente nos homens e o décimo terceiro entre as mulheres, segundo dados do Instituto Nacional de Câncer – INCA. O programa de epidemiologia e vigilância do câncer e seus fatores de risco estimou para o triênio de 2023-2025 a ocorrência anual de 10.900 casos de câncer de cavidade oral em homens e 4.200 em mulheres (INCA, 2023).

Dentre as neoplasias malignas orais o Carcinoma Espinocelular (CEC), é o tipo histológico mais comum, representando 94% de todas as malignidades orais e 40% dos tumores malignos da cabeça e pescoço (Kowalski et al, 2000). Ocorre mais frequentemente em pessoas entre a quinta e a sexta décadas de vida, sendo mais comum em homens. Contudo, nos últimos anos, vem sendo observado uma diminuição na relação entre homens e mulheres e um aumento da ocorrência em pessoas mais jovens (Stewart et al, 2014; Curado et al. 2016). Quando avaliado a localização na cavidade oral o sítio de maior acometimento de tumores malignos na cavidade oral é a língua, seguida pelo assoalho bucal (Moore *et al*, 2008; Curado *et al*, 2016).

Quando observado o perfil epidemiológico desses pacientes, cerca de 70% dos casos têm como fatores de risco o consumo de tabaco e de álcool, sendo considerados os principais agentes associados ao desenvolvimento dessa neoplasia (Barnes *et al*, 2004; Curado *et al*, 2016). Nos últimos anos observou-se uma mudança no perfil epidemiológico, notando-se um aumento do número de casos entre jovens não expostos aos fatores de risco clássicos (Kaminagakura *et al*, 2012; De Cicco *et al*, 2020).

- Células do microambiente tumoral e sua relação com prognóstico

O conceito de que o sistema imune poderia erradicar as células mutadas que surgem continuamente em seres humanos foi proposto em 1909 por Ehrlich, sendo que esse processo ocorre inúmeras vezes antes que essas mutações consigam ser avaliadas clinicamente. Posteriormente, Burnet e Thomas, introduzem o conceito de vigilância imunológica, onde as células tumorais teriam antígenos reconhecíveis diferentes das células normais e, portanto, potenciais alvos de depuração imunológica

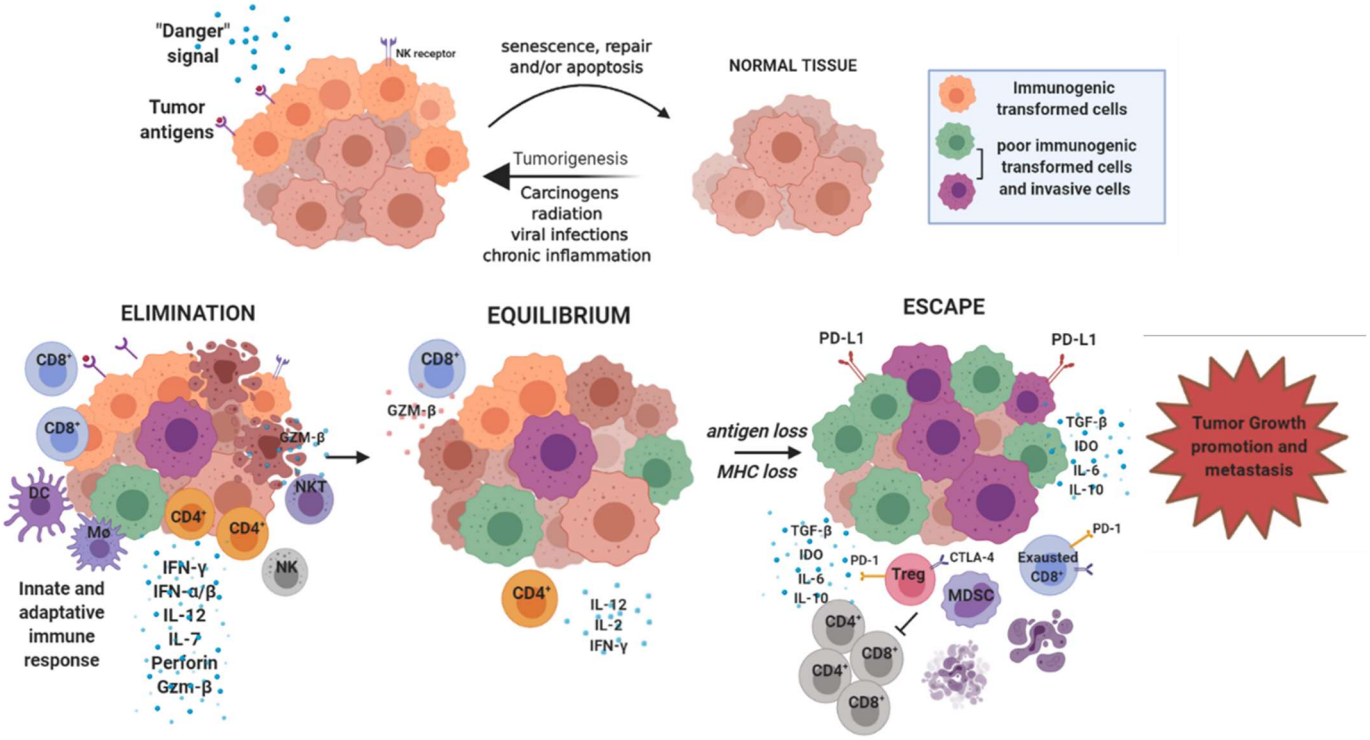
(Kim, 2007; Ferris, 2015). Contudo, com o advento da biologia molecular, os estudos focaram em compreender os sistemas das células tumorais, observando as características próprias delas, como mitose, apoptose, diferenciação celular, entre outras. Mas esses estudos não conseguiram esclarecer algumas questões, isso porque as células epiteliais neoplásicas encontram-se em um estado dinâmico inter-relacionado com o microambiente do tumor (MAT) (Salo *et al*, 2014).

Esse MAT contém uma variedade de componentes como células não cancerosas (células imunes, fibroblastos e células vasculares angiogênicas), matriz extracelular (fibras de colágeno e fibronectina) e fatores solúveis (enzimas, fatores de crescimento e quimiocinas). Assim, o MAT passou a ser considerado parte do tecido maligno (Salo *et al*, 2014). Com isso, o conceito de vigilância imunológica do câncer foi retomado, demonstrando-se o papel funcional das células apresentadoras de antígeno na iniciação tumoral e na ativação das células T (Ferris, 2015). Para o CEC de boca, a presença de diferentes populações de células T e células B vem sendo encontradas na fronte de invasão tumoral; no entanto, ainda é controverso se a presença dessas células no MAT refletirem um bom ou mau prognóstico (Hui and Chen, 2015).

Assim, compreender a relação do sistema imune e o microambiente tumoral parece ser promissora para o desenvolvimento de novas formas de tratamento (Linette *et al*, 2019). Em nosso estudo recente, avaliamos o microambiente imune em CEC de língua, através de imuno-histoquímica, e identificamos células imunes associadas a um melhor prognóstico, como os linfócitos T (CD3), linfócitos T citotóxicos (CD8), linfócitos B (CD20) e as células dendríticas maduras (CD83), além do CD3 ter sido associado a um melhor valor de prognóstico de forma independente. Ficou claro que o microambiente imune ativo em pacientes com CEC oral pode melhorar a sobrevida desses pacientes. (Sales-Sá, 2019).

Outros fatores devem ser considerados, e é de conhecimento que o microambiente circundante imunossupressor mantem o ambiente propenso a células reguladoras, à expressão de citocinas como IL-6, VEGF, IL-10 e TGF-β, e à polarização de macrófagos em relação a um fenótipo de macrófagos imunossupressor (M2) (Ferris *et al.,* 2015). As células Treg são mais frequentes no sangue periférico, e são “mais potentes” quando comparadas às células T infiltrantes do tumor (TILs), isso favorece a uma maior imunossupressão local (Alhamarneh *et al.* 2008) (Figura 1).

Figura 1. Imunoedição do câncer



Fonte: *Nayane Galdino*, modificado de SA, G. Cancer immunoediting: Integrating the role of immunity in cancer suppression and promotion. **Science Signalling**, v. 331, n. March, p. 78, 2011.

- Imunoterapia em CEC de cabeça e pescoço e oral

O tratamento cirúrgico para câncer oral avançou consideravelmente nos últimos anos, assim como, terapias anti-neoplásicas em combinação com a radioterapia e/ou quimioterapia. Contudo, a taxa de sobrevivência a 5 anos não apresentou melhoras significativas nas últimas décadas, sendo estimada em 50% para o câncer oral (Curado *et al*, 2016; J.-N. *et al*, 2019). Assim, inúmeros estudos vêm desenvolvendo novas terapias e tratamentos mais efetivos para contribuir com a melhora nas taxas de sobrevida dos pacientes com CEC de cabeça e pescoço incluído a cavidade oral. Nos anos recentes diversos avanços no campo da imunologia tumoral, a imunoterapia surgiu como uma opção promissora para tratamento de doença recidivada ou metastática (Quan *et al*, 2016; Moskovitz and Ferris, 2018).

A imunoterapia veio como uma nova proposta terapêutica, onde usa-se o sistema imune do próprio paciente como uma nova opção para o tratamento oncológico (Solomon *et al*, 2018). Uma das principais descobertas para tratamento com imunoterapia foi a proteína de morte programada 1 (PD-1) expressa pelas células T, que ao se ligar a seu ligante (proteína de morte programada 1 (PD-L1)) sobre

células tumorais ou antígenos que apresentam células, induzem um sinal de supressão à célula T que a impede de eliminar o tumor. Assim, a interação do PD-1 e PD-L1 formam um complexo de receptores que impedem a ativação das células T (Chen and Mellman, 2013; Muenst *et al*, 2015; Gong *et al*, 2018).

Em 2016, a Food and Drug Administration (FDA) dos EUA concedeu as primeiras aprovações para tratamento com a imunoterapia com os inibidores do ponto de verificação imune anti-PD-1 (nivolumab e pembrolizumab) em pacientes com CEC de cabeça e pescoço recorrente e que não respondiam à quimioterapia à base de platina. Em 2017, a Comissão Europeia aprovou o uso do nivolumab para o tratamento da mesma população de pacientes, e posteriormente aprovou a monoterapia com pembrolizumab para o tratamento de CEC de cabeça e pescoço recorrente ou metastático em adultos cujos tumores expressam PD-L1 numa proporção ≥ 50%. Em 2019, o FDA concedeu aprovação para a inibição da PD-1 como tratamento de primeira linha para pacientes com CEC de cabeça e pescoço recorrente, metastático ou inoperável, aprovando o pembrolizumab em combinação com platina e fluorouracil para os pacientes com CEC de cabeça e pescoço (Moskovitz and Ferris, 2018; Przybylski *et al*, 2018; J.-N. *et al*, 2019). O KEYNOTE-048 foi um estudo randomizado de fase 3 com participantes com carcinoma de células escamosas de cabeça e pescoço recorrente ou metastático localmente incurável não tratado (NCT02358031). Os participantes foram alocados em três braços, para pembrolizumabe (PEMBRO), pembrolizumabe mais platina e 5-fluorouracil (PEMBRO + QT) ou cetuximabe mais platina e 5 -fluorouracil (EXTREME).

Apesar destes estudos incluírem pacientes com tumores de cavidade oral, não foram publicados estudos clínicos que avaliem apenas a resposta nesse grupo específico. Quando avaliamos os estudos de CEC oral observamos que buscam compreender a função do sistema imune e associá-los com as taxas de sobrevida e com o risco de metástases. O desenvolvimento de técnicas de imunohistoquímica e os novos marcadores vem auxiliando para compreender mais o comportamento desse tipo de tumor (Zancope *et al*, 2010; Quan *et al*, 2016; Hadler-Olsen and Wirsing, 2019). Em nosso estudo recente, observamos a importância dessas células imunes como CD8+ citotóxico e CD3+ como bons fatores de prognósticos e a sua correlação com a expressão de PD-1/PD-L1 (Sales-Sá, et al. 2019). Protocolos imunoterapêuticos específicos para CEC orais não estão estabelecidos, necessitando de estudos que

avaliem os melhores protocolos que possam ser utilizados para cada paciente (Wolf *et al*, 2015; Solomon *et al*, 2018; Zhou *et al*, 2018; Hadler-Olsen and Wirsing, 2019).

2. ARTIGO

2.1 PD-L1 expression patterns in oral cancer as an integrated approach for further prognostic classification

Running Title: PD-L1 Expression Patterns in Oral Cancer

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Abstract

Background

Despite the well-known role of programmed cell death ligand 1 (PD-L1) in promoting immune resistance in oral squamous cell carcinoma (OSCC), its potential utility as a

prognostic biomarker is undetermined. We evaluated PD-L1 expression as predictor of survival in OSCC patients and explored PD-L1 expression patterns.

Methods

PD-L1 expression was assessed through immunohistochemistry in 123 surgical specimens of OSCC. A first approach evaluated tumor proportion scores (TPS) and combined proportion scores (CPS). Next, expression patterns were examined by evaluating PD-L1 localization in tumor nests and in TC—IC interfaces in the tumor microenvironment.

Results

High-level PD-L1 expression determined by TPS and CPS using variable cutoffs was not associated with survival. Immunohistochemistry revealed that TC expressed PD-L1 in either patchy or diffuse patterns. The patchy pattern predicted worse survival. Furthermore, expression patterns in the tumor immune microenvironment showed that most cases expressed PD-L1 on both TC and IC, while PD-L1 non-expressors had the lowest overall survival.

Conclusion

PD-L1 expression patterns in the context of localization in tumor nests and TC—IC interactions represent antitumor immune responses better than either TPS or CPS. Our suggested classification system may have important implications for the characterization of OSCC and for the use of PD-L1 as a prognostic biomarker.

Keywords

Oral cancer, programmed cell death ligand 1, score, expression pattern, imune microenvironment.

Introduction

Immune evasion is a driving force that enables neoplastic cells to survive, proliferate, and disseminate, and is emerging as a recognized hallmark of cancer [1]. A major immune resistance strategy of tumor cells (TC) exploits the expression of programmed cell death ligands 1 and 2 (PD-L1 and PD-L2) to target the programmed cell death 1 receptor (PD-L1) in activated T cells [2]. PD-L1 expression is upregulated in various solid tumors; its binding to T cell PD-1 receptors inhibits cytotoxicity and cytokine production [3]. Moreover, local secretion of interferon-γ by activated T cells upregulates PD-L1 expression by TC; macrophages; antigen-presenting cells; and T cells; promoting further immune suppression [4]. Targeted anti-PD-1 (e.g., nivolumab, pembrolizumab) and anti-PD-L1 (e.g. durvalumab, atezolizumab) therapies to block

suppressive signaling and restore immune function [5] [6], have emerged as effective modalities for a variety of cancers, including head and neck squamous cell carcinoma (HNSCC) [7].

Despite the well-known role of PD-L1 in the pathogenesis of oral squamous cell carcinoma (OSCC), its role as a prognostic biomarker is undetermined. Previous studies have associated PD-L1 overexpression with either reduced [8-10], or prolonged survival [11]. In contrast, other reports have found no prognostic significance of PD-L1 expression [12-14]. The contradictory findings expand to assess PD-L1 as a predictor of response to immunotherapy. Studies to assess PD-L1 expression as a predictor of immunotherapeutic response have also yielded conflicting results. Clinical trials of pembrolizumab highlighted improved survival rates in PD-L1-positive patients [15-17], while studies of other immunotherapeutics such as nivolumab reported no prognostic value of PD-L1 expression [18].

Discrepant results might be attributed in part to the lack of standardized methods for PD-L1 immunostaining, e.g., the use of different antibodies; alternative definitions of positivity (e.g., membranous or cytoplasmic staining); and variations in assay cutoffs [19, 20]. [19, 20]. Furthermore, aspects of tumor biology such as intertumor and intratumor hetero geneity of PD-L1 expression in both TC and tumor-infiltrating IC may also produce incongruous data [21, 22]. Previous studies have scored PD-L1 expression by determining the percentage of positively stained TC (tumor proportion score [TPS]) [23].

However, a different approach proposed an aggregate score of TC and IC (combined proportion score [CPS]), due to emerging evidence suggesting its greater clinical relevance [24]. Based on recently reported tumor classification systems focused on TC and IC PD-L1 expression patterns in melanoma and lung cancer tumor microenvironments (TME) [25-27], we hypothesized that PD-L1 expression patterns at TC/IC interfaces may be clinically relevant in OCCS. Encouraged by this hypothesis, we aimed to evaluate the prognostic utility of PD-L1 expression in a large cohort of OSCC patients by using a standardized methodology; to determine the impacts of diferente scoring methods and assay cutoffs on prognostic accuracy; and to explore the prevalence of TME TC/IC PD-L1 expression patterns in OSCC and their associations with survival.

Patients and Methods

Study Population and Tissue Samples.

A retrospective cross-sectional study was performed by retrieving surgical specimens of OSCC included in a previously-described tissue microarray (TMA) (Cohort 1) [28]. To expand our sample size and to evaluate PD-L1 expression by tumorinfiltrating IC, we evaluated whole tissue sections from an independent patient cohort diagnosed and treated for OSCC at the A.C. Camargo Cancer Center (São Paulo, Brazil) and the Instituto de Anatomia Patologica–IAP (Santa Barbara d'Oeste, Brazil) (Cohort 2). The study was conducted in accordance with ethical guidelines of the Declaration of Helsinki. Samples and clinicopathological data were de-identified and coded. Approval was obtained from the Research Ethics Committees of the institutions (2.481.465).

The inclusion criterion was the diagnosis of previously untreated OSCC. Patients with distant metastases, tumors of the lips or oropharynx, a second primary tumor, and cases without a formalin-fixed paraffin-embedded (FFPE) sample were excluded. FFPE surgical specimens from eligible patients were retrieved, and hematoxylin and eosin slides were reviewed by a certified pathologist to confirm original diagnoses.

Patient medical charts were reviewed to collect sociodemographic characteristics (e.g., gender, age, risk factors); clinical data (e.g., tumor site, TNM stage-7th edition, HPV status); pathologic features (e.g. histologic differentiation, status of surgical margins); therapeutic modality (surgery, radiotherapy, and/or chemotherapy); and follow-up status. Tumor and clinical stages were categorized as initial (I and II) or advanced (III and IV) [29]. Histologic differentiation was designated as either well- (grade I), moderately- (grade II), or poorly differentiated (grade III) [30]. Microscopic examination of resected surgical margins was categorized according to the distance between the tumor and the surgical resection into negative (≥ 5 mm) and positive (< 5 mm). Survival outcomes were evaluated as overall survival (OS); the time interval between treatment and death due to any cause or the last follow-up; and disease-free survival (DFS), the interval between treatment and first recurrence, or last follow up.

Immunohistochemical Staining.

PD-L1 expression was evaluated by immunohistochemical staining of 4-µm histologic sections. Samples from Cohort 1 were evaluated using the Ventana BenchMark XT automated system (Ventana Medical Systems, Inc., Tucson, AZ, USA) following the manufacturer's instructions. The TMA slide was dewaxed using the

EZprep solution (cat. 950-102, Ventana Medical Systems), followed by antigen retrieval using a cell conditioning 1 reagent (cat. 950-224, Ventana Medical Systems) at 95°C for 1 hour. Subsequently, the slide was incubated with anti-PDL1 antibody (1:50, clone 28-8, ab205921; Abcam), followed by the Ultraview detection kit (cat. 760-500, Ventana Medical Systems). Antigen retrieval from Cohort 2 was carried out with EDTA/TRIS pH 9.0 for 15 minutes. Slides were incubated with anti-PD-L1 antibody (1:500, clone 73-10, ab228415; Abcam), followed by detection with the Advance kit (Dako, Hamburg, Germany) according to manufacturer's instructions. Adequate positive control (Human tonsil) was implemented, and the negative control was obtained by omitting the primary specific antibody.

Immunohistochemical Analysis.

Digital images of the immunohistochemically stained slides were obtained at low and high magnifications. Staining characteristics were evaluated by two certified pathologists using reported parameters for the specific antibody clones [31]. TC membranes and cytoplasm may bind anti-PD-L1 immunohistochemical stains, however, only membranous positivity was considered for scoring purposes. Complete circumferential or partial membranous staining of TC and IC at any intensity was considered PD-L1-positive (+). TPS was calculated by determining the percentage of TC (+) within a minimum of 100 viable TC (Cohorts 1 and 2), while CPS disclosed the percentage of TC (+) and IC (+) (Cohort 2). To evaluate the impact of different scoring methods previously reported in the literature, the percentages of positive TC were classified into four groups: (1) ≤1%, (2) 1-5%, (3) 5-10%, and (4) >10% (Cohort 1). To better understand TC—IC interactions, tumors from cohort 2 were categorized into four patterns (I-IV) according to PD-L1 expression: Pattern I, TC (+) IC (+); Pattern II, TC (-) IC (-); Pattern III, TC (+) IC (-); and Pattern IV, TC (-) IC (+) **(Table 1)**. For statistical purposes, cases were split into low (≤1%) or high (> 1%) PD-L1 expression (Cohorts 1 and 2).

Table 1. Patterns of PD-L1expression according to the tumor cells and immune cells interfaces in the tumor microenvironment

Pattern[a]	PD-L1 expression	OSCC[b] prevalence	Biological significance	Immunotherapy implication[c]
I	TC (+), IC (+)	49%	Genetic modifications and adaptive immune resistance	Good responders
II	TC (–), IC (–)	17%	Immune ignorance	Non-responders
III	TC (+), IC (–)	11%	Intrinsic induction	Non-responders
IV	TC (–), IC (+)	23%	Immune tolerance	Good responders

Note: Abbreviations: IC, tumor-infiltrating immune cells; OSCC, oral squamous cell carcinoma; PD-L1, Programmed cell death ligand 1; TC, tumor cells.

[a]Based on Taube 2012, revised by Teng 2015 and modified considering all the immune cells in the tumor microenvironment.

[b]Based on our results.

[c]Data reported for melanomas (Teng, 2015).

Statistical Analysis.

A descriptive analysis of clinicopathologic characteristics was performed using absolute and relative frequencies. Chi-square, Yates's correction for continuity, and Fisher´s exact tests were used to correlate clinicopathologic features and PD-L1 expression. The Kaplan-Meier method was used to construct survival curves, and a Logrank test was conducted to evaluate the prognostic significance of PD-L1 expression. The Cox proportional hazard model was employed for univariate survival analysis. Data analysis was performed using SPSS Statistics, version 23.0 (IBM Corp., Armonk, NY, USA), and a 95% confidence level (p-value ≤ 0.05) was considered statistically significant.

Results

High PD-L1 expression in TC is not a predictor of survival.

Seventy-six patients from Cohort 1 and 46 patients from Cohort 2 met inclusion criteria, for a total of 123 study subjects. Cohort 1 comprised tumors of the floor of the mouth, retromolar area, gingiva, and other oral anatomic sites, while Cohort 2 included tumors located only on the mobile tongue. Clinical and pathological characteristics are presented in **Supplementary Table 1**. TPS using cutoffs of <1%, 1-5%, 5-10% and >10% detected PD-L1 in 40.8% (n=31), 5.3% (n=4), 10.5% (n=8) and 43.4% (n=33) of Cohort 1 patients, respectively. Of note is that most of cases were classified into the <1% and >10% categories **(Figure 1A)**. Over half of Cohort 1 tumors expressed PD-L1 (59.2%, n=45) **(Figure 1B)**. Quantification of Cohort 2 TPS confirmed the previous

result; 59.6% of cases (n=28) showed PD-L1 expression levels of >1% **(Figure 1C)**. **Figure 1D** provides representative images of immunohistochemical staining. PD-L1 expression did not correlate with gender, alcohol consumption, HPV status, tumor size, lymph node metastasis, clinical stage, histologic differentiation, or surgical margins **(Table 2)**. Although PD-L1 expression was similar in smokers and nonsmokers, a significant difference was found between smokers and ex-smokers (p=0.04). Our analysis also failed to demonstrate any significant differences in PD-L1 expression related to assay cutoffs (5%, 10%) and clinicopathological features **(Supplementary Table 2)**.

There were no significant survival differences between patients with PD-L1-expressing and non-expressing tumors in both cohorts. In Cohort 1, the median DFS was 37.5 months in patients with low PD-L1 expression (HR 24.66, 95% confidence interval [CI], 0 - 85.9) *vs* 26.9 months in the high-expression group (HR 10.4, CI 6.5 - 47.3) (p=0.69) **(Figure 1 E)**. Cohort 2 displayed a median DFS of 49.7 months in patients with low PD-L1 expression (HR 8.4, CI, 33.22–66.19) *vs* 64.97 months in the high-expression group (HR 9.1, IC 46.95–82.99) (p=0.47). **(Figure 1 F)**. The median OS of Cohort 1 patients with low PD-L1 expression was 32.8 months (HR 21.2, IC 0–74.6), and 47.2 months (HR 19.8, CI 8.38 – 86.15) in patients with high PD-L1 expression (p=0.65) **(Figure 1 G)**. Patients in Cohort 2 with low PD-L1 expression showed a median OS of 74.75 months (HR 6.4, IC 62.08 – 87.42) *vs* 76.7 months in the high-expression group (HR 7.2, CI 62.49–91.03) (p=0.93) **(Figure 1 H)**. In addition, no significant diferences in DFS and OS were detected when PD-L1 expression was assayed by using diferente cutoffs of 5% and 10% **(Supplementary figure 1)**. *A reactive patchy PD-L1 pattern is associated with a worse prognosis.* Two patterns of PD-L1 staining in TC were identified by immunohistochemical staining: 1) positive cells segregated in patchy, irregular distributions, or 2) homogeneously stained and diffusely distributed positive cells **(Figure 2A)**. Among OSCC with a PD-L1 expression level ≥ 1%, 57.1% (n=16) showed the diffuse pattern, while the patchy pattern was present in 42.9% of cases (n=12) **(Figure 2B)**. The most striking result emerged from the survival analysis; the patchy pattern was associated with a lower median OS (52.8 months, HR 12.2, CI 28.81–76.88) compared with the diffuse pattern (90.9 months, CI 79.59–102.31) (p=0.03) **(Figure 2C)**. Furthermore, the Cox proportional hazard model showed that the risk of death in patients with the patchy pattern was 2.58 times higher

than in patients with the diffuse pattern (HR 0.258, CI 0.078- 0.853) (p=0.026) **(Figure 2D)**.

Figure 1. PD-L1 expression in tumor cells is unrelated to survival (A) Quantification of PD-L1 expression in OSCC using different cutoffs showed that most tumors were classified in categories <1% (40.8%) and >10% (43.4%). **(B, C)** TPS detected high-level PD-L1 expression (>1%) in over half of both cohorts (Cohort 1, 59.2%, and cohort 2, 59.6%). **(D)** Representative images of PD-L1-positive tumors showing membranous immunostaining on >1% of tumor cells, and low expression (<1%). **(E, F)** Kaplan-Meier analysis revealed that TC PD-L1 expression did not impact disease-free survival (Cohort 1, p=0.69 and Cohort 2, p=0.47), **(G, H)** or overall survival (Cohort 1, p=0.65 and Cohort 2, p=0.93). *Abbreviations:* OSCC, oral squamous cell carcinoma; PD-L1, Programmed cell death ligand-1; TPS, Tumor proportion score.

Figure 1

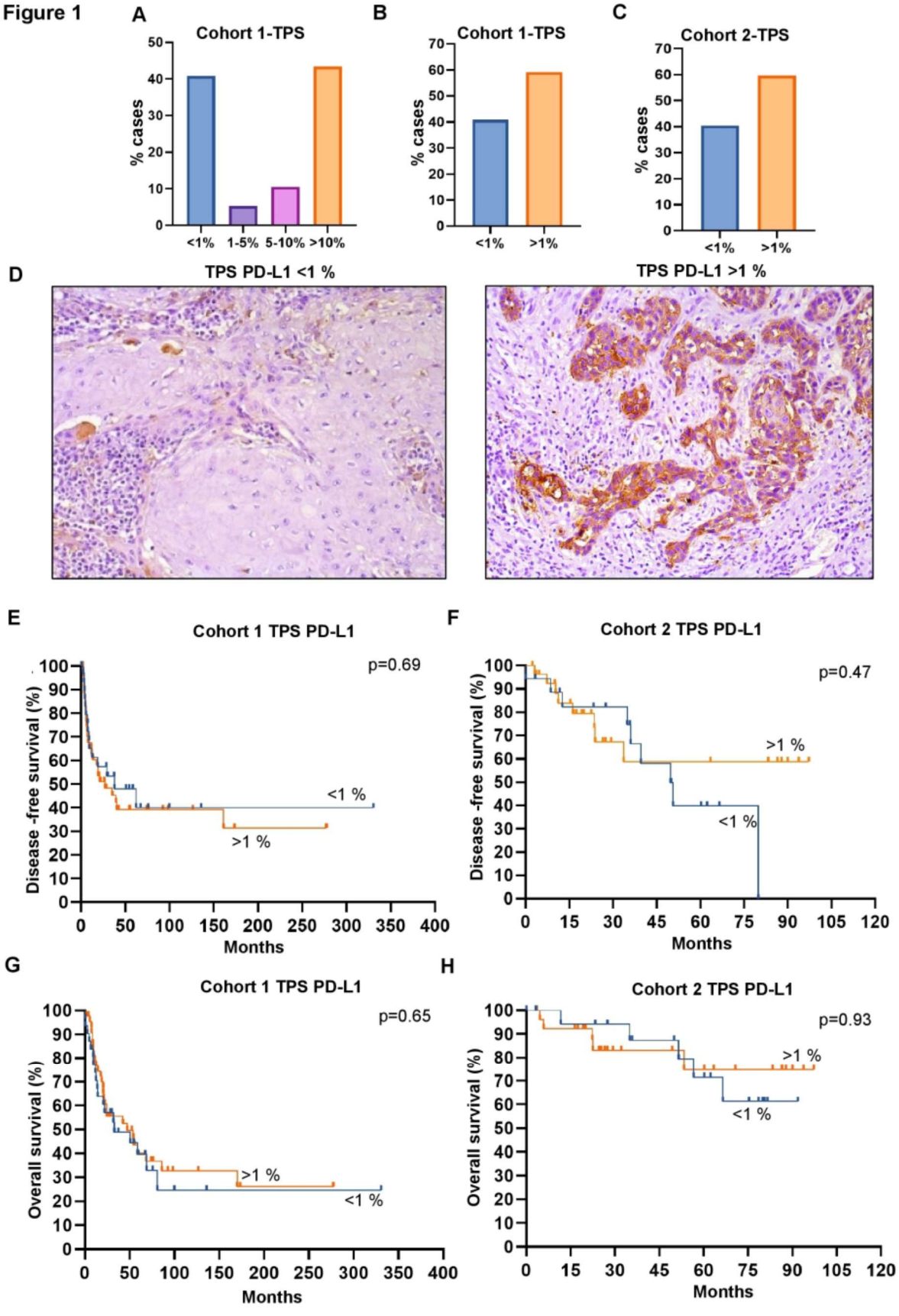
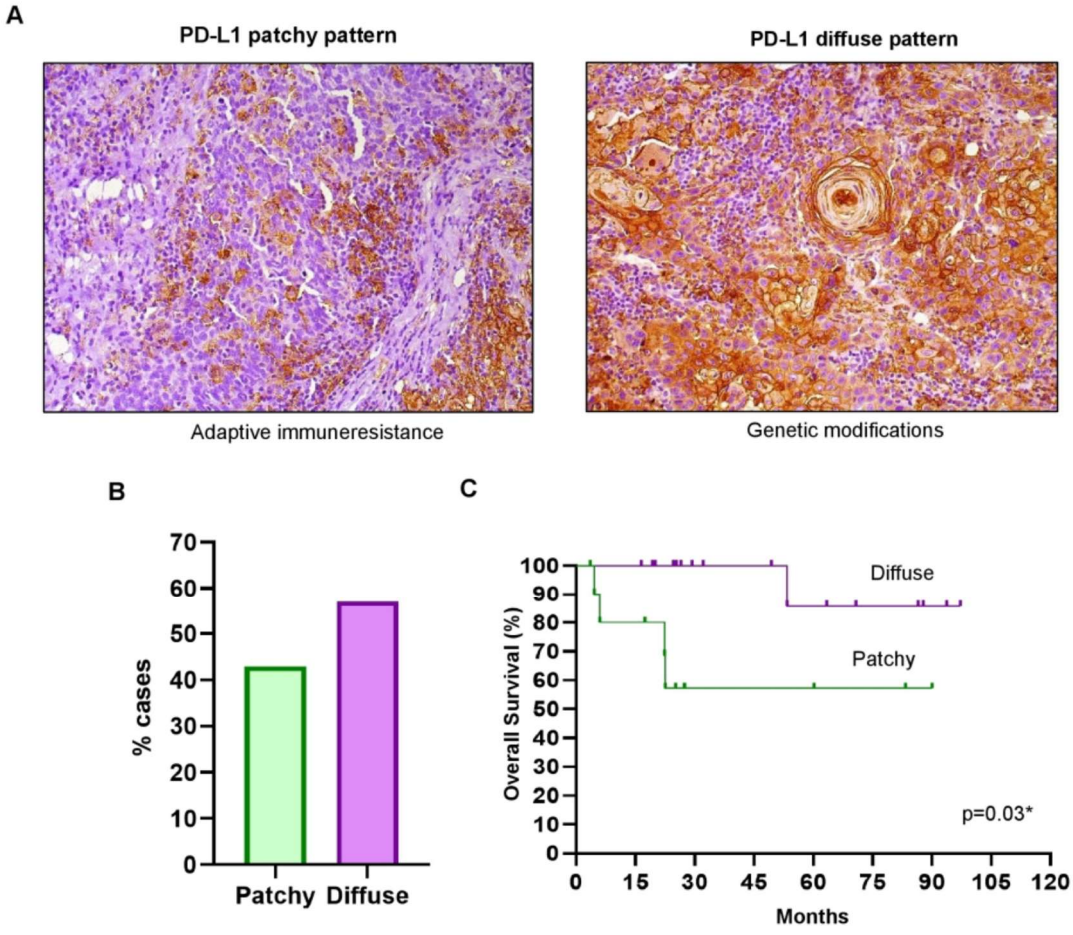

A
Cohort 1-TPS
% cases
<1%
1-5%
5-10%
>10%
B
Cohort 1-TPS
% cases
<1%
>1%
C
Cohort 2-TPS
% cases
<1%
>1%
D
TPS PD-L1 <1 %
TPS PD-L1 >1 %
E
Cohort 1 TPS PD-L1
p=0.69
Disease -free survival (%)
<1 %
>1 %
Months
F
Cohort 2 TPS PD-L1
p=0.47
Disease -free survival (%)
>1 %
<1 %
Months
G
Cohort 1 TPS PD-L1
p=0.65
Overall survival (%)
>1 %
<1 %
Months
H
Cohort 2 TPS PD-L1
p=0.93
Overall survival (%)
>1 %
<1 %
Months

Figure 2. PD-L1 expression in the patchy pattern carries a worse prognosis. (A) Representative images demonstrating that OSCC express PD-L1 in two patterns: positive cells segregated in irregular foci (patchy), or homogeneously stained and widely distributed positive cells (diffuse). These patterns result from two underlying biological mechanisms: adaptive immune resistance (patchy), or genetic modifications (diffuse). **(B)** Quantification of PD-L1 patterns showed that 57.1% of cases exhibited a diffuse pattern, while the patchy pattern was present in 42.9% of tumors. **(C)** Comparison of survival rates according to PD-L1 patterns revealed that the patchy pattern was associated with a lower median OS (52.8 months, HR 12.2, CI 28.81–76.88) compared with the diffuse pattern (90.9 months, CI 79.59–102.31) (p=0.03). **(D)** The previous results were confirmed through the Cox proportional hazard model (HR 0.258, CI 0.078- 0.853, p=0.026). *Abbreviations:* CI, Confidence interval; HR, Hazard ratio; OS, Overall survival; OSCC, Oral squamous cell carcinoma; PD-L1, Programmed cell death ligand-1.

Figure 2



D

Cox proportional hazard model

Pattern	p-value	HR univariate (95% CI)
Patchy	0.026	0.258 (0.078-0.853)
Diffuse	0.105	5.492 (0.702-42.936)

Survival is unrelated to PD-L1 expression evaluated by CPS.

Under physiological conditions, PD-L1 is expressed on macrophages and dendritic cells and bind to PD-1 in order to avoid autoimmune responses, through the reduction of proliferation of antigen-specific T-cells, and decreasing apoptosis in regulatory T cells [3]. Although TC has the ability to express PD-L1 in their membranes, they are also capable to stimulate antigen-presenting cells to express this protein, contributing to the dysregulation and evasion of the antitumor immune activity [4] **(Figure 3)**. PD-L1-positive cells in the TME were characterized as lymphocytes,

macrophages, and dendritic cells in intratumoral and peritumoral sites (**Figure 4A).** CPS revealed that 48.9% of the cases (n=23) showed high expression (>1%), while in 51.1% cases (n=24) the expression was <1% **(Figure 4B)**. Representative photos of immunostaining categorized as CPS low (<1%) and high (>1%) PD-L1 expression are provided in **Figure 4C**. Consistent with results obtained from TPS analysis, PD-L1 expression measured by CPS did not influence any clinicopathological variable **(Table 2)** or survival **(Figure 4D and E)**. The median DFS was 79.9 months in patients with low expression (HR 21.74, CI 37.27–122.52) compared with 55.3 months in the subjects with high expression (HR 10.3, CI 35.15–75.54) (p=0.52) **(Figure 4D)**. Moreover, the medians OS were not significantly different between the groups (78.3 months in PD-L1 <1% and 73.3 months in PD-L1 >1%) (p=0.74) **(Figure 4E)**.

TABLE 2 Simple and multiple Cox proportional hazards model for overall survival

		Simple Cox regression model			Multiple Cox regression model		
Variable	Category	HR	CI (95%)	p	HR	CI (95%)	p
Gender	Male	Ref					
	Female	1.32	0.39–5.05	0.67			
Tobacco Consumption	Yes	Ref					
	Former	1.09	0.31–3.76	0.88			
Alcohol Consumption	Yes	Ref					
	Former	0.52	0.13–2.06	0.35			
T Classification	T1/T2	Ref					
	T3/T4	6.70	1.64–27.3	**0.01***			
N Classification	N0/N1	Ref					
	N2/N3	6.01	1.70–21.21	**0.01***			
Clinical Stage	I/II	Ref			Ref		
	III/IV	3.55	0.91–13.8	0.06	1.64	0.30 – 8.82	0.56
Histological differentiation	I	Ref			Ref		
	II	2.35	0.27–20.2	0.43	9.30	0.68 – 126.8	0.09
	III	10.37	1.20–89.6	**0.03***	12.61	0.84 – 187.8	0.06
PD-L1 TPS	≤1%	Ref					
	>1%	1.05	0.31–3.46	0.93			
PD-L1 CPS	≤1%	Ref					
	>1%	0.41	0.12–1.36	0.14			
Pattern I	Yes	Ref					
	No	0.81	0.24–2.72	0.74			
Pattern II	Yes	Ref			Ref		
	No	0.31	0.09–1.02	0.05	0.11	0.01 – 1.10	0.06
Pattern III	Yes	Ref					
	No	1.45	0.18–11.4	0.72			
Pattern IV	Yes	Ref					
	No	*N/A*	*N/A*	*N/A*			
Patchy Pattern	No	Ref			Ref		
	Yes	3.8	0.07–0.85	**0.02***	35.5	2.8 – 445.2	**0.01***
Diffuse Pattern	Yes	Ref					
	No	5.49	0.70–42.93	0.10			

Note: CI, confidence Interval; CPS, combined proportion score; HPV, human papillomavirus; HR, Hazard ratio; N/A, no available (It was not possible to estimate due to the small number of patients—few events); PD-L1, Programmed death ligand 1; Ref, reference category; TPS: tumor proportion score.
*Statistically significant difference.

Figure 3. PD-L1 expression in tumor cells and infiltrating immune cells. (A) OSCC TC dysregulates antitumor immunity through the expression of PD-L1 that targets the PD-1 receptor of activated T cells. The assessment of PD-L1 expression using TPS is based on the percentage of TC showing membranous staining. **(B)** However, TC can also stimulate antigen-presenting cells to express PD-L1, thus amplifying immunosuppression. CPS quantifies PD-L1 expression by the aggregate of TC and IC. *Abbreviations:* APC, Antigen-presenting cell; CPS, Combined proportion score; NK, Natural killer; OSCC, oral squamous cell carcinoma; PD-1, Programmed cell death 1; PD-L1, Programmed cell death ligand-1; RGMb, repulsive guidance molecule b; TAM, Tumor-associated macrophages; TPS, Tumor proportion score.

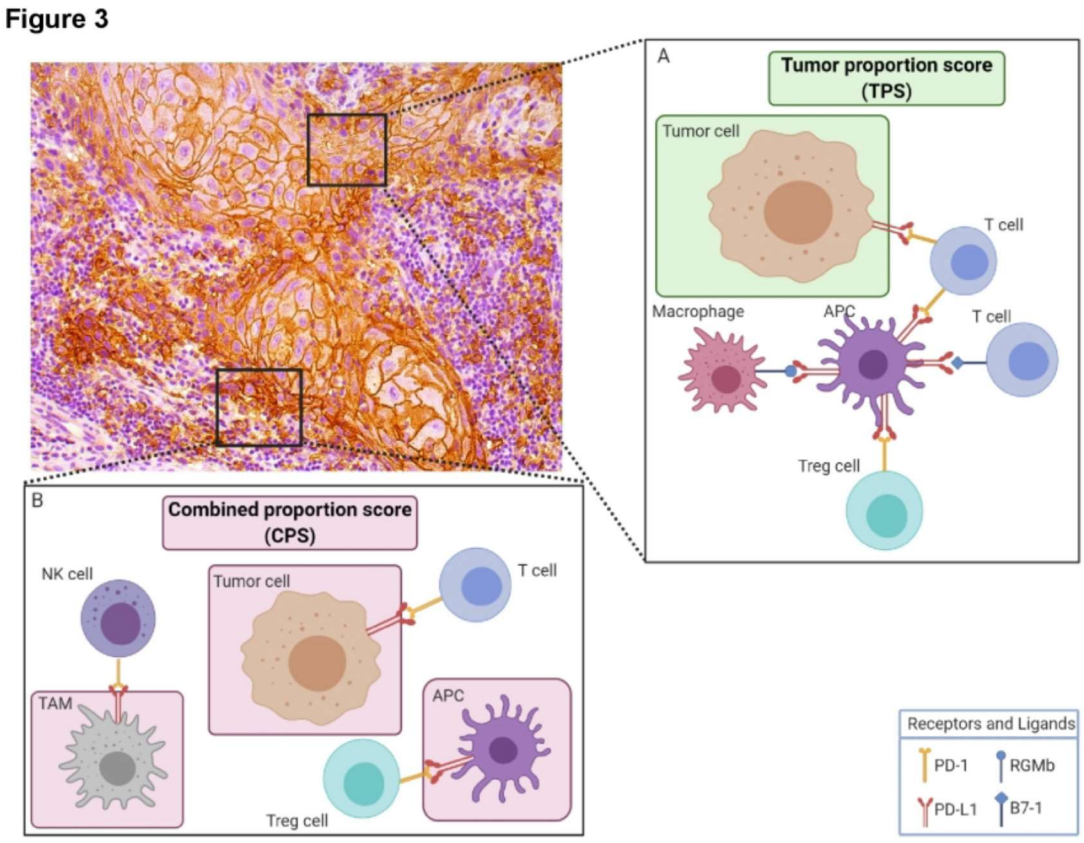
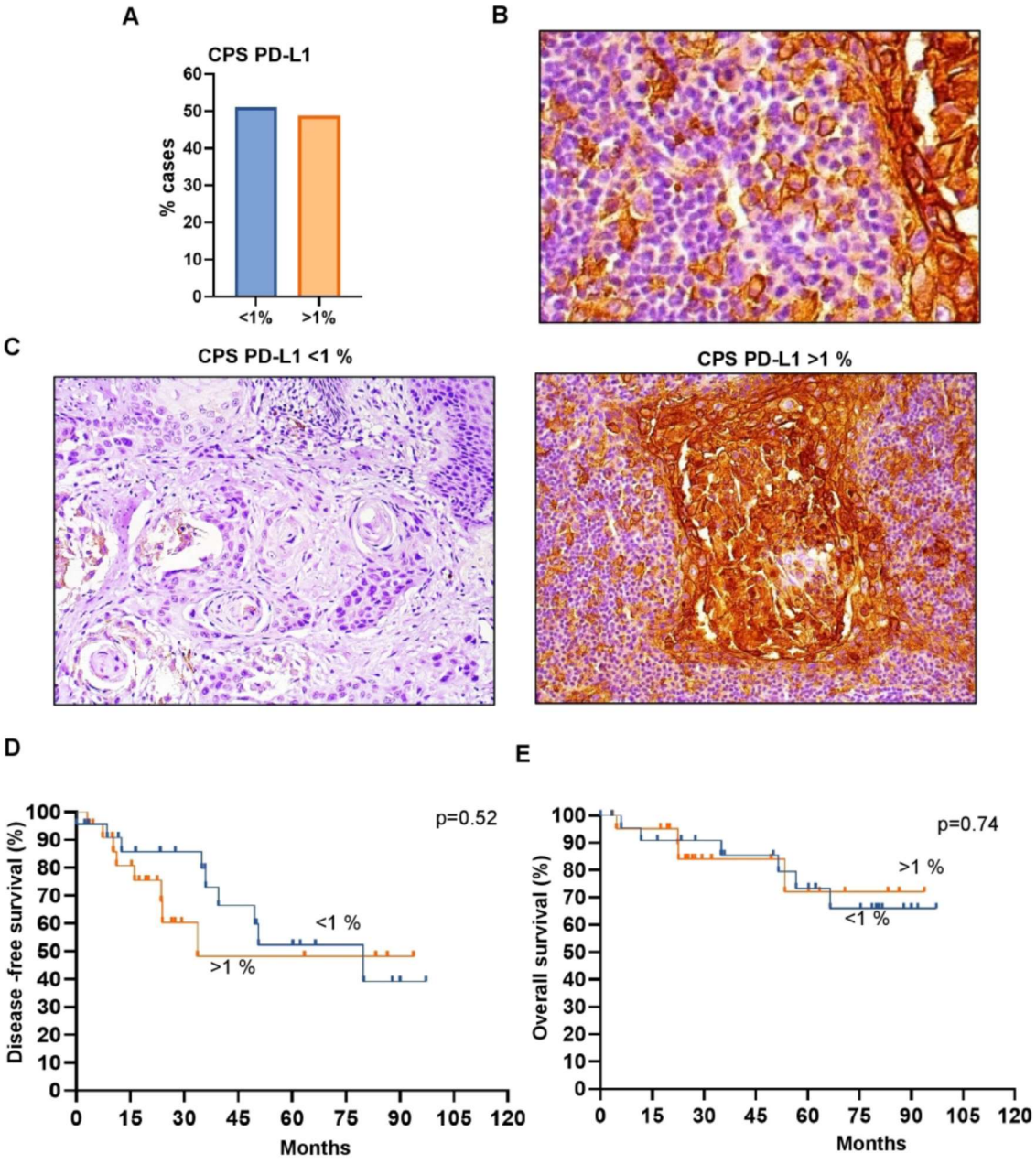


Figure 4. OSCC outcomes are not associated with CPS of PD-L1 expression. (A) PDL1 immunostaining in tumor microenvironmental cells morphologically characterized as lymphocytes, macrophages, and dendritic cells in intratumoral and peritumoral sites. **(B)** CPS revealed that 48.9% of the cases showed high expression (>1 %), while in 51.1% of cases the expression was <1%. **(C)** Representative images of immunostaining categorized as CPS low (<1%) and high (>1%) expression. **(D)**

Kaplan-Meier analysis revealed that CPS was unrelated to disease-free survival (p=0.52) and overall survival (p=0.74). *Abbreviations:* CPS, Combined proportion score; OSCC, oral squamous cell carcinoma; PD-L1, Programmed cell death ligand-1.

Figure 4



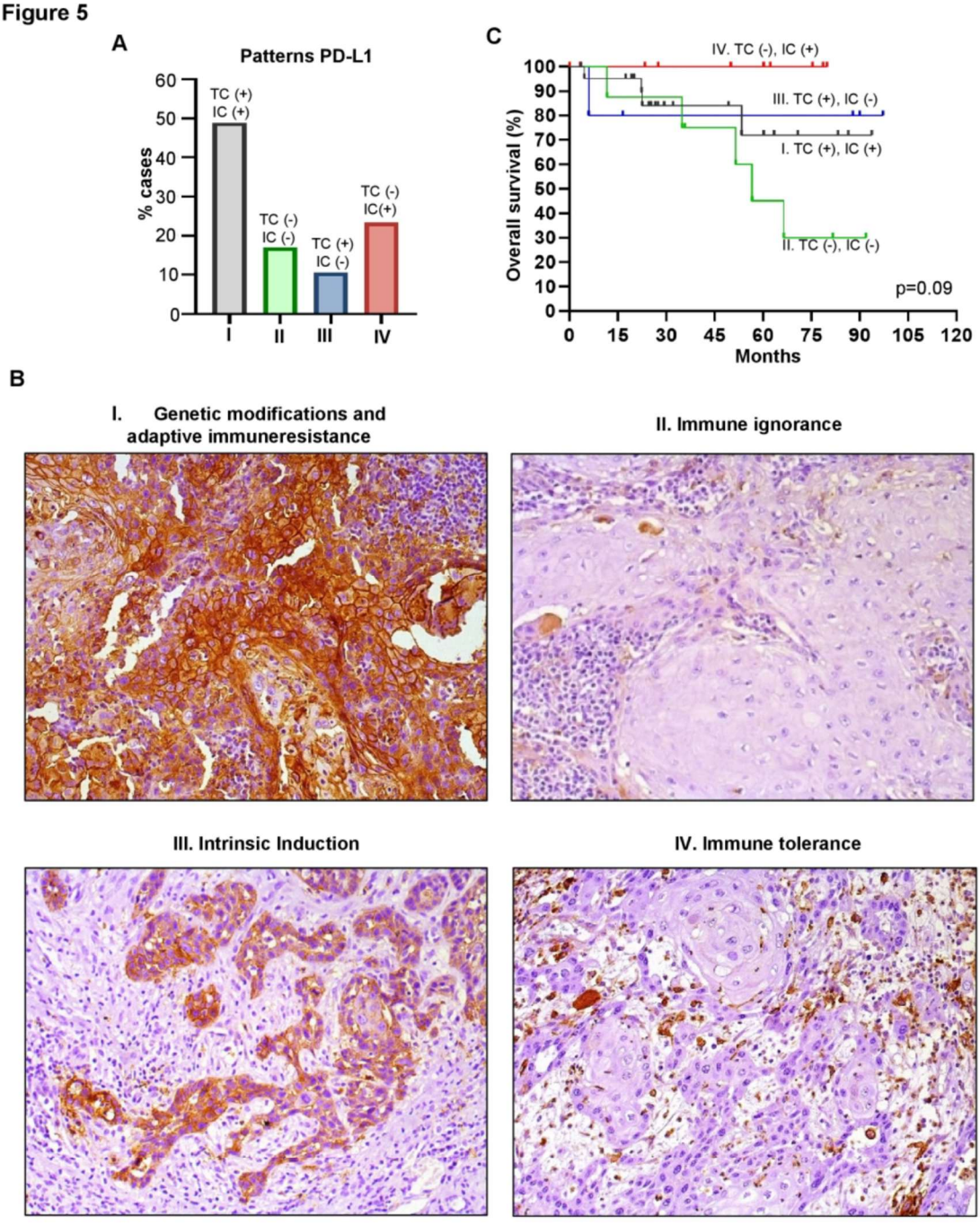
Patterns of PD-L1 expression based on the interaction between TC and imune microenvironment.

Cases categorized as Pattern I showed a diffuse and intense expression of PD-L1 on the membranes of TC and IC. In pattern II, PD-L1 was not expressed in TC or IC. Pattern III showed intense membranous expression in TC and negative expression

in adjacent IC. In Pattern IV, any expression was observed in tumor islands or individual cells, while a strong membrane positivity was identified in stromal IC **(Table 1)**. Pattern I constituted the highest proportion (48.9%). Interestingly, the second most common pattern was PD-L1 expression only in IC (Pattern IV) (23.4%), followed by Pattern II (17%). The least frequent pattern was Pattern III (10.6%) **(Figure 5A)**. Representative immunohistochemical photos of PD-L1 staining patterns are shown in **Figure 5B.** Kaplan-Meier curves based on PD-L1 patterns are presented in **Figure 5C.** Interestingly, Pattern II showed the lowest median OS (59.61 months [HR 9.60, CI 40.79- 78.43]). The median OS in patients with Pattern I was 73.37 months (HR 7.88, CI 57.91– 88.83), while that of Pattern III was 78.97 months (HR 16.32, 46.98-110.95) and any death was reported in the Pattern IV group.

Figure 5. Patterns of PD-L1 expression based on interactions of tumor cells and immune microenvironment. Tumors were categorized into four patterns based on the interaction between TC and the immune microenvironment. **(A)** PD-L1 expression by both TC and IC (Pattern I) constituted the highest proportion (48.9%), followed by PDL1 expression only in IC (Pattern IV) (23.4%). Less frequent patterns were lack of expression in TC and IC (Pattern II) (17%), and PD-L1 positivity only in TC (Pattern III) (10.6%). **(B)** Representative images of the four PD-L1 staining patterns. **(C)** Kaplan-Meier curves indicated that Pattern II (PD-L1 double negative) was associated with the lowest overall survival, with a median of 59.61 months (HR 9.60, CI 40.79-78.43), compared with the other patterns (I, median 73.37 months, HR 7.88, CI 57.91–88.83; III, 78.97 months, HR 16.32, 46.98-110.95; and any death was reported in the Pattern IV group). *Abbreviations:* CI, Confidence interval; HR, Hazard ratio; IC, immune cells; OSCC, oral squamous cell carcinoma; PD-L1, Programmed cell death ligand-1; TC, tumor cells

Figure 5



Discussion

Despite the recognition of the PD-L1 signaling pathway as a major immunologic escape mechanism exploited by TC [32], studies of the prognostic value of PD-L1 expression in OSCC have yielded inconsistent findings [33]. Herein we demonstrated

that PD-L1 expressions by TC and infiltrating IC determined by TPS and CPS are unrelated to clinicopathologic characteristics and survival rates. Nevertheless, to our knowledge, the present study is the first attempt to analyze PD-L1 expression patterns in TC—IC interactions in the TME, and suggests that these patterns may serve as prognostic biomarkers.

HNSCC arises from oral, oropharyngeal, hypopharyngeal, and laryngeal mucosas, and is highly associated with smoking and alcohol consumption [30]. Recently, a subset of tumors related to high-risk HPV, primarily affecting the oropharynx, has been recognized with unique features at molecular (TP53 wild-type), clinical (improved prognosis), and pathologic levels (basaloid morphology) [34]. From a microenvironmental perspective, the improved clinical response of HPV-related tumors may be explained by adaptive immune responses against viral antigens that stimulate potent antitumor immunity [35]. A major limitation of previous studies is the pooled evaluation of oral and oropharyngeal tumors. Thus, studies evaluating the interplay between TC and the immune microenvironment should subcategorize tumors according to anatomic site and HPV status. Our sample, derived from two independent cohorts, was composed exclusively of OCCS. Although the prevalence of HPV in OCCS is low [36], we found that PD-L1 expression was unrelated to HPV-DNA status and tumor site.

Whereas our literature review did not identify studies assessing the interplay between HPV and PD-L1 in OSCC, a subgroup of PD-L1+ and HPV+ oropharyngeal tumors has

been recognized for its excellent prognosis [37]. This may be due to HPV-mediated viral

oncogenesis and the immunogenicity of HPV antigens in this tumor subset. We aimed to assess the clinicopathological and prognostic significance of PD-L1 in OSCC by using different scoring methods through standardized criteria [31]. First, we evaluated TC PD-L1 expression in two independent cohorts by using four separate cutoffs, and showed that PD-L1 expression is unrelated to clinicopathological characteristics and survival rates. Because a recent approach assesses PD-L1 expression by CPS with improved response to immunotherapy in HNSCC [24], we ALSO used a second technique to assay PD-L1 expression. Our results revealed that PD-L1 expression in both TC and IC populations did not influence survival in OSCC. These results are supported by the use of two clones, 28-8 and 73-10, both validated

as diagnostic biomarkers for PD-L1 expression to select patient candidates for nivolumab and avelumab therapies, respectively. In contrast to previous reports that identify PD-L1 expression as a predictor of survival [10, 11], our results support literature not linking PD-L1 with survival in OSCC [12-14].

Despite the multiplicity of PD-L1 immunostaining techniques, it is important to consider a recent consensus that PD-L1 positivity >1% by CPS should be used to select patients for immunotherapy, as superior outcomes were obtained by patients with higher CPS, while TPS was not predictive [38]. Indeed, immunotherapy has benefited HNSCC patients regardless of PD-L1 expression, however, with long-term follow-up, PD-L1 expression (>1%) was associated with further improvement in OS [17]. In contrast to the broad literature investigating the prognostic value of PD-L1, data are limited regarding PD-L1 expression patterns and their underlying biological mechanisms. We observed that OSCC TC express PD-L1 in two patterns, patchy and diffuse, and found an almost equal distribution of PD-L1- positive tumors among the two pattern groups. A previous study described intratumoral PD-L1 peripheral and diffuse staining patterns throughout tumor nests, and reported that most cases displayed the peripheral pattern [14] Notably, the patchy pattern described in our report differs from the peripheral pattern, because the patchy pattern features positive cells in both the center and periphery of tumor nests. One of our most relevant findings related clinical outcomes to mechanisms of PDL1 upregulation. Chromosome 9 copy number gains drive the constitutive expression of PD-L1 by TC [39], resulting in a homogenous and diffuse pattern. In contrast, interferon-γ secretion by activated T cells induces PD-L1 expression by TC in a heterogeneous patchy

pattern, usually in TME regions infiltrated by T cells, such as the invasive margin [40].

We demonstrated that OS is significantly shorter in patients with tumors expressing PDL1 in a patchy pattern. Our data support the hypothesis that high intratumoral heterogeneity in PD-L1 expression promotes adaptive immune resistance [22]. Dynamic interactions between TC and non-malignant cells in the TME drive PDL1 expression [3]. In search of a more accurate predictive model of clinical outcome, we explored PD-L1 expression based on TC and IC interactions in OSCC. A previous study classified human melanocytic lesions according to the presence or absence of PD-L1 expression in melanocytes and tumor-infiltrating lymphocytes [25-27]. Because local T cell secretion of interferon-γ upregulates PD-L1 expression by both TC and IC

[4, 41], we stratified tumors into four groups according to PD-L1 expression in TC and IC. The most prevalent pattern, accounting for almost half of cases, was PD-L1 expression by both TC and IC (Pattern I). These findings support the concept of OSCC as an immunosuppressive disorder in which neoplastic cells produce cytokines that suppress cell-mediated antitumor immunity [42]. Less frequently observed patterns were PD-L1 expression exclusively in IC (Pattern IV), PD-L1 double negative tumors (Pattern II), and the expression of PD-L1 only in TC (Pattern III). Whereas patterns II and IV are associated with immune ignorance or tolerance, respectively, constitutive PD-L1 expression by TC is driven by poorly characterized oncogenic signaling pathways [27].

Our most surprising finding was the association of expression patterns with clinical outcomes. Although a significant p-value was not established, the early separation of survival curves and worse OS in the double-positive (Pattern I) and double-negative (Pattern IV) groups indicate the clinical impacts of adaptive immune resistance and immunologic ignorance.

Our study has several limitations. First, patients were treated with conventional therapy (surgery, radiotherapy, and chemotherapy), precluding an analysis of immunotherapy response. Second, the sample size of cohort 2 was small. Nevertheless, our results encourage further studies of larger cohorts using well-designed methodologies to validate our findings and to assess PD-L1 patterns as prognostic biomarkers of immunotherapy response.

Conclusion

Our novel findings indicate that PD-L1 expression patterns in the context of TC—IC interactions represent antitumor immune responses better than TPS or CPS. Our suggested stratification may have important implications for the characterization of OSCC; for the use of PD-L1 expression patterns as predictors of outcome; and for the design of new strategies to improve efficacies of established therapies.

Conflict of Interest

The authors declare no conflict of interest

Acknowledgments

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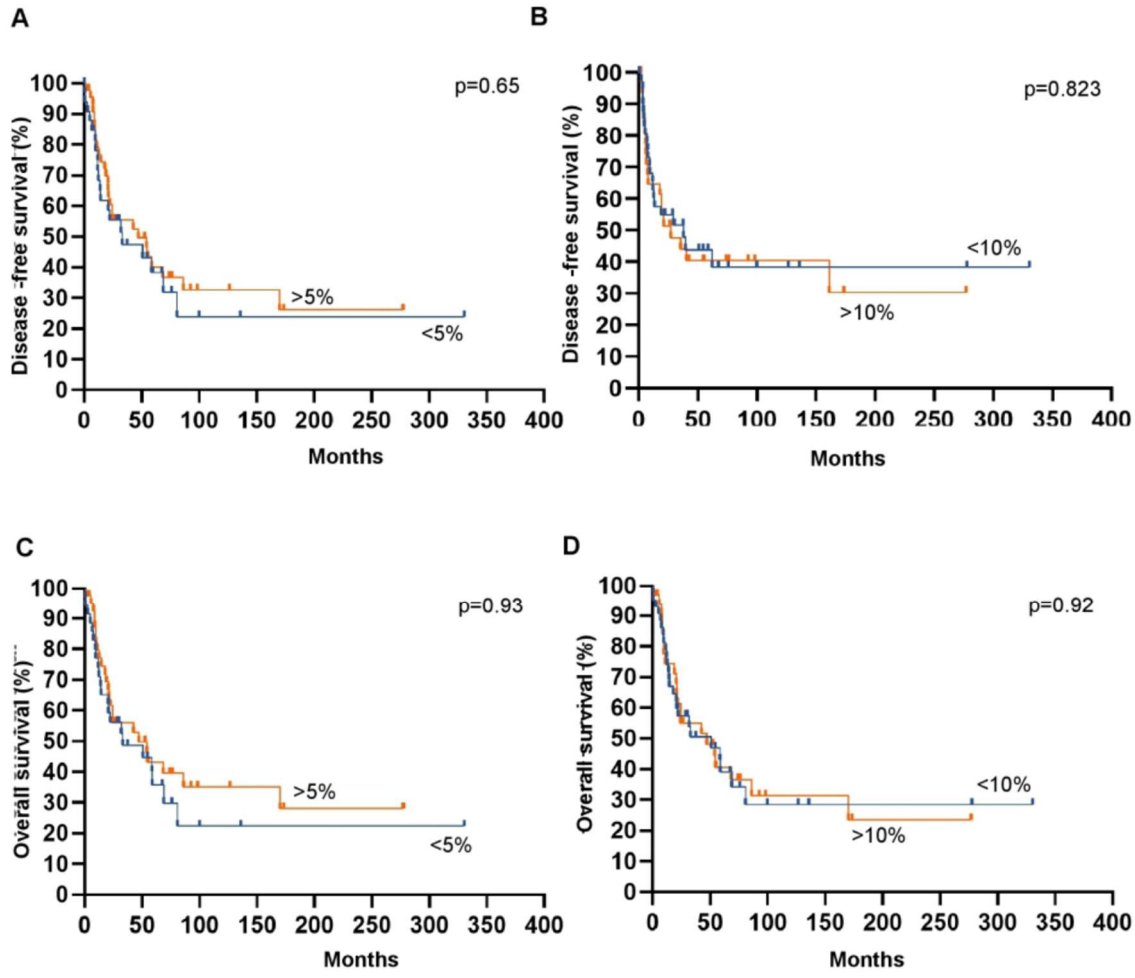
Supplementary figure 1



2.2. Evaluation of PD-L1 Expression and Soluble Immune Profile Interaction in OSCC

Running Title: Soluble Immune profile in Oral Cancer

Artigo submetido ao periódico Oral Diseses

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ABSTRACT

Background

The immune profile of OSCC and its relationship with PD-L1 expression is crucial to identifying the most suitable candidates for therapy with immune checkpoint inhibitors.

Methods

A prospective study involving 23 patients was conducted, during which plasma samples and paraffin-embedded blocks were collected. Subsequently, we

characterized the soluble immune profile by analyzing 32 analytes and correlating them with clinicopathological characteristics. Following this, we assessed the immunoexpression of PD-L1. We distinguished between these two expression profiles and correlated them with the soluble immune profile.

Results

Increased plasma levels of IL-1RA, IL-8, IL-12p40, IP-10, MIG, PFGD-aa/bb, and FGF-2 were linked to some worse clinical features, according to the analysis of the soluble immune profile of OSCC patients. In the meantime, improved clinical-pathological features in OSCC were linked to higher levels of IL-4 and IL-10. Subsequently, using different cut-off points, we evaluated PD-L1 expression in paraffin tissue determined by TPS and CPS and associated it with growth factor, chemokine, and cytokine levels. It was discovered that patients with immune cell expression of PD-L1 had an immunological ignorance profile, whereas those without immune cell expression of PD-L1 displayed an immune tolerance profile.

Conclusion

High levels of specific cytokines and chemokines are associated with worse clinical characteristics in patients with OSCC, while higher levels of IL-4 and IL-10 are linked to better characteristics. PD-L1 expression also influences the patient's immune profile

Keywords

Oral cancer, programmed cell death ligand 1, imune microenvironment, cytokine, chemokine

INTRODUCTION

Recent advances in the treatment of cancer, especially head and neck cancer, have brought attention to the importance of individualized approaches including immunotherapy and targeted therapies. Although these therapies have demonstrated notable advantages, particularly in situations of recurrence or metastasis, their efficacy has to be maximized (Hussain *et al*, 2021; Harrington *et al*, 2022, 2023; Ruffin *et al*, 2023). Research that focuses on defining and personalizing patient reactions to these treatments is essential, especially in light of immune checkpoint inhibitor side effects.

Several studies have shown the role of the PD-1/PD-L1 pathway in immune resistance in head and neck squamous cell carcinoma (HNSCC), highlighting the necessity to understand how the tumor escapes immune resistance during malignant transformation. (Lin *et al*, 2015; Patel and Kurzrock, 2015a, 2015b; De Vicente *et al*,

2019; Zhou *et al*, 2021). Furthermore, there is a negative correlation between the prognosis of oral squamous cell carcinoma (OSCC) and high PD-L1 expression, highlighted the need to discover biomarkers such as PD-L1 to predict treatment outcomes.

Immune escape mechanisms and lymphocyte depletion are two issues in the tumor microenvironment that need to be addressed in addition to the potential of immune checkpoint inhibitors as a treatment for oral cavity squamous cell carcinoma (Eckert *et al*, 2016; Munn and Bronte, 2016; Chen *et al*, 2020). Knowing the immunological profile of OSCC patients and how it links to PD-L1 expression will help to determine which patients are most appropriate for immune checkpoint inhibitor therapy. In summary, a better knowledge of the immunological landscape of tumors and the unique responses to these drugs is crucial for the effective use of immune checkpoint inhibitors in the treatment of OSCC.

MATERIALS AND METHODS

All patients were under the care of the Head and Neck Surgery and Otorhinolaryngology Departments of the ACCamargo Cancer Center, São Paulo, Brazil. This is a prospective, non-interventional clinical study. The diagnosis, treatment, and follow-up of the patients were carried out independently of the decision to include the patient in this study. The study was conducted in accordance with Brazilian ethical guidelines and approved by the local Research Ethics Committee under protocol no. 44673821.0.0000.5432

We included 23 patients treated at the AC Camargo Cancer Center with a diagnosis of primary OSCC. All patients had surgery as the initial treatment. Exclusion criteria included those with other previously treated or synchronous cancers and taking immune checkpoint inhibitors. Patients with distant metastasis or debilitating chronic diseases, autoimmune diseases, and concomitant chronic infections have also been excluded from the study.

Immune profile analysis:

- Determination of the soluble immune profile of 27 analytes from the plasma of OSCC patients, analyzing inflammatory and anti-inflammatory activities.

The soluble immune profile was assessed using blood samples collected in an EDTA tube and processed for plasma separation by centrifugation at 400×g, 10 min, 20°C, and then stored at -80°. The assay was performed using the MILLIPLEX® Human

Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel kit (HCYTA-60K 32), at a 1:2 dilution in diluent (Serum Matrix + Assay Buffer). The kit contains capture sets to detect: FGF-2/FGF-basic, G-CSF, GM-CSF, IFNα2, IFNγ, IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-7, IL-8/CXCL8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A/CTLA8, IL-18, IP-10/CXCL10, MCP-1/CCL2, MCP-3/CCL7, M-CSF, MIG/CXCL9, MIP-1α/CCL3, MIP-1β/CCL4, PDGF-AB/BB, RANTES/CCL5, TNFα, TNFβ/Lymphotoxin-α, VEGF-A. To this end, the plasma samples were added to a mixture of magnetic beads, each of which is specific to an analyte, which acts as a catch for the analytes present in the sample. After the interaction between the magnetic beads and the specific analyte, detection is carried out using an antibody conjugated with a fluorescent marker. The reading is performed on the Magpix machine using software from the Luminex platform. The amount of each analyte detected in pg/ml is calculated based on a standard curve.

Evaluation of PD-L1 immunoexpression in patients with OSCC

Twenty-two samples were included for immunohistochemistry. PD-L1 expression was evaluated by immunohistochemical staining of 4 µm histological sections. The slides were incubated with the anti-PD-L1 monoclonal antibody (1:500, clone 73-10, ab228415; Abcam), followed by detection using the Advance kit (Dako, Hamburg, Germany) according to the manufacturer's instructions. An appropriate positive control (human tonsil) was implemented, and the negative control was obtained by omitting the primary specific antibody. The staining characteristics were evaluated by the pathologist, who was blinded to the clinical and pathological characteristics, using parameters reported for specific antibody clones (Tsao *et al*, 2017). Digital images of the immunohistochemical stained slides were obtained at low (×100) and high magnification (×200). Representative fields were selected at a magnification of ×200 for cell counting. Complete or partial membranous staining of TC and IC at any intensity was considered positive for PD-L1+. The TPS was calculated by determining the percentage of positive TC (+) within a minimum of 100 viable TCs, while the CPS revealed the percentage of positive TC (+) and IC (+). To evaluate the impact of PD-L1 expression patterns, the evaluation protocol described in the study by Miranda-Galvis et al. (2021) was used, where four patterns (I–IV) of PD-L1 expression were assessed: Pattern I, TC (+) IC (+); Pattern II, TC (−) IC (−); Pattern III, TC (+) IC (−); and Pattern IV, TC (−) IC (+) (Miranda-Galvis *et al*, 2020).

- Statistical Analysis

Statistical analyses were conducted using GraphPad Prism version 8.3. The Kolmogorov-Smirnov normality test was applied to identify whether the data groups followed a normal distribution. Following this analysis, comparisons between the two groups were made using the Student's t-test or the Mann-Whitney test, depending on the data distribution. Values of $p < 0.05$ were considered statistically significant. However, data with $p < 0.1$, considered to show a "trend," were also presented in this work.

For the construction of heatmaps, Principal Component Analysis (PCA) was utilized through the online tool ClustVis (https://biit.cs.ut.ee/clustvis/), which performs unsupervised clustering of multivariate data via an R software interface. To construct ROC curves, univariate logistic regression was performed using GraphPad Prism.

- Bioinformatic Processing

For the correlation analyses, RStudio software was used to develop a correlation matrix based on the data distribution using the corrplot package. Pearson correlation was the statistical test used, as most of the data followed a parametric distribution. The results are presented in the correlation matrix with the respective r values and a range from 1 to -1, indicating positive or negative correlation. The hclust method was employed for data organization, allowing the matrix to be reordered according to correlation coefficients through hierarchical clustering.**3.**

RESULTS

3.1 Demographic Data of the Study Patients

We recruited 23 patients with OSCC across all clinical stages. The patients analyzed in this study had a mean age of 70 years (ranging from 35 to 91 years), with 12 (52%) being female. In this cohort, 13 (57%) of the patients were non-smokers, and 11 (48%) consumed alcohol.

In clinical aspects,9 patients had tongue tumors (n = 9; 39.1%), followed by 5 with buccal mucosa (21.7%), 5 with alveolar ridge (21.7%), 3 with floor of mouth (13.0%), and 1 with hard palate (4.3%) tumors. Concerning clinical staging (CS), 5 (21.7%) had CS I, 6 (26.1%) had CS II, 3 (13.0%) had CS III, and 39 (9.1%) had CS IV. The presence of lymph node metastasis (N+) was detected in 7 (30.4%) patients, while 16 (69.6%) of the patients had no lymph node metastasis (N0). As for the pathological size of the tumor, we had 5

(21.7%) pT1 cases, 8 (34.8%) pT2 cases, 5 (21.7%) pT3 cases, and 5 (21.7%) pT4 cases (Table 1).

Table 1. Clinicopathological features of OSCC.

	N	%
Gerder		
Male	11	47.8
Famele	12	52.2
Tumor site		
Tongue	9	39.1
Buccal mucosa	5	21.7
Hard palate	1	4.3
Alveolar ridge/gengive	5	21.7
buccal floor	3	13.0
Tabacco consumption		
Yes	4	17.4
No	12	52.2
Ex	6	26.1
NI	1	4.3
Alchool consumption		
Yes	11	47.8
No	11	47.8
NI	1	4.3
pT		
1	5	21.7
2	8	34.8
3	5	21.7
4	5	21.7
pN		
0	16	69.6
1	2	8.7
2	3	13.0
3	2	8.7
pM		
0	23	100
Clinical stage		
I	5	21.7
II	6	26.1
III	3	13.0
IV	9	39.1
RT		
No	11	47.8
Yes	12	52.2

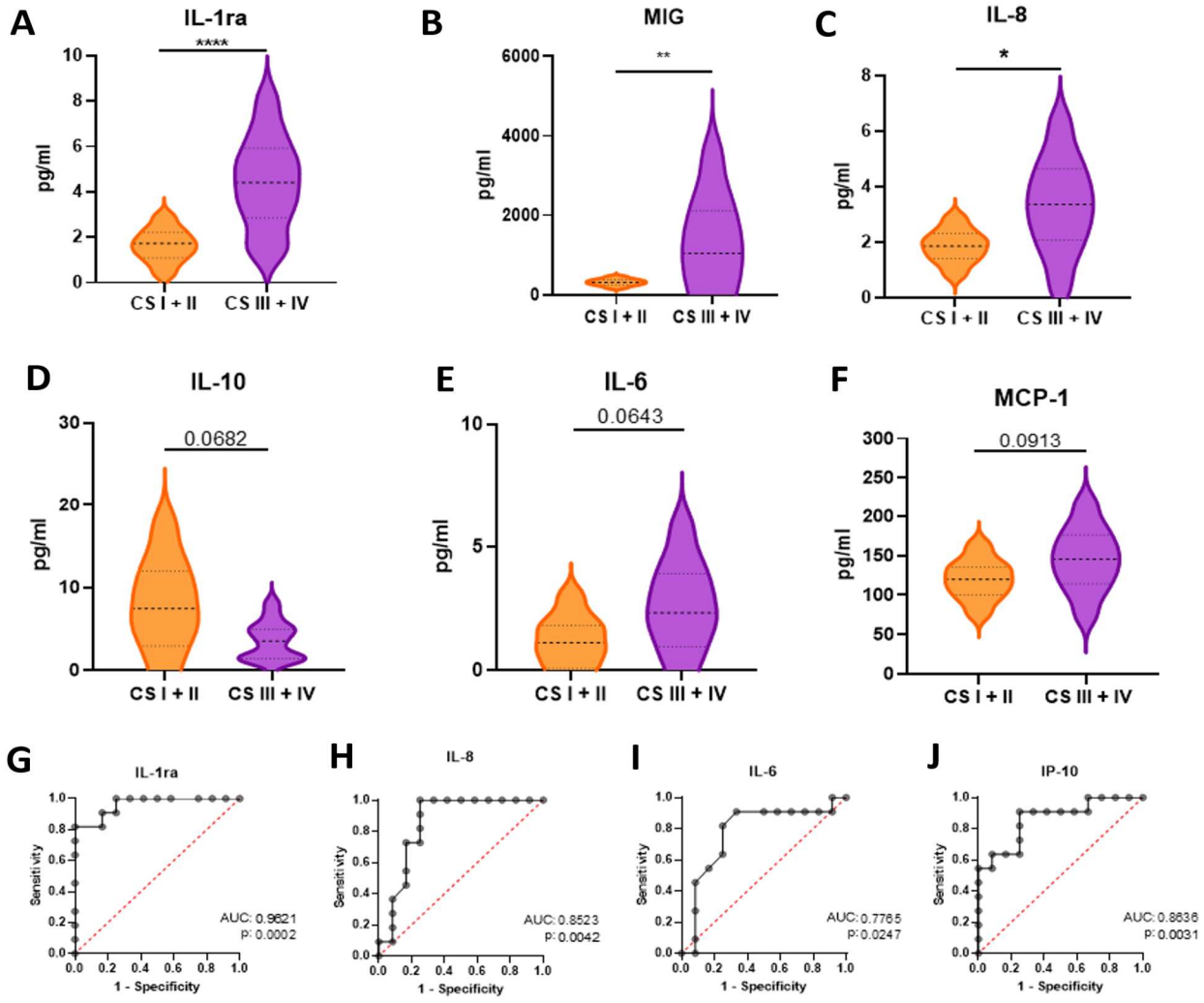
3.2 Evaluation of the soluble factor levels in patients with oral SCC and their correlation with clinical staging

Multiplex immunoassay analysis was employed to evaluate the soluble immune profile of plasma samples from 23 OSCC patients. Among the 32 analytes analyzed, only three cytokines—IL-1RA, MIG, and IL-8—showed statistically significant differences when stratified by clinical staging (Figure 1 A-C). Additionally, three cytokines—IL-10, IL-6, and MCP-1—showed trends toward statistical significance (Figure 1 D-F).

To explore whether these variables could differentiate between groups, we constructed a heatmap using the ClustVis online application. This analysis included cellular populations and soluble proteins that exhibited statistically significant differences or trends ($p > 0.1$) between groups across all populations and analytes. The results indicated that only the soluble factors were able to form a distinct immunological pattern (Supplementary Figure 1).

To assess the impact of these cytokines on outcomes and clinical staging, logistic regression analysis was performed to identify potential biomarkers for predicting tumor development. ROC curve analysis was also conducted to evaluate the precision of these biomarkers. Cytokines associated with both pro- and anti-inflammatory profiles, such as IL-6, IL-8, and IP-10, with an area under the curve (AUC) value of 0.7 or higher and $p < 0.05$, demonstrated good predictive capacity for clinical staging (Figure 1G-J).

Figure 1. Analysis of the soluble immune profile in the plasma of patients with oral squamous cell carcinoma (OSCC) at different clinical stages. Analysis of soluble components in plasma from OSCC patients classified into initial clinical stage (n = 11) and advanced stage (n = 12) showed statistical significance. ROC curves of soluble factors and immune cell populations associated with clinical outcome in OSCC patients. AUC values above 0.7 were considered, with statistical significance set at $p < 0.05$.

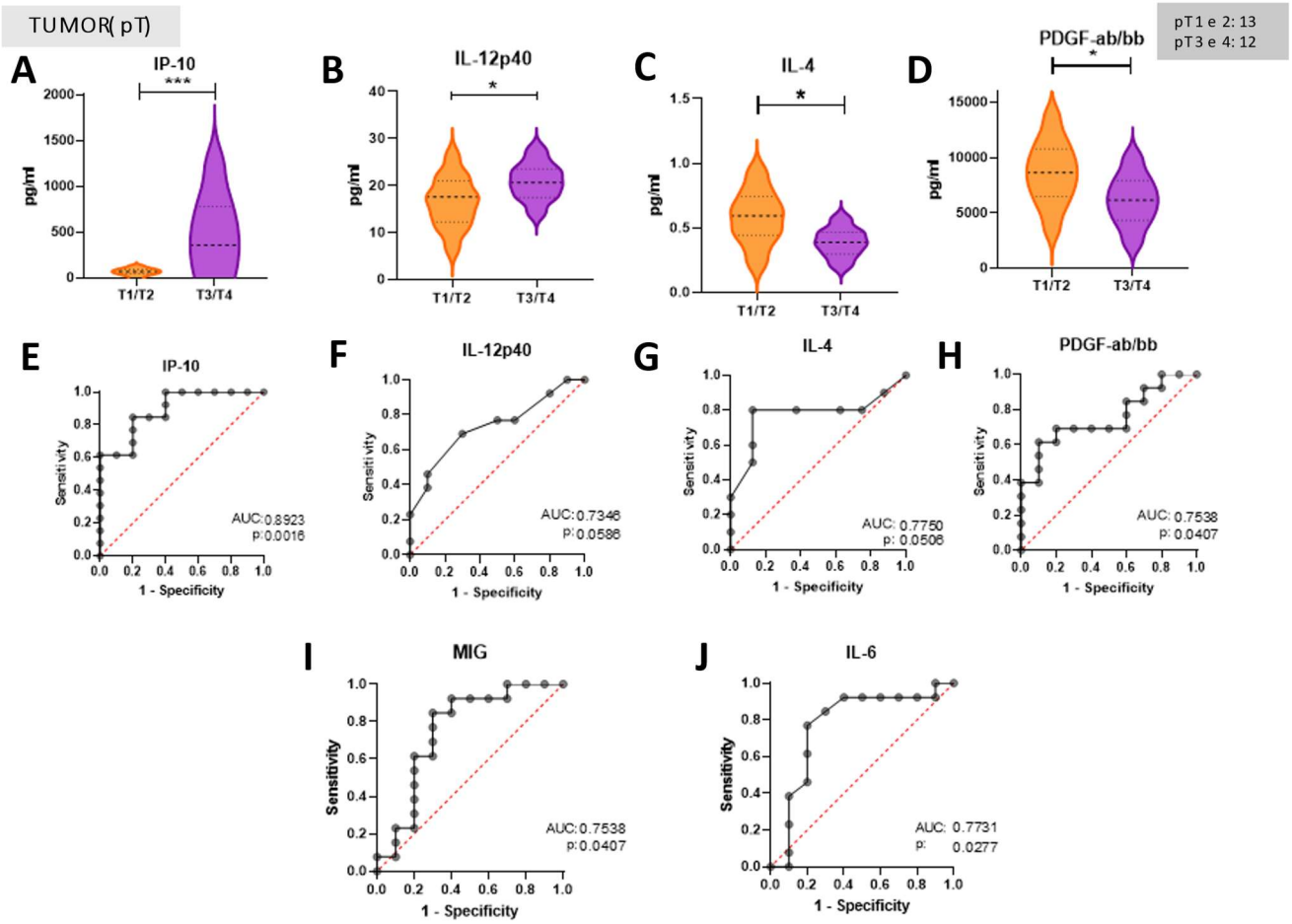


3.3 Evaluation of the frequency of soluble factor levels in patients with oral squamous cell carcinoma and their correlation with pathological tumor size (pT)

To analyze the soluble immune factors in the plasma of OSCC patients, we categorized our data into two groups: early tumors (pT1 and pT2) and advanced tumors (pT3 and pT4). We found that four cytokines IL-4, IL-12p40, IP-10, and PDGF-aa/bb exhibited statistically significant differences when examining the pT categories. Specifically, IP-10 levels were positively correlated with more advanced malignancies (Figure 2A-D). Additionally, G-CSF and IL-6 showed a statistical trend (Supplementary Figure 2).

Subsequently, we performed ROC curve analysis to evaluate the accuracy of these cytokines as biomarkers for pT. Cytokines with an area under the curve (AUC) value of at least 0.7 and a p-value less than 0.05 were considered to have strong predictive capacity. IL-6, IP-10, MIG, and PDGF-ab/bb demonstrated significant predictive potential in relation to pT (Figure 2E-J).

Figure 2. Analysis of the soluble immune profile in the plasma of patients with OSCC at different pT. Analysis of the soluble components present in the plasma of OSCC patients who had pT1 and 2 (n = 13) and pT3 and 4 (n = 10) showed statistical significance. ROC curves of soluble factors and immune populations associated with clinical outcome in OSCC patients. The AUC value was considered to be above 0.7, and the p-value considered to be statistically significant was p<0.05

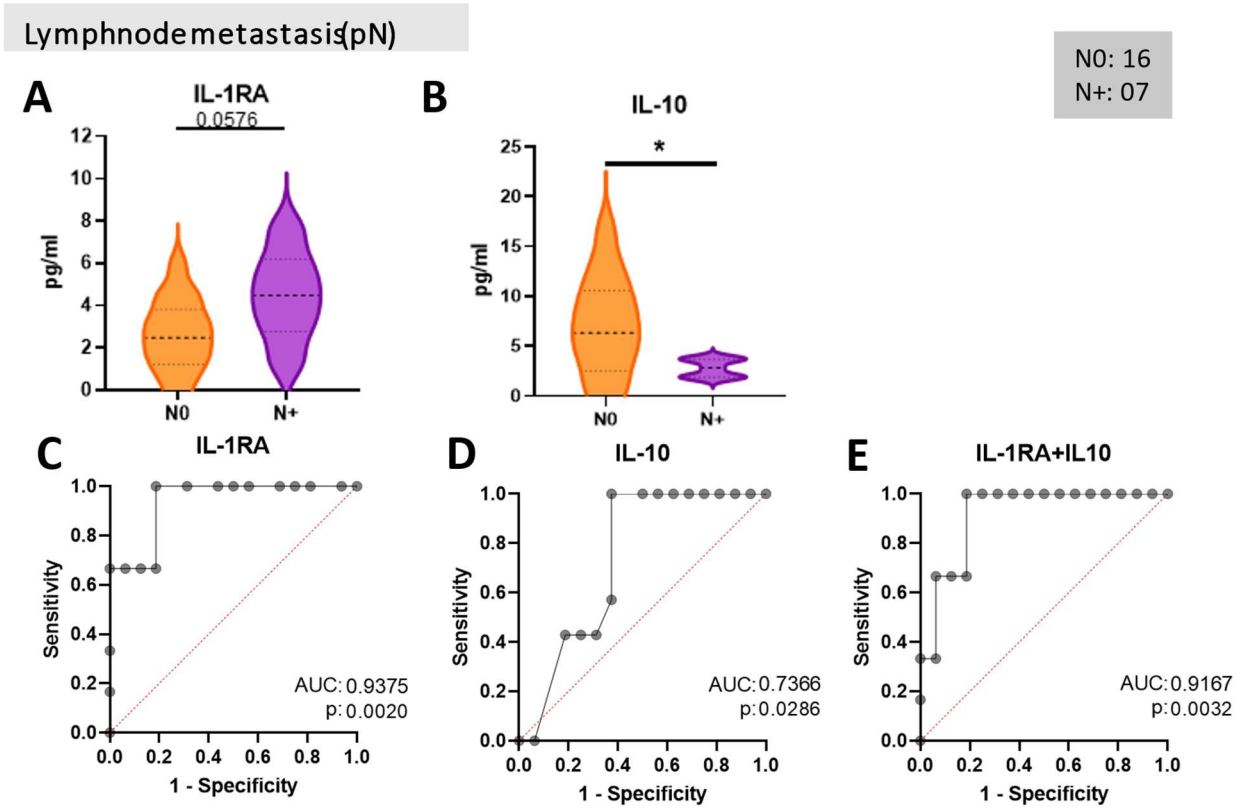


3.4 Evaluation of the soluble factor levels in patients with OSCC and their correlation with lymph node metastasis

We investigated the association of soluble factors with OSCC and identified two cytokines with statistical significance: IL-1RA and IL-10 (Figure 3 A-B). IL-10 was associated with a protective effect against lymph node metastasis, while IL-1RA was linked to an increased incidence of lymph node metastasis in OSCC patients.

To evaluate the accuracy of IL-1RA as a biomarker, we performed ROC curve analysis. An area under the curve (AUC) value of 0.7 or greater, with $p<0.05$, indicated that IL-1RA is a strong predictor, achieving an AUC of 0.9375 and $p=0.02$. Furthermore, in a multivariate analysis that included IL-1RA and IL-10, IL-1RA maintained its predictive power, demonstrating its robustness as a predictor of lymph node metastasis in OSCC patients (Figure 3 C-E).

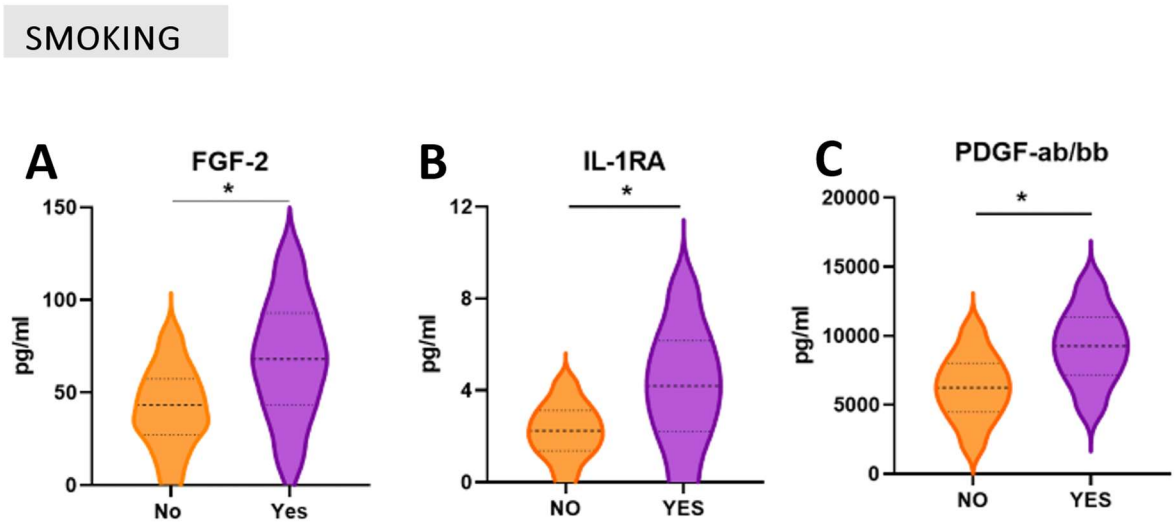
Figure 3. Analysis of the soluble immune profile in the plasma of patients with OSCC in relation to lymph node metastasis. Analysis of the soluble components present in the plasma of OSCC patients who has N0 (16) and N+ (n = 7) showed statistical significance. ROC curves of soluble factors and immune populations associated with clinical outcome in OSCC patients. The AUC value was considered to be above 0.7, and $p<0.05$ was considered to be statistically significant.



3.5 Evaluation of soluble factor levels in patients with OSCC and their correlation with smoking

Smoking is a risk factor for OSCC. However, in our cohort, we noticed a low incidence of smokers. However, our cohort exhibited a low incidence of smokers. To investigate whether smoking influenced the inflammatory response, we assessed the frequency of soluble factors in these patients. We observed an increase in the levels of factors such as FGF-2, PDGF-ab/bb, and IL-1RA in smokers (Figure 4 A-C). Notably, the violin plot for IL-1RA indicated significantly higher plasma levels in smokers compared to non-smokers. Interleukin-1 receptor antagonist (IL-1RA) is a key modulator of the inflammatory response, and its elevated levels in smokers may represent an adaptive response to the heightened inflammation associated with smoking. Additionally, platelet-derived growth factor (PDGF), which is crucial for angiogenesis and modulating the inflammatory response, was also increased in smokers. This may be related to tissue repair and remodeling processes stimulated by smoking (Figure 4 A-C).

Figure 4 - Analysis of the soluble immune profile in the plasma of patients with OSCC in relation to smoking. Analysis of the soluble components present in the plasma of OSCC patients who were smokers (n=10) and non-smokers (n=13) that showed statistical significance.

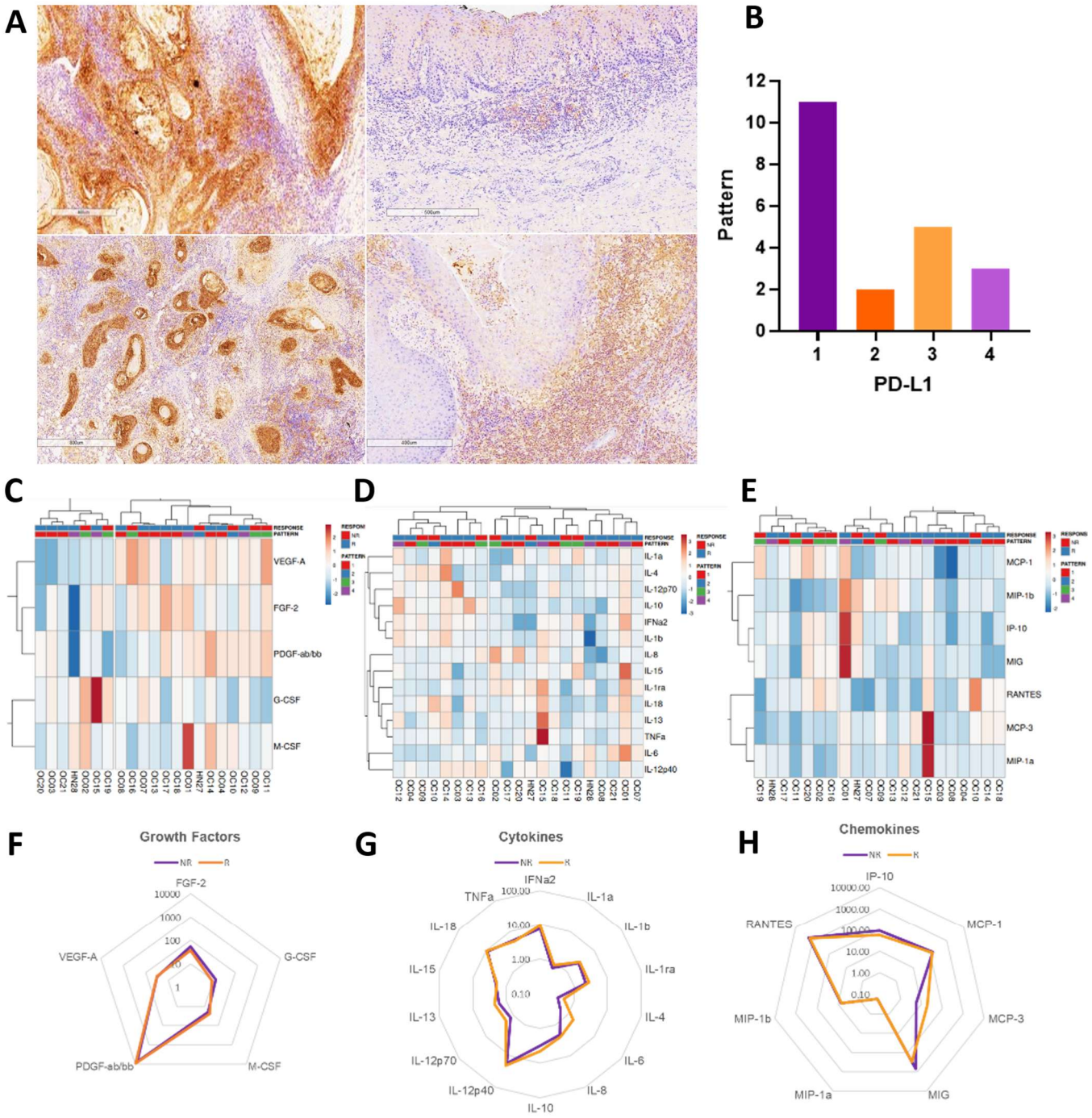


3.6 PD-L1 immunoexpression in patients with oral SCC and its relationship with prognosis and immune profile

Building on our previous research (Miranda-Galvis *et al*, 2020), we identified four distinct PD-L1 expression patterns using a ratio between tumor cells (CT) and immune cells (IC) to categorize the tumors. The most common pattern (52.4%) was PD-L1 expression in both CT and IC (Pattern I), followed by positive PD-L1 expression only in CT (Pattern III) (23.8%), and exclusive PD-L1 expression in IC (Pattern IV) (14.3%). The least common pattern was the absence of PD-L1 expression in both CT and IC (Pattern II) (9.5%) (Figure 5A-B).

We found that these patterns corresponded to two patient groups: one with an active immune profile responsive to immunotherapy, showing PD-L1 expression in IC (responder group), and another with a non-responsive immune profile, lacking PD-L1 expression in IC (non-responder group). Using this data, we established correlations between soluble variables in our patient population. Biomarkers analyzed included VEGF-A, MCP-4, IL-12p70, IL-1β, M-CSF, IFNa2, and others. Heat map and scatter plot analyses revealed distinct expression patterns among these biomarkers. Additionally, radar graphs illustrated the relative differences in expression levels, highlighting differential expression of cytokines, particularly IL-6/IL-4, and chemokines, such as MCP-3, in the responder and non-responder groups.

Figure 5: Patterns of PD-L1 immunoexpression in OSCC based on interactions between tumor cells and the immune microenvironment. Heatmap and radar chart of soluble factors in patients segregated by PD-L1 immunoexpression. Heatmap from unsupervised clustering using all soluble factors that showed statistical significance or trend ($p > 0.01$).



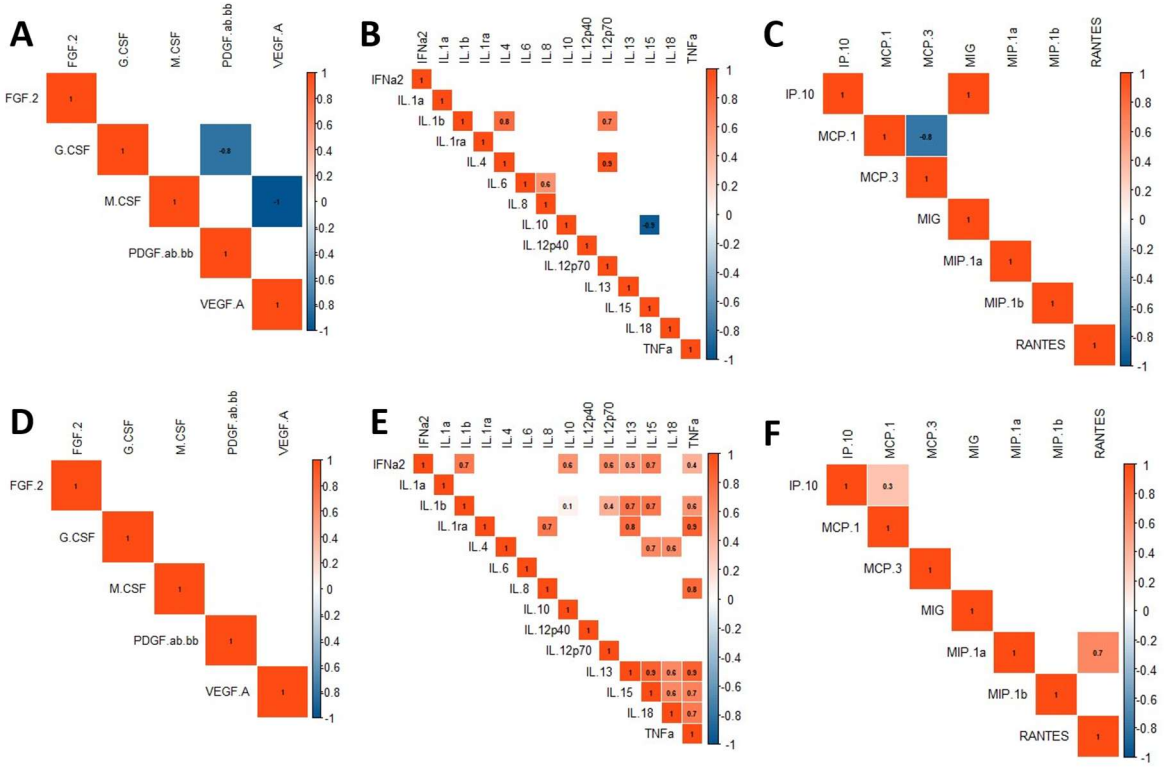
3.7 Analysis of the correlation between the soluble immune profile and the PD-L1 immunoexpression response in OSCC patients

Given that the PD-L1 expression profile reveals differences in the distribution, frequency, and immunological profile of immune populations, a correlation analysis

was conducted among all populations exhibiting statistically significant differences ($p < 0.05$). The data were categorized by cytokines, chemokines, and growth factors and further divided into responder (Figure 6 A-C) and non-responder (Figure 6 D-F) groups. Correlations among these variables (both positive and negative) were analyzed. A Spearman's correlation matrix was generated using the R platform with the corrplot tool.

In the responder group, certain growth factors, such as G-CSF with PDGFab.bb and M.GSF with VEGF.A, exhibited negative correlations (Figure 6A). In contrast, no significant correlations were found between growth factors in the non-responder group (Figure 6C). When analyzing cytokine interactions, the non-responder group showed more frequent positive correlations. This pattern may reflect immune profile exhaustion, characterized by increased co-expression of cytokines. For chemokines, MCP-3 demonstrated a strong negative correlation with MCP-1, while MIG was strongly positively correlated with IP-10 in the responder group. In the non-responder group, a strong positive correlation was observed between RANTES and MIP-1A.

Figure 6: Correlation matrix between soluble immune profile and patient response to PD-L1 labeling in OSCC patients. Spearman correlation matrix between populations with $p > 0.05$ in the blood of OSCC patients. The test was carried out on the R platform using the Corrplot package. The p-value considered for statistical significance was $p<0.05$. Positive correlations are indicated in red, negative correlations in blue, and correlations without statistical significance in white.



Discussion

The tumor microenvironment (TME) is highly complex, encompassing a multitude of cytological, proteomic, and immunological interactions, among others. These interactions can elicit both anti-tumor and pro-tumor inflammatory effects, significantly impacting disease progression. Understanding the immune microenvironment in specific tumor types can provide valuable insights into predicting a patient's immune response, which is crucial for the development of individualized treatment strategies. In this context, our study characterizes the soluble immune profile of patients with oral squamous cell carcinoma (OSCC) and explores its association with PD-L1.

In this study, we characterized soluble factors in the plasma of patients with OSCC, focusing on growth factors, cytokines, and chemokines, and correlated these with clinicopathological characteristics, including clinical staging, pathological tumor size (pT), and the presence of lymph node metastasis (pN). When stratifying these factors into those associated with lower versus higher risk, we observed distinct associations between certain cytokines and these characteristics. For instance, IL-4 and IL-10 were linked to lower-risk factors for tumor progression. In contrast, IL-1RA, IL-8, IL-12p40, IP10, and MIG were more frequently associated with higher-risk features indicative of OSCC progression.

Several studies have shown that in HNSCC, plasma levels of various cytokines are elevated compared to those in healthy donors. These cytokines include IFN-γ, IL-6, IL-2, IL-10, and TNF-α, collectively reflecting a profile characterized by both pro-inflammatory and anti-inflammatory responses. (Ruffin *et al*, 2023; Adil et al. 2019; Nisar et al., 2021). Understanding the intricate networks of cytokines and chemokines within the tumor microenvironment (TME) of head and neck squamous cell carcinoma (HNSCC) is essential. Nisar et al. (2021) highlighted the critical role these networks play in activating signaling pathways that promote tumor progression, metastasis, and resistance to therapy. Additionally, Kondoh and Mizuno-Kamiya (2022) underscored that immune-modulatory cytokines within the TME regulate the malignant phenotypes of HNSCC, with cytokines such as IFN-γ demonstrating both anti-tumoral and pro-tumoral activities. Yao et al. (2020) further emphasized the prognostic value of immune-related genomic biomarkers in HNSCC, underscoring the significance of the immune response within the TME in tumorigenesis and clinical outcomes.

We evaluated growth factors, cytokines, and chemokines in our cohort of OSCC patients and correlated them with a range of pathological and clinical features. Notably, the presence of IL-1RA was able to stratify patients effectively. The interleukin-1 (IL-1) family includes both pro-inflammatory and anti-inflammatory proteins, with the interleukin-1 receptor antagonist (IL-1RA) acting as a natural anti-inflammatory antagonist to the pro-inflammatory cytokines within this family. IL-1RA plays critical roles in various pathological conditions, including cancer. For instance, reduced levels of IL-1RA have been reported in several cancer types, such as leukemia, colorectal cancer, and prostate cancer, and have been negatively associated with the development of premalignant oral dysplasia (Mantovani et al., 2017). Yuan et al. (2023) investigated the potential of IL-1RA as a clinical marker for OSCC progression and patient outcomes. Furthermore, the cellular mechanisms of IL-1RA in OSCC malignancy have been explored both in vitro and in vivo, shedding light on the development of novel therapeutic strategies that target vulnerabilities in the IL-1RA-associated mitochondrial metabolic pathway (Yuan *et al*, 2023).

In patients with HNSCC, elevated levels of Th2 cytokines such as IL-4, IL-6, and IL-10 contribute to an immunosuppressive state, posing a significant challenge for targeted immunotherapy in oncogenic treatments. Studies have shown that patients with HNSCC exhibit significantly higher plasma levels of IL-4, IL-6, and IL-10 compared to controls (Fialová et al., 2020; Elmusrati et al., 2021). Additionally, serum levels of IL-4, along with other components, have been reported to be markedly elevated in HNSCC patients (Kondoh & Mizuno-Kamiya, 2022). The key cytokines identified within the HNSCC microenvironment include IL-4, IL-6, IL-8, and IL-10 (Bruchhage et al., 2018). In our cohort, we observed that patients with elevated levels of IL-4 and IL-8 were associated with more favorable clinicopathological characteristics.

IP-10, also known as CXCL10, is a chemokine crucial for immune responses, particularly in the presence of interferon-gamma (IFN-γ). This potent chemoattractant is produced by various cell types in response to IFN-γ and plays a key role in recruiting cytolytic lymphocytes to tumor sites (Georganaki et al., 2018). Research has shown that IP-10 is specifically expressed in lymphocyte-infiltrating primary and metastatic melanoma tumors, highlighting its distinct role within the tumor microenvironment (Georganaki et al., 2018; Wu et al., 2016). The IP-10 score has proven to be an effective independent predictive biomarker, accurately forecasting outcomes in patients with HNSCC, including overall survival, progression-free survival, disease

recurrence, and metastatic events (Berszin et al., 2022). In our cohort, we observed significantly higher IP-10 levels in patients with more advanced tumors.

Following the characterization of the soluble immune profile based on clinicopathological characteristics, patients were divided into two subgroups according to our previous work. The first subgroup, consisting of potential responders, exhibited an active immune profile, as indicated by PD-L1 expression in immunohistochemistry (IHC). The second subgroup, comprising non-responders, exhibited a non-responsive immune profile, characterized by the absence of PD-L1 expression in IHC. The patient cohort was initially analyzed to segregate growth factors, cytokines, and chemokines. Radar graph analysis and heat map clustering revealed distinct differences in cytokine profiles, with the most significant divergence observed in IL-6/IL-4 ratios and MCP-3 chemokine concentrations between the good and poor responder groups.

IL-4 and IL-6 are cytokines that play significant roles in cancer biology, including inflammation, tumor progression, and modulation of the immune response. In OSCC, elevated IL-6 expression levels have been linked to disease severity, with high IL-6 levels being associated with a greater prevalence of lymph node or distant metastases (Cho et al., 2018; Miyake et al., 2017). Additionally, there is growing evidence suggesting that IL-4 and IL-6 are involved in inflammation-mediated carcinogenesis (Gundamaraju et al., 2018). Studies have shown that patients with head and neck squamous cell carcinoma (HNSCC) undergoing chemoradiotherapy exhibit higher salivary levels of inflammatory cytokines, including IL-6 (Ala et al., 2022; Jing et al., 2020). IL-4, on the other hand, is recognized for its role in inducing T-cell differentiation and its presence in the tumor microenvironment of various cancers (Terrematte et al., 2022). Furthermore, IL-4 has been associated with enhancing the inflammatory competence of macrophages, which can, in turn, limit the invasiveness of cancer cells (Salmiheimo et al., 2016). Understanding the relationship between immune system-related factors, such as cytokines like IL-4 and IL-6, and the response to immunotherapy can provide valuable insights into predicting treatment efficacy and patient outcomes across various cancer types.

Our findings indicate that assessing the expression patterns of cytokines and chemokines is crucial for identifying individuals who may benefit from immunotherapy. The significant differences observed in cytokine and chemokine levels between responder and non-responder groups emphasize the need to consider the patient's immune response when developing personalized therapeutic strategies. Identifying

immunological markers, such as the cytokines and chemokines analyzed, aids in predicting treatment responses and stratifying patients, which allows for a more targeted and effective approach. Understanding these immune profiles can help optimize therapy and improve clinical outcomes for patients with varying immune response profiles. Future studies, particularly those with larger cohorts and those correlating with clinical trial data, will further underscore the importance of personalizing treatment for patients with OSCC.

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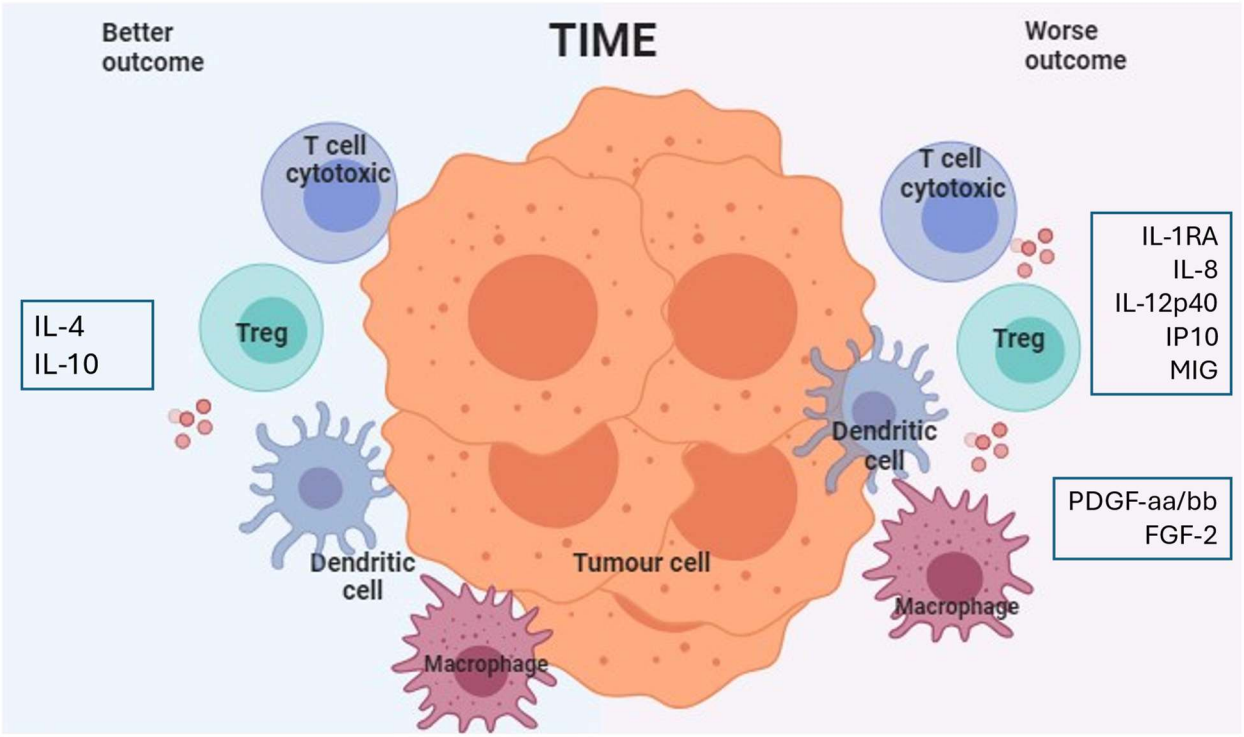
Graphical abstract: Tumor immune microenvironment in a patient with OSCC. IL-4 and IL-10 promote an anti-inflammatory environment associated with better outcomes, while IL-1RA, IL-8, IL-12p40, IP-10, and MIG are linked to worse prognoses. Additionally, growth factors such as PDGF-aa/bb and FGF-2 favor angiogenesis and tumor progression.


Better outcome
TIME
Worse outcome
T cell cytotoxic
T cell cytotoxic
IL-1RA
IL-8
IL-12p40
IP10
MIG
IL-4
IL-10
Treg
Treg
Dendritic cell
Dendritic cell
PDGF-aa/bb
FGF-2
Tumour cell
Macrophage
Macrophage

3. Discussão

O microambiente imune tumoral é extremamente complexo, com inúmeras interações citológicas, proteicas, imunológicas, entre outras. Os efeitos inflamatórios anti e/ou pró-tumorais causados por estas interações podem ter repercussões na progressão da doença. Apesar do reconhecimento da via de sinalização PD-L1 como um importante mecanismo de escape imunológico [32]. O reconhecimento do microambiente imunitário em certos tipos de tumores pode prever a resposta imunitária do doente. Isto é essencial quando pensamos nas perspectivas de individualização do tratamento. Com base nisto, o nosso estudo caracterizou o perfil imune solúvel dos doentes com CEC oral e associa-o à imunoexpressão de PD-L1.

Estudos têm demonstrado que, no CEC de cabeça e pescoço (CECCP) há uma elevação nos níveis plasmáticos de várias citocinas quando comparados aos de doadores saudáveis. Estas citocinas incluem IFN-γ, IL-6, IL-2, IL-10 e TNF-α e, coletivamente, indicam um perfil de citocinas pró-inflamatórias e anti-inflamatórias. (Ruffin et al, 2023; Adil et al. 2019; Nisar et al., 2021). Nisar et al. (2021) destacaram o papel dessas redes na ativação de vias de sinalização que impulsionam a progressão do tumor, metástase e resistência à terapia (Nisar et al., 2021). Além disso, Kondoh e Mizuno-Kamiya (2022) enfatizaram que as citocinas imunomoduladoras dentro do TME regulam os fenótipos malignos dos HNSCCs, com citocinas como IFN-γ exibindo atividades antitumorais e pró-tumorais (Kondoh & Mizuno-Kamiya, 2022).

Nesse contexto, em nosso estudo caracterizamos fatores solúveis através do plasma de pacientes com CECO, avaliando fatores de crescimento, citocinas e quimiocinas e correlacionamos com as características clínico-patológicas. Entre essas características destacamos, estadiamento clínico, tamanho tumor patológico (pT), presença de metástase linfonodal (N). Quando dividimos esses fatores em com menor risco e maior risco, notamos que algumas citocinas estavam mais associadas com cada característica. Como, IL-4 e IL10 segregaram a menores fatores de risco para progressão tumoral. Enquanto, IL-1RA, IL-8, IL-12p40, IP10 e MIG foram mais associadas as características relacionadas a maior risco de progressão tumoral para CECO.

A IL4 e a IL10 são citocinas que têm sido implicadas em vários aspectos da biologia do câncer, incluindo inflamação, progressão tumoral e modulação da resposta imunológica. Além disso, há cada vez mais evidências que sugerem o envolvimento

da IL4 e na carcinogênese mediada por inflamação (Gundamaraju et al., 2018). A IL4 foi reconhecida por seu papel na indução da diferenciação de células T e por sua presença no ambiente tumoral de vários tipos de câncer (Terrematte et al., 2022). A IL4 também tem sido associada à promoção da competência inflamatória dos macrófagos, que, por sua vez, pode restringir a invasividade das células cancerígenas (Salmiheimo et al., 2016). Compreender a correlação entre fatores relacionados ao sistema imunológico, como citocinas como IL4, e a resposta à imunoterapia pode fornecer informações sobre a previsão da eficácia do tratamento e os resultados dos pacientes em vários tipos de câncer.

IL-10, uma interleucina conhecida por suas propriedades anti-inflamatórias, tem sido implicada em vários aspectos do câncer, incluindo câncer oral. Estudos têm mostrado que IL-10 desempenha um papel no microambiente tumoral ao afetar a função das células imunes. As células T reg desempenham funções imunossupressoras em tumores por meio da secreção de citocinas inibitórias, como IL-10, IL-35 e TGFβ, regulação positiva de receptores inibitórios, interrupção do metabolismo via CD39 e CD73 e privação do TME local de IL-2 por meio da alta expressão de CD25. As células T_{reg} estão presentes no sangue periférico e em tumores de pacientes com HNSCC (Ruffin et al., 2023). Além disso, os níveis de IL10 foram investigados em relação à leucoplasia oral, onde IL10 foi encontrado elevado em pacientes com essa condição (Melguizo-Rodríguez et al., 2020). Além disso, os genótipos de IL10 foram associados à suscetibilidade ao carcinoma espinocelular oral em certas populações (Goud et al., 2019). No contexto de macrófagos associados a tumores, um aumento de IL10 e uma redução de TNFα foram observados em modelos de câncer oral, contribuindo para um microambiente imunossupressor (Malfitano et al., 2020). Em resumo, a IL10 desempenha um papel multifacetado no câncer oral, influenciando a função das células imunológicas, o microambiente tumoral e potencialmente impactando a suscetibilidade à doença. Entender a interação da IL10 na patogênese do câncer oral pode oferecer insights sobre novas estratégias terapêuticas visando a resposta imune.

A família da interleucina-1 (IL-1) inclui proteínas pró e anti-inflamatórias. O antagonista do receptor de interleucina-1 (IL-1Ra) é um antagonista anti-inflamatório natural da família de citocinas pró-inflamatórias da interleucina-1. A IL-1RA desempenha várias funções em diversas condições patológicas, inclusive no câncer. Por exemplo, níveis mais baixos de IL-1RA foram encontrados em vários tipos de

câncer, como leucemia, câncer colorretal e câncer de próstata, e foram negativamente associados ao desenvolvimento de desordens orais potencialmente malignas. (Mantovani et al., 2017). Yuan et al. 2023, avaliaram o potencial da IL-1RA como um marcador clínico para a progressão do CECO e os resultados dos pacientes demostraram que os mecanismos celulares da IL-1RA na malignidade do CECO in vitro e in vivo, fornecendo informações sobre o desenvolvimento de novas estratégias terapêuticas para o CECO que visam vulnerabilidades na via metabólica mitocondrial associada à IL-1RA (Yuan et al., 2023). Entre as citocinas que em nossa coorte, notamos que o aumento na expressão de IL-1RA esteve mais prevalente nos piores fatores de prognósticos, mesmo em parâmetro multivariados, sendo capaz de segregar os fatores.

IP-10, frequentemente chamada de CXCL10, é uma quimiocina essencial para as respostas imunológicas, especialmente quando o interferon-gama (IFN-γ) está ativo. Produzida por vários tipos de células em reação ao IFN-γ, essa poderosa proteína quimioatrativa atrai linfócitos citolíticos para lesões tumorais (Georganaki et al., 2018). Estudos mostram que a IP-10 tem uma função específica no microambiente tumoral, sendo expressa especificamente em tumores de melanoma primários e metastáticos (Georganaki et al., 2018; Wu et al., 2016). A expressão de IP-10 demonstrou sua eficácia como biomarcador preditivo independente ao prever corretamente os resultados em pacientes com HNSCC, inclusive sobrevida global, sobrevida livre de progressão, recorrência da doença e ocorrências metastáticas. (Berszin et al., 2022). Em nossa coorte, os níveis de IP-10 foram muito mais altos em pacientes com tumores mais avançados, mostrando que em CECO essa quimiocinas também pode ser um bom biomarcador preditivo.

Após a caracterização do perfil imunológico solúvel com base nas características clinicopatológicas, os pacientes foram divididos em dois subgrupos com base em nosso trabalho anterior (artigo 1). No nosso estudo, avaliando a imunoexpressão de PD-L1 para CECO, o nosso objetivo foi avaliar o significado clinicopatológico e prognóstico do PD-L1 no CCEO, utilizando diferentes métodos de pontuação através de critérios padronizados. Em primeiro lugar, avaliámos a expressão de PD-L1 nas células tumorais (TC) em duas coortes independentes, utilizando quatro pontos de corte distintos, e mostrámos que a expressão de PD-L1 não está relacionada com as características clinicopatológicas e as taxas de sobrevivência. Uma vez que uma abordagem recente avalia a expressão de PD-L1

por CPS com uma melhor resposta à imunoterapia no CECO, utilizámos também uma segunda técnica para avaliar a expressão de PD-L1. Os nossos resultados revelaram que a expressão de PD-L1 nas populações de CT e CI não influenciou a sobrevivência no CECO. Estes resultados são apoiados pela utilização de dois clones, 28-8 e 73-10, ambos validados como biomarcadores de diagnóstico da expressão de PD-L1 para selecionar pacientes candidatos a terapias com nivolumab e avelumab, respetivamente.

Apesar da multiplicidade de técnicas de imunocoloração de PD-L1, é importante considerar um consenso recente de que a positividade de PD-L1 >1% por CPS deve ser utilizada para selecionar doentes para imunoterapia, uma vez que foram obtidos resultados superiores em doentes com CPS mais elevada, enquanto a TPS não foi preditiva. De facto, a imunoterapia beneficiou os doentes com CECO independentemente da expressão de PD-L1; no entanto, com o seguimento a longo prazo, a expressão de PD-L1 (>1%) foi associada a uma melhoria adicional da sobrevida global [17]. Em contraste com a vasta literatura que investiga o valor prognóstico do PD-L1, os dados são limitados no que respeita aos padrões de expressão do PD-L1 e aos seus mecanismos biológicos subjacentes. Observámos que CECO expressa PD-L1 em dois padrões, irregular e difuso, e encontrámos uma distribuição quase igual de tumores PD-L1-positivos entre os dois grupos de padrões. Um estudo anterior descreveu padrões de coloração intratumoral de PD-L1 periféricos e difusos ao longo dos ninhos tumorais, e relatou que a maioria dos casos apresentava o padrão periférico [14]. Notavelmente, o padrão irregular descrito no nosso relatório difere do padrão periférico, porque o padrão irregular apresenta células positivas tanto no centro como na periferia dos ninhos tumorais. Uma das nossas descobertas mais relevantes relaciona os resultados clínicos com os mecanismos de regulação positiva de PD-L1. Os ganhos no número de cópias do cromossoma 9 conduzem à expressão constitutiva de PD-L1 por CT [39], resultando num padrão homogéneo e difuso. Em contrapartida, a secreção de interferão-γ por células T ativadas induz a expressão de PD-L1 por CT num padrão heterogéneo e irregular [40].

Demonstramos que a OS é significativamente mais curta em doentes com tumores que expressam PDL1 num padrão irregular. Os nossos dados apoiam a hipótese de que a elevada heterogeneidade intratumoral na expressão de PD-L1 promove a resistência imunitária adaptativa [22]. As interacções dinâmicas entre o CT

e as células não malignas no TME determinam a expressão de PDL1 [3]. Em busca de um modelo preditivo mais preciso do resultado clínico, explorámos a expressão de PD-L1 com base nas interacções entre CT e CI no CECO [25-27]. Como a secreção local de células T de interferon-γ regula positivamente a expressão de PD-L1 tanto por CT quanto por IC [4, 41], estratificamos os tumores em quatro grupos de acordo com a expressão de PD-L1 em CT e IC. O padrão mais prevalente, responsável por quase metade dos casos, foi a expressão de PD-L1 tanto por CT como por CI (Padrão I). Estes resultados apoiam o conceito de CCEO como uma doença imunossupressora em que as células neoplásicas produzem citocinas que suprimem a imunidade antitumoral mediada por células [42]. Os padrões menos frequentemente observados foram a expressão de PD-L1 exclusivamente em CI (Padrão IV), tumores PD-L1 duplamente negativos (Padrão II) e a expressão de PD-L1 apenas em CT (Padrão (Padrão III). Enquanto os padrões II e IV estão associados à ignorância ou tolerância imunitária, respetivamente, a expressão constitutiva de PD-L1 por CT é impulsionada por vias de sinalização oncogénicas mal caracterizadas [27].

Posteriormente, o estudo sobre a caracterização do padrão de expressão de PD-L1. Na busca que responder questionamentos, principalmente como em relação ao microambiente tumoral e a expressão de PD-L1. Avaliamos o perfil imune solúvel e identificamos os padrões de PD-L1. O primeiro subgrupo, por um perfil imunológico ativo, conforme indicado pela marcação PD-L1 nas células imunes (CI). O segundo subgrupo, por um perfil imunológico não responsivo, conforme indicado pela ausência de marcação de PD-L1 no CI. A coorte de pacientes foi analisada inicialmente para segregar fatores de crescimento, citocinas e quimiocinas. A análise de gráficos de radar e o agrupamento de mapas de calor revelaram diferenças nos perfis de citocinas, com a maior divergência observada nas concentrações de quimiocinas IL-6/IL-4 e MCP-3 entre os grupos com boa e má resposta.

A IL-4 e a IL-6 são citocinas que têm sido implicadas em vários aspectos da biologia do câncer, incluindo inflamação, progressão tumoral e modulação da resposta imunológica. No CECO, os níveis de expressão da IL6 foram associados à gravidade da doença. A alta expressão de IL6 no CCEO foi associada a uma maior prevalência de linfonodos ou metástases distantes (Cho et al., 2018; Miyake et al., 2017). Além disso, há cada vez mais evidências que sugerem o envolvimento da IL4 e da IL6 na carcinogênese mediada por inflamação (Gundamaraju et al., 2018). Estudos

demonstraram que pacientes com CECP submetidos à quimiorradioterapia apresentam níveis salivares mais altos de citocinas inflamatórias, como a IL6 (Ala et al., 2022; Jing et al., 2020). Por outro lado, a IL4 foi reconhecida por seu papel na indução da diferenciação de células T e por sua presença no ambiente tumoral de vários tipos de câncer (Terrematte et al., 2022). A IL4 também tem sido associada à promoção da competência inflamatória dos macrófagos, que, por sua vez, pode restringir a invasividade das células cancerígenas (Salmiheimo et al., 2016). Compreender a correlação entre fatores relacionados ao sistema imunológico, como citocinas como IL4 e IL6, e a resposta à imunoterapia pode fornecer informações sobre a previsão da eficácia do tratamento e os resultados dos pacientes em vários tipos de câncer.

Os nossos novos resultados indicam que os padrões de expressão de PD-L1 no contexto das interacções TC-IC representam melhor as respostas imunitárias antitumorais do que os TPS ou CPS. A estratificação sugerida pode ter implicações importantes para a caraterização do CECE; para a utilização de padrões de expressão de PD-L1 como preditores de resultados; e para a conceção de novas estratégias para melhorar a eficácia das terapias estabelecidas.

Em resumo, nossas descobertas, a avaliação dos padrões de expressão de citocinas e quimiocinas pode ser essencial para identificar indivíduos que podem se beneficiar da predição e possibilidade de um tratamento direcionado. A presença de diferenças significativas nos níveis de citocinas e quimiocinas entre os grupos que PD-L1 positivos em CI e os PD-L1 negativos em CI destacam a importância de considerar a resposta imunológica do paciente ao planejar estratégias terapêuticas personalizadas. A identificação de marcadores imunológicos, como as citocinas e quimiocinas analisadas, pode ajudar a prever a resposta ao tratamento e estratificar os pacientes, permitindo uma abordagem personalizada. A compreensão desses perfis imunológicos ajuda a otimizar a terapia e melhorar os resultados clínicos em pacientes com diferentes perfis de resposta imunológica. Estudos voltados para a investigação desses dados, especialmente com pontos de corte maiores e correlacionados com pacientes em ensaios clínicos, colaborarão e reforçarão a importância da personalização do tratamento de pacientes com CECO.

4. CONCLUSÃO

- **Importância dos Padrões de Expressão de PD-L1**: Os padrões de expressão de PD-L1, especialmente considerando sua localização nos ninhos tumorais e as interações entre células tumorais (CT) e células imunes (CI), proporcionam uma representação mais precisa das respostas imunes antitumorais do que as metodologias tradicionais TPS e CPS.
- **Análise do Perfil Imune Solúvel**: A análise detalhada do perfil imune solúvel foi fundamental para a compreensão da patologia do carcinoma espinocelular oral. Níveis elevados de citocinas e quimiocinas, como IL-1RA, IL-8, IL-12p40, IP-10, MIG, PFGD-aa/bb e FGF-2, foram associados a características clínicas adversas, enquanto níveis mais altos de IL-4 e IL-10 correlacionaram-se com melhores características clínicas. Essas descobertas sublinham a importância do perfil imune solúvel na predição da progressão da doença e na identificação de pacientes com pior prognóstico.
- **PD-L1 como Biomarcador Prognóstico**: A utilização dos padrões de expressão de PD-L1, aliada à análise do perfil imune solúvel, tem o potencial de se tornar um biomarcador prognóstico importante. Esta abordagem integrada permite prever os desfechos clínicos de forma mais eficaz e orienta decisões terapêuticas personalizadas.
- **Estratificação e Personalização Terapêutica**: A estratificação sugerida, que considera tanto a expressão de PD-L1 quanto o perfil imune solúvel, pode influenciar diretamente o desenvolvimento de novas estratégias terapêuticas. Essa personalização no tratamento do CECO tem o potencial de melhorar a eficácia das terapias existentes e promover abordagens mais direcionadas e eficazes para cada paciente.

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Anexo 1

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ORIGINAL ARTICLE

ORAL DISEASES WILEY

PD-L1 expression patterns in oral cancer as an integrated approach for further prognostic classification

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Abstract

Background: Despite the well-known role of programmed cell death ligand 1 (PD-L1) in promoting immune resistance in oral squamous cell carcinoma (OSCC), its potential utility as a prognostic biomarker is undetermined. We evaluated PD-L1 expression as predictor of survival in patients with OSCC and explored PD-L1 expression patterns.

Methods: We conducted a retrospective cohort study that assessed PD-L1 expression through immunohistochemistry in 123 surgical specimens of OSCC. A first approach evaluated tumor proportion scores (TPS) and combined proportion scores (CPS). Next, expression patterns were examined by evaluating PD-L1 localization in tumor nests, as well as the interfaces of tumor cells (TC) and immune cells (IC) in the tumor microenvironment.

Results: High-level PD-L1 expression determined by TPS and CPS using variable cutoffs was not associated with survival. Immunohistochemistry revealed that TC expressed PD-L1 in either patchy or diffuse patterns. The patchy pattern was an independent risk factor for overall survival. Furthermore, expression patterns in the tumor immune microenvironment showed that most cases expressed PD-L1 on both TC and IC, while PD-L1 non-expressors had the lowest overall survival.

Conclusion: PD-L1 expression patterns in the context of localization in tumor nests and TC–IC interactions represent antitumor immune responses better than either TPS or CPS. Our suggested classification system may have important implications for the characterization of OSCC and for the use of PD-L1 as a prognostic biomarker.

KEYWORDS
expression pattern, immune microenvironment, oral cancer, programmed cell death ligand 1, score

1 | INTRODUCTION

Immune evasion is a driving force that enables neoplastic cells to survive, proliferate, and disseminate and is emerging as a recognized hallmark of cancer (Hanahan & Weinberg, 2011). A major immune resistance strategy of tumor cells (TC) exploits the expression of programmed cell death ligands 1 and 2 (PD-L1 and PD-L2) to target the programmed cell death 1 receptor (PD-1) in activated T cells (Blank

Anexo 2

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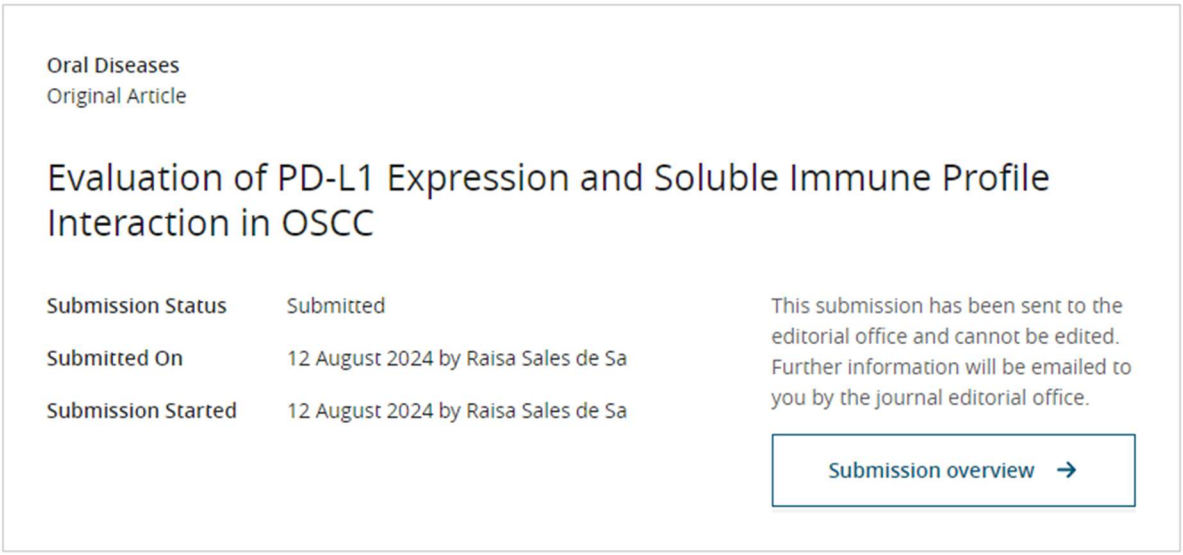
ANEXO 3



Oral Diseases
Original Article

Evaluation of PD-L1 Expression and Soluble Immune Profile Interaction in OSCC

Submission Status	Submitted
Submitted On	12 August 2024 by Raisa Sales de Sa
Submission Started	12 August 2024 by Raisa Sales de Sa

This submission has been sent to the editorial office and cannot be edited. Further information will be emailed to you by the journal editorial office.

Submission overview →

Anexo 4

FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNIVERSIDADE DE CAMPINAS - FOP/UNICAMP

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Personalização de tratamento com imunoterapia em pacientes com carcinoma espinocelular oral: Um estudo 3D in vitro com "microfluidic chip"

Pesquisador: Luiz Paulo Kowalski

Área Temática:

Versão: 2

CAAE: 44573821.0.3004.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 6.089.298

Apresentação do Projeto:

O parecer inicial é elaborado com base na transcrição editada do conteúdo do registro do protocolo na Plataforma Brasil e dos arquivos anexados à Plataforma Brasil. Os pareceres de retorno, emendas e notificações são elaborados a partir do último parecer e dos dados e arquivos da última versão apresentada. O arquivo do projeto de pesquisa adotado na elaboração do parecer foi o arquivo "Projeto_raisa_doutorado.pdf" de 25/07/2022. O arquivo do registro do protocolo na PB adotado foi o "PB_INFORMAÇÕES_BÁSICAS_1989003_E3.pdf" de 27/02/2023.

Trata-se de PROTOCOLO em Coparticipação, originalmente aprovado pelo CEP do Centro Proponente (FUNDAÇÃO ANTÔNIO PRUDENTE - A.C. CAMARGO CANCER CENTER), na versão em tramitação (E3) em 30/03/2023 para avaliação junto ao CEP-FOP-UNICAMP.

A EQUIPE DE PESQUISA citada na capa do projeto de pesquisa inclui LUIZ PAULO KOWALSKI (Médico, Docente do PPG em Estomatopatologia da FOP-UNICAMP, Docente da FMUSP-SP, Pesquisador responsável, Orientador), RAÍSA SALES DE SÁ (Cirurgiã-dentista, Doutoranda no PPG em Estomatopatologia da FOP-UNICAMP, Orientanda), KENNETH JOHN GOLLOB (Graduação em Molecular Cellular and Developmental Biology, Pesquisador do Instituto Israelita de Ensino e

Anexo 5

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ORIGINALITY REPORT

9%	4%	9%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Marisol Miranda-Galvis, Alicia Rumayor Piña, Raísa Sales de Sá, Amanda Almeida Leite et al. "PD-L1 Expression Patterns in Oral Cancer as an Integrated Approach for Further Prognostic Classification", Oral Diseases, 2020 Publication	3%
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