Universidad Rey Juan Carlos

TESIS DOCTORAL

Expression of immunoregulatory molecules in oral squamous cell carcinoma microenvironment

Autor:

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Directores:

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Dr. José Luís Cebrián Carretero
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All the illustrations in this doctoral thesis are self-made.

No se puede vencer a alguien que nunca se rinde,
George Herman Ruth, Jr. "Babe Ruth"

A mí abuelo Cardelles,
en el cielo

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A mis amigos, los de siempre y los de ahora. Parte importante del camino hasta aquí.

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ABBREVIATIONS

AJCC	American Joint Committee on Cancer
APC	Antigen-presenting cell
CCL	C–C motif chemokine ligand
CN	Neoplastic cell
CPS	Combined positive score
CSF-1	Colony stimulating factor 1
CSF1R	Colony stimulating factor 1 receptor
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DOI	Depth of invasion
EGF	Epidermal grow factor
FDA	Food and Drug Administration
FOXP3	Forkhead Box P3
HNSCC	Head and neck squamous cell carcinoma
HPV	human papillomavirus
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IL	interleukin
INF-γ	Interferon gamma
IS	Immune system
LAG3	lymphocyte activation gene 3
LHR	lymphocytic host response
MHC	Major histocompatibility complex
NKs	Natural killers
NSCLC	Non-small cell lung carcinoma
OPMD	Oral potentially malignant disorders
OSCC	Oral squamous cell carcinoma
PCR	Polymerase Chain Reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PNI	Perineural invasion
TAM	Tumour-associated macrophages
TIM3	T-cell immunoglobulin and mucin protein-3
TNM	Tumour (T), nodes (N), and metastases (M)
TCR	T-cell receptor
TGF-β	Transforming growth factor-beta
Th	T- Helpers

TIL	Tumour-infiltrating lymphocytes
TME	Tumour microenvironment
TNF	Tumour necrosis factor
TPS	Tumour proportion score
Tregs	Regulatory T cells
UICC	Union for International Cancer Control
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
WPOI	Worst pattern of invasion

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responsible for stabilising the interaction of the TCR and, on the other hand, the immune tolerance receptor co-stimulatory PD-1 on the CD8 T lymphocyte also interact with its ligand on the surface of the tumour cell. When PD-1 is stimulated by PD-L1, the immune tolerance process is activated, leading to a ceasing of the immune response of the CD8 T-lymphocyte against the tumour cell.

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Antecedentes

El cáncer es una patología que representa una de las principales protagonistas en la muerte del ser humano. El carcinoma oral de células escamosas (COCE) se trata del carcinoma epidermoide más común de los cánceres de cabeza y cuello (Warnakulasuriya, 2009).

A pesar de que la cavidad oral es una pequeña región del organismo, existe dentro de ésta, diferentes áreas donde puede desarrollarse la enfermedad. El origen primario del COCE se halla, en la mayoría de los casos, en el epitelio de la región anterior de la lengua y en el suelo de la boca (Bagan y cols., 2010; Sundermann y cols., 2017).

El diagnóstico en estadios iniciales es complejo debido a la ausencia de signos y síntomas. Por ello, su diagnóstico no suele ser precoz y es detectado en la mitad de los casos en estadios más avanzados, hecho que no ha mejorado con el paso del tiempo (Awan, 2014; van der Wall y cols., 2011).

La morbildidad de su tratamiento es muy alta (Sacco & Cohen, 2015), así como su mortalidad, con una supervivencia de los pacientes con esta enfermedad a los cinco años de aproximadamente el 60% (Chi y cols., 2015; Rapidis y cols., 2009) y desafortunadamente, la media de supervivencia de los pacientes con recidiva de enfermedad o metástasis es de aproximadamente 8-10 meses (Zandberg & Strome, 2014).

Aunque existen estudios sobre el microambiente tumoral en el cáncer de cabeza y cuello (Peltanova et al., 2019), el microambiente en torno al COCE no ha sido investigado en profundidad (Mohan et al., 2019). Desde la aparición de la inmunoterapia, han tenido lugar diferentes ensayos clínicos con diana terapéutica en los puntos de control inmunológicos con resultados favorables (Cohen y cols., 2019; Ferris y cols., 2016; Mehra y cols., 2018; Segal y cols., 2019; Weiss y cols., 2017), por lo que el conocimiento del microambiente del COCE puede ser determinante para tratamientos futuros.

El COCE tiene la capacidad de producir una serie de moléculas que regulan de forma negativa la actividad de la respuesta inmunológica, impactando principalmente sobre el

reconocimiento por parte de los linfocitos de las células tumorales, dando lugar a una tolerancia inmunológica (Chen y cols., 2013).

Es por ello que la inmunoterapia enfocada al bloqueo del punto de control inmunológico PD-1/PD-L1 supone un cambio de paradigma en el tratamiento del cáncer, las observaciones realizadas en estudios de diferentes cánceres, como el melanoma, el cáncer de pulmón no microcítico, el carcinoma de células renales, el cáncer de vejiga, el carcinoma de células de Merkel y el carcinoma hepatocelular, entre otros (Boussiotis, 2016; Meng y cols., 2015; Sharpe & Pauken, 2017) han tenido como resultado una gran mejora en el contexto de la inmunoterapia oncológica. El último fármaco aprobado por la *Food and Drug Administration* como agente de inmunoterapia contra el cáncer de cabeza y cuello en el punto de control PD-1/PD-L1 ha sido el pembrolizumab (Cohen y cols., 2016).

Objetivos

Conocer la relación del immunecheckpoint PD-1/PD-L1 con la evolución clínica del COCE.
Evaluar la supervivencia del COCE en base a las características del microambiente tumoral y el *histologic risk score.*
Evaluar la relación clínica e histopatológica del COCE con su microambiente tumoral inmunológico.

Metodología

Estudio retrospectivo llevado a cabo en muestras de 65 pacientes con diagnóstico de COCE en suelo de boca y lengua móvil del Servicio de Cirugía Oral y Maxilofacial del Hospital Universitario La Paz en Madrid entre los años 2010 y 2015.

Los pacientes incluidos fueron pacientes con diagnóstico anatomopatológico de COCE primario tras resección quirúrgica en localizaciones de lengua móvil y/o suelo de boca.

Se excluyeron pacientes que hayan sido tratados previamente a la extirpación quirúrgica del tumor con terapia oncológica ya sea farmacológica o radioterapéutica, casos cuyo diagnóstico fue un carcinoma oral de tipo microinfiltrante, casos con en los que faltó información relevante y casos con insuficiente material histológico para poder realizar el análisis histopatológico.

Se recogieron las variables clínicas de sexo, edad, hábito tabáquico, hábito enólico, la localización primaria del tumor, las fechas de diagnóstico y de tratamiento, la presencia de recidivas (locales, regionales y a distancia) y la presencia de trastornos potencialmente malignos previos al diagnóstico del COCE en base a la última clasificación (Warnakulasuriya y cols., 2020).

Las características de la neoplasia como el tamaño del tumor, la presencia de metástasis regionales o a distancia fue registrada según la última clasificación TNM de la región de la cabeza y cuello de la *American Joint Committee on Cancer* (Amin y cols., 2017).

Todas las muestras fueron analizadas mediante microscopio óptico de forma simultánea e independiente por tres observadores. Previo al análisis de las muestras, con el fin de establecer una equiparación para determinar las características de las muestras, se siguió una evaluación basada en el modelo de riesgo histológico de recurrencia (Brandwein-Gensler y cols., 2005; Brandwein-Gensler y cols., 2010). El estudio de cada muestra se llevó a cabo al azar y con cegamiento de la información clínica del paciente.

Dentro del modelo de riesgo histológico se contempla la respuesta linfoplasmocitaria del huésped, el peor patrón de invasión y la invasión perineural, clasificándose en tres grupos (bajo riesgo (0-1), riesgo moderado (2-3), alto riesgo (4-7)).

También se registró el grado histológico del tumor, clasificándolo como pobre, moderado o bien diferenciado según criterios de la *World Health Organization,* así como si el tumor presentaba invasión vascular y/o linfática (El-Naggar y cols., 2017; Thomson, 2006).
Se registró la profundidad de invasión (DOI, por sus siglas en inglés), clasificándose como lesiones invasivas leves (≤ 5 mm), invasión moderada (> 5 mm y ≤ 10 mm) e invasión profunda (> 10 mm).

Se realizó el estudio inmunohistoquímico para el análisis las expresiones de PD-1, PD-L1, FoxP3, CD4, CD8, CSF1R y p16.

Para la recogida objetiva de la expresión de los marcadores, se observaron las muestras bajo microscopio óptico a una magnificación de 10x, 20x y 40x calculando el porcentaje de expresión, a excepción del PD-L1 en el que se utilizaron los siguientes sistemas de puntuación:

-TPS (*Tumor Proportion Score),* definido como el porcentaje de células tumorales con tinción parcial o completa a nivel de membrana en relación con todas las células tumorales presentes en la muestra (positivas o negativas). Se excluyen de la puntuación aquellas células con tinción únicamente citoplasmática, así como la infiltración por células inmunitarias, cualquier otra célula o tejido celular necrótico.

-El CPS (*Combined Positive Score*), se define como el número de células con tinción a nivel de membrana de PD-L1 (Células tumorales, linfocitos, macrófagos) dividido entre el número total de células tumorales, multiplicado por 100. Aunque el resultado del cálculo puede exceder 100, la puntuación máxima se define como CPS 100.
El registro de la expresión en CPS se realizó con la clasificación en tres grupos: < 1 CPS, ≥ 1 CPS, y ≥ 20 CPS, con el fin de poder comparar los resultados de este estudio con los hallazgos obtenidos por los ensayos clínicos (Oliva et al., 2019).

-Intensidad, el registro de intensidad de expresión se realizó con la clasificación clásica: 0 (Negativo), 1+ (Débil), 2+ (Moderado), 3+ (Intenso).

Además, en el caso de PD-1 y PD-L1 se crearon sendas variables dicotómicas para definir la positividad. En el caso de PD-1 se consideraron positivos aquellos tumores con porcentajes de expresión >0%. Se consideraron positivos para PD-L1 aquellos tumores con ≥1% de expresión.

Supervivencia

Los datos de supervivencia de los pacient*es* se recogieron de la historia clínica. Desde la fecha del diagnóstico de la enfermedad hasta el evento estudiado (muerte y/o recidiva) hasta el mes más cercano del registro. Los eventos se definieron como: Muerte por COCE, muerte por otra causa, recurrencia (local, regional o a distancia) y supervivencia sin recurrencia. En base a estos eventos, se consideraron tres tipos de supervivencia: Supervivencia específica de la enfermedad (*Disease Specific Survival* (DSS)), siendo considerada únicamente la muerte por COCE como evento; Supervivencia libre de enfermedad (*Disease Free Survival* (DFS)), cuando tanto la muerte por COCE o cualquier recurrencia tienen lugar, considerando ambas como evento; y la supervivencia global (*Overall Survival* (OS)), cuyo evento se ha definido como muerte por cualquier causa.

Resultados

Durante una media de seguimiento de 73 meses, tuvieron lugar 32 muertes (49%) por cualquier causa y un 42% por COCE.

La expresión media de PD-1 fue del 1%, siendo el 80% de los tumores analizados (75% en hombres y 88% en mujeres) positivos (>0%).

La positividad a PD-1 se asoció a una mayor proporción de infiltración linfoplasmocitaria (p-value= 0.03), con una mayor proporción en la categoría *Nil/Low* (46%% vs. 85%%, p-valor= 0.019), con una menor profundidad de invasión (mediana del DOI 8 vs. 12, p-value=0,017), con una mayor proporción en la categoría *less invasive* (39% vs. 17%, p-value=0.087), y con un menor tamaño tumoral (67% vs 33% en T1 or T2, p-valor= 0.073).

Los valores de expresión de PD-L1 (TPS ≥5%) se asociaron con una mayor profundidad mediana de invasión (10mm vs. 7 mm, p-valor=0.17), pero con un patrón invasivo (*Worst Pattern Of Invasive)* WPOI-5 (0% vs. 16%, p-valor=0.085) más favorable. Cuando el criterio utilizado se basó en CPS (>1) se observó una relación similar con el patrón invasivo (2.9% vs. 20%, p-valor=0.043), una menor puntuación en el *histologic risk score* (4-7: 47% vs 26%, p-valor 0.043) y una mayor proporción de recurrencias locales (40% vs. 17%, p-valor=0.074).

La expresión de FoxP3 por encima del 10% tuvo lugar en tumores T2. La muestra presentó porcentajes de expresión de CD4 mayores al 35%. La expresión de CD8 ha sido superior en hombres que en mujeres y el CSF1R se ha observado en aquellos casos con grado histológico pobremente diferenciados.

El análisis univariado indicó que la expresión positiva de PD-1 es un factor protector de la supervivencia (DDS, HR 0.43 [0.19,0.98], p=0.044; OS, HR 0.47 [0.22,1.02], p=0.05; DFS, HR 0.47 [0.22-0.99], p=0.047), así mismo, la expresión positiva de PD-L1 con la categorización (TPS≥5%) también se asoció a un mejor pronóstico (DSS, HR 0.42 [0.17,1.05], p=0.063; OS, HR 0.41 [0.17,0.95], p=0.038). Se obtuvieron resultados similares cuando la positividad de PD-L1 se basó en CPS (>1%) (DSS, HR 0.53 [0.24,1.17], p=0.12; OS, HR 0.44 [0.213,0.927], p=0.031).
El análisis multivariado también confirmó que la positividad de PD-1 es un factor protector, especialmente en el modelo DFS (HR 0.36 [0.14,0.93], p=0.034).

Además, el análisis multivariado indica que la presencia de metástasis, así como el grado histológico moderado o pobremente diferenciado se ha asociado a un peor pronóstico en todos los modelos de supervivencia.

Conclusiones

PD-1 es un factor protector de supervivencia que se mantiene independientemente de la expresión de PD-L1. Los valores altos de expresión de PD-L1 también mejoran la supervivencia, mientras que los valores bajos se asocian con un peor pronóstico. Se observa una mayor expresión de PD-1 en tumores más pequeños y una mayor expresión de PD-L1 en mujeres.

2. No se encuentra relación entre el microambiente tumoral y el *histologic risk score* que influya en la supervivencia del COCE.

3. No se evidencia una relación entre las características histopatológicas y los marcadores estudiados, aunque los casos positivos de PD-1 y PD-L1 tienden a asociarse con patrones de invasión favorables y la expresión de PD-1 positiva se asocia con un DOI más leve.

SUMMARY

SUMMARY

Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent neoplasm among head and neck carcinomas, this group is one of the most frequent groups of cancers globally. The morbidity of its treatment is very high, as is mortality, and the survival of patients with this disease at five years is approximately 60%. Unfortunately, the average survival of patients with recurrence of the disease or metastasis is approximately 8-10 months.

The histopathological characteristics of OSCC have been studied, and a histologic risk assessment system has been developed, motivated by the high rate of relapses of this neoplasia. Despite that there are studies on the microenvironment in head and neck cancer, the tumour microenvironment that exists around OSCCs has not yet been studied in depth, which would improve the understanding of this tumour in the face of the emergence of immunotherapy since clinical trials conducted in recent years have evaluated drugs whose therapeutic targets are various immunological checkpoints.

Objectives

To know the relationship of the immune-checkpoint PD-1 / PD-L1 with the clinical evolution of OSCC; to assess survival in OSCC based on the characteristics of TME and histologic risk score; and to evaluate the clinical and histopathological relationship of OSCC with immunological TME.

Methods

A retrospective study was carried out on 65 samples from patients with OSCC on the floor of the mouth or tongue. Clinicopathological variables and the expression of the biomarkers PD-1, PD-L1, FoxP3, CD4, CD8, CSF1R, and p16 were recorded. The relationship of the clinical and histological variables with the expression of the biomarkers was evaluated, and survival was studied.

Results

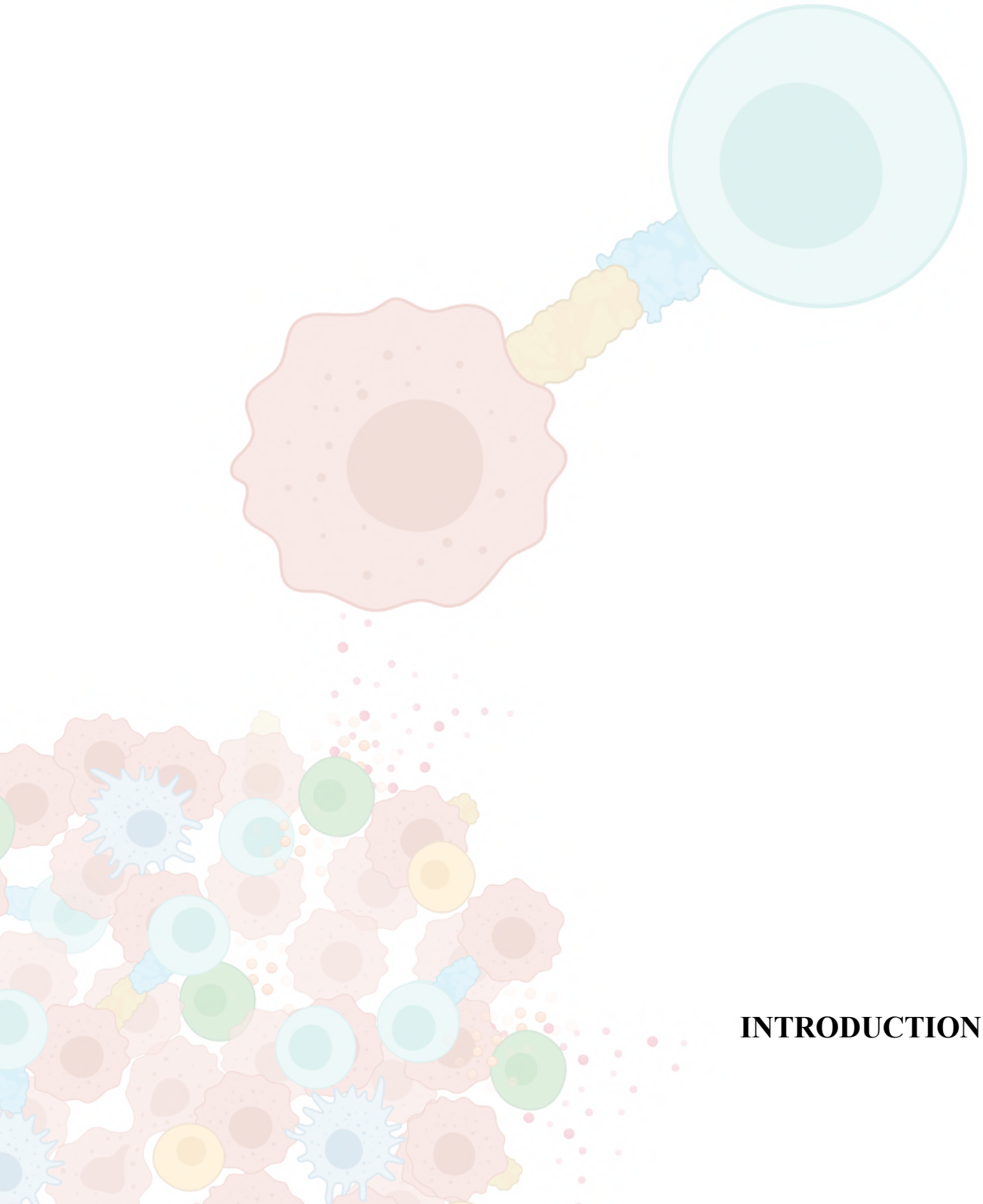
The univariate and multivariate analysis indicated that positive PD-1 expression was an independent protective factor for survival (overall, disease-free, disease-specific survival) and that high PD-L1 also improved survival. Poorly differentiated histological grade and metastasis were associated with a worse prognosis.

Conclusion

PD-1 is a protective survival factor that is maintained independently of PD-L1 expression. High values of PD-L1 expression also improve survival, while low values are associated with a worse prognosis. Higher expression of PD-1 is observed in smaller tumours, and higher expression of PD-L1 is more likely in women.

No relationship between the tumour microenvironment and histologic risk score was found to influence the survival patterns studied in the OSCC.

There is no evidence of a relationship between the histopathological features and the studied markers, although the positive PD-1 and PD-L1 cases have a lower risk of a high WPOI score, and positive PD-1 expression was associated with a lower DOI.



INTRODUCTION

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Oral squamous cell carcinoma

Cancer is a pathology that represents one of the major protagonists in human death. Oral squamous cell carcinoma (OSCC) is the most common epidermoid carcinoma of head and neck cancers (Warnakulasuriya, 2009).

In 2018, 19 million cancers were diagnosed, and 354,864 were OSCC, with a net increase from previous years, 2012 and 2015 (Bray et al., 2018) (Figure 1). Cancer is more prevalent in highly developed countries perhaps due to lifestyle and an ageing population (Torre et al., 2016). Although cancer prevalence is high in developed countries, mortality is higher in developing countries. It is estimated that by 2030, cancer prevalence will increase between 81% and 100% (Fidler et al., 2018).

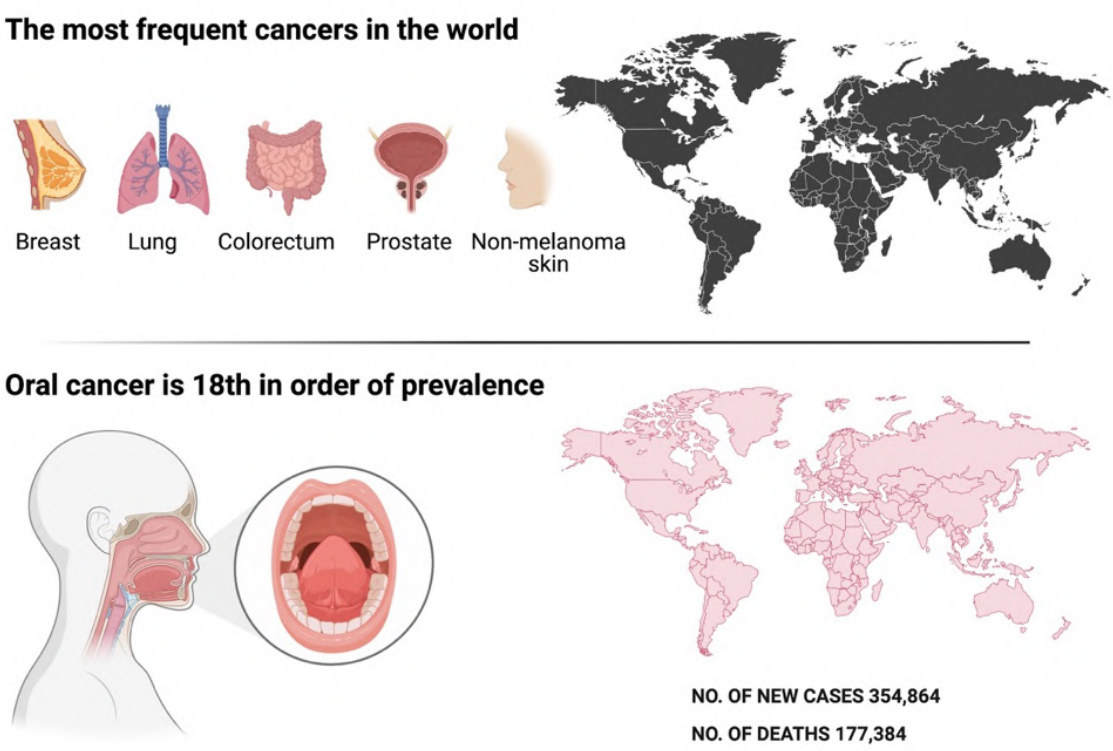


Figure 1. During 2018, a total of approximately 18,078,957 cancer cases occurred worldwide, with total mortality of approximately 9,555,027, thus demonstrating the high mortality of this disease. The five most frequent cancers were breast, lung, colorectum, prostate, and non-melanoma skin. Oral cancer, although not among the most common cancers, ranking 18th in prevalence, is a cancer with high mortality and significant morbidity.

Although the oral cavity is a small region of the body, there are different areas within it where the disease can develop. The primary origin of OSCC is found in most cases in the anterior region of the tongue (40%) and on the floor of the mouth (30%), being the worst prognosis areas (Bagan et al., 2010; Ng et al., 2017; Rivera, 2015; Sundermann et al., 2017).

Oral cancer has always been a disease that affected men with classics risk factors such as smoking, alcohol and betel nut, although, the latest publications indicate a tendency of incidence in younger population and females without classics factors (Ng et al., 2017).

Diagnosis at early stages is critical but complex due to the absence of signs and symptoms, a fact that has not improved over the years (van der Wall et al., 2011; Awan, 2014). However, only one third are diagnosed in initial states (I and II) that are related to a curation of 80% and 60% respectively. Therefore, two third diagnosis occur in advanced states with a worst therapeutic respond (Rivera, 2015).

Both, disease mortality and morbidity after treatment are very high (Sacco & Cohen, 2015), with survival at five years approximately 60% (Chi et al., 2015; Rapidis et al., 2009). Unfortunately, after recurrence or metastasis, the average survival is approximately 8-10 months (Zandberg & Strome, 2014).

Treatment

The main treatment for OSCC is surgery, mainly in the early stages, where complete resection of the tumour with free margins can be achieved. However, in advanced disease, free margins are difficult to achieve, so the use of chemoradiation adjuvant to surgery reduces the risk of recurrence and its use is indicated in locoregionally advanced disease (Fridman et al., 2018, Omura, 2014).

In OSCC, radiotherapy is usually applied postoperatively, due to the complexity of performing surgical excision in previously irradiated tissues. Usually, radiotherapy is used in the affected area, however, in more advanced tumours or those with suspected cervical metastases, radiotherapy is also performed in the cervical region. External beam radiation is commonly used and usually begins within the first 6 weeks after surgery, and

although the radiation dose can vary, the total dose is approximately 60 Gy (Omura, 2014; Wong & Wiesenfeld, 2018).

The indicated treatment is usually surgery alone or in combination with radiation therapy, and in more advanced stages the use of adjuvant chemotherapy, usually consists of the use of cisplatin, carboplatin, 5-flurouroracil, paclitaxel and docetaxel. The choice of treatments in addition to the stage will depend on the patient's comorbidities (Rivera, 2015). Neoadjuvant chemotherapy to surgery has a limited role, so its use is not common (Vishak, et al., 2015).

In the case of surgical treatment, this is not limited only to the primary tumour lesion, since in most cases, T1 tumours larger than 3 mm, and any T2, T3 and T4, are also performed a cervical dissection and extirpation of lymph nodes (Wong & Wiesenfeld, 2018).

Despite these treatments, some patients progress during or after therapies. Those who show resistance to platinum treatment face limited treatment options. The most used at present are cetuximab, methotrexate, docetaxel, and paclitaxel, however, the emergence of drugs to inhibit immune checkpoints opens the door to new treatments (Oliva et al., 2019).

Staging of OSCC

The staging of the OSCC has been developed considering several important aspects as:

-Its anatomical location and the adjacent structures in this area.

- The routes of dissemination. Of particular note are the cervical lymph nodes, whose lymphatic drainage comes directly from the region of the floor of the mouth, a structure that is often significantly affected by this disease.

The American Joint Committee on Cancer (AJCC) uses the TNM classification (primary tumour, regional lymph node, metastasis) as a classification system together with the Union for International Cancer Control (UICC) to classify the disease and determine the

most appropriate therapy for head and neck squamous cell carcinoma (HNSCC) (Amin et al., 2017).

Tumours of the head and neck region encompass the location of the lip and oral cavity, as well as the mucosal surface of the upper airways, pharynx (oropharynx, nasopharynx, hypopharynx), maxillary sinuses, nasal cavity, ethmoid sinuses, malignant mucosal melanoma, major salivary glands and thyroid gland (Amin et al., 2017).

The TNM classification attempts to precisely define the disease at the time of diagnosis before the first treatment takes place. Such a diagnosis may include, in addition to clinical examination, complementary tests to reach as complete a diagnosis as possible (Amin et al., 2017).

The majority of squamous cell carcinomas on mucosal surfaces affect the head and neck in four main anatomical regions: the oral cavity, the sinonasal cavity, the pharynx and the larynx (Amin et al., 2017).

The latest consensus of the AJCC on the TNM classification of the head and neck region corresponds to the eighth edition, defined in 2018 (Table 1). In this update, the AJCC presented important histological factors to be considered in the oral cavity region such as depth of invasion (DOI), as well as changes regarding cancer in the oropharyngeal region when it presents human papillomavirus (HPV) aetiology.

The OSCC stage classification is shown below (Amin et al., 2017):

- Stage I: T1, N0, M0.

- Stage II: T2, N0, M0.

- Stage III: T3, N0, M0; T1-T3, N1, M0.

- Stage IVA: T4a, N0 or N1, M0; T1 to T4a, N2, M0.

- Stage IVB: Any T, N3, M0; T4b, any N, M0; Any T, any N, M1.

The TNM classification of p16-positive oropharyngeal carcinomas shows differences concerning p16-negative carcinomas. P16 has been shown to be an excellent marker for oropharyngeal carcinomas since p16-positive/ correlate with the better survival prognosis. It should be highlighted that, although the classification has used p16 as an HPV marker, other detection methods (in situ hybridisation, polymerase chain reaction (PCR) of viral DNA) have shown similarities concerning the better survival prognosis shown by p16-positive patients (Chow, 2020)

Given this superior prognosis, the TNM classification for this tumour was divided in 2017 into p16-positive and p16-negative. Paradoxically p16-positive neoplasms with a size or lymph node involvement that is superior to p16-negative neoplasms, have a better prognosis. Thus, "more advanced" tumours with HPV aetiology have a better prognosis than less advanced tumours unrelated to HPV (Chow, 2020).

DOI

The AJCC tumour staging, in its eighth edition, incorporated the assessment of DOI. Although tumour thickness could be a prognostic factor for squamous cell carcinomas, recent studies have indicated that DOI is a more accurate predictor than the measurement of tumour thickness (Shim et al., 2015; Spiro et al., 1986).

DOI is a quantitative length measurement whose magnitude used is millimetres (mm). Quantification is performed on the most representative histological slice of the tumour. It must be determined whether the tumour presentation is exophytic or ulcerated, in which case, the basement membrane (horizontal line) must be determined and then a vertical line ("plumb line") must be drawn from the basement membrane to the invasion front of the tumour, classifying the tumours as mild invasive lesions (≤ 5 mm), moderate invasive lesions (> 5 mm and ≤ 10 mm) and deep invasive lesions (> 10 mm) (Shim et al., 2015; Spiro et al., 1986).

The AJCC advocates DOI rather than tumour thickness, as the difference between the two measurements can affect the pT category in up to 5.7% of patients with OSCC (Berdugo et al., 2019).

Potentially malignant oral disorders

An important aspect to highlight in the etiology of OSCC is that there may be lesions that may appear before cancer; these oral lesions are referred to as potentially malignant oral disorders (OPDM). In 2007, a workshop was held by the WHO Collaborating Centre for Oral Cancer and Precancer in London, in which different aspects were discussed concerning lesions susceptible to malignancy in the oral mucosa and to evaluate the adoption of the term OPDM described in the WHO monograph on Head and Neck Tumours (2005) (Warnakulasuriya et al., 2007).

Currently, lesions diagnosed as oral leukoplakia, erythroplakia, proliferative verrucous leukoplakia, palatal lesions in reverse smokers, oral lichen planus, lupus erythematous, congenital dyskeratosis, actinic cheilitis, lichenoid lesions, graft-versus-host lesions are considered OPDM (Warnakulasuriya, 2020). However, due to limited scientific evidence, chronic hyperplastic candidiasis, exophytic verrucous hyperplasia and oral epidermolysis bullosa are not currently considered OPDM (Warnakulasuriya et al., 2020).

According to current knowledge, the gold standard for confirmation of the clinical diagnosis of an OPDM is to present a representative biopsy to discern whether malignancy is present or not.

Review observational studies indicate a rate of malignant transformation ranging from 0.13% to 34% in oral leukoplakia and 0-3.5% in lichen planus (Warnakulasuriya, 2020).

The time from the onset of an OPDM to the development of a malignant process is approximately 2 years, although it may extend up to 10-15 years (Warnakulasuriya, 2020).

Histological features of OSCC

OSCC is a malign epithelial neoplasm with different grades of squamous differentiation. It is usually originated in the stratified squamous epithelium of the oral mucosa and with a high prevalence of localisation on the tongue and floor of the mouth.

Grades of differentiation can be poorly differentiated, moderately differentiated and well-differentiated (Edge et al., 2010), with keratin bead formation and different invasions patterns resulting from disruption of the basement membrane by OSCC and extension into the subepithelial connective tissue (Barnes et al., 2005).

Histologically, well-differentiated OSCCs resemble the epithelium of origin. Moderately differentiated OSCCs are characterized by nuclear pleomorphism, mitotic activity and usually a lower degree of keratinisation. Poorly differentiated OSCCs exhibit a predominance of immature cells with numerous mitoses and virtually no keratinisation (Barnes et al., 2005).

The histological features and the risk associated with them can be assessed by the classification proposed by Brandewein-Gesler et al. (2005). This classification is based on the analysis of the worst pattern of invasion (WPOI), the presence of perineural invasion (PNI) and the host lymphocytic response to the tumour (Table 2) (Brandwein-Gensler et al., 2005; Brandwein-Gensler et al., 2010).

Cancer and immunotherapy

Cancer is an entity with a multifactorial etiology and its evolution depends on the host response. When a neoplastic cell (NC) appears, following a mutation, the cell itself produces a series of specific signals that are recognised by the cells of its environment or tumour microenvironment (TME). The responses of the TME will be determinant for the evolution of the NC and the development of cancer.

The characteristics of cancer described by Hanahan and Weinberg over the years have proven to be fundamental in the understanding of cancer traits and the design of therapeutic strategies for cancer. These authors suggested in 2000 six characteristics (called "Hallmarks") present in the pathogenesis of NCs (Hanahan & Weinberg, 2000). These were the production of growth signallings, the not recognition of growth-inhibitory signals, ability to evade programmed death, angiogenesis, unlimited replicative potential, and metastatic capacity (Hanahan et al., 2000). See figure 2.

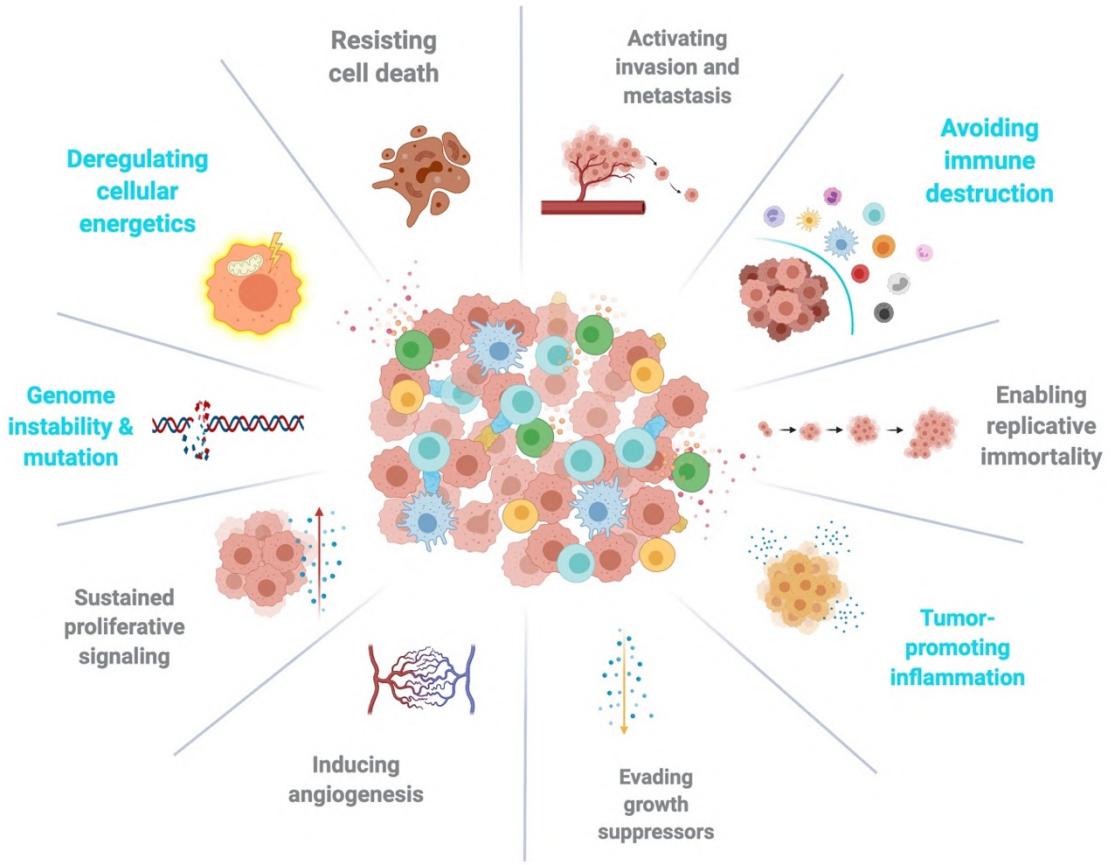


Figure 2. Characteristics of NCs according to Hanahan and Weinberg. The first six described characteristics (in grey) of NCs were self-sufficiency of growth signals, which are determined, for example, by overexpression of receptors for growth factors or Ras proteins. Insensitivity to growth-inhibitory signals, which they do mainly through their cell cycle, via proteins such as retinoblastoma (pRb), or transforming growth factor-beta (TGF-b) molecules. The ability to evade programmed death, such as through p53 mutation, among others. Angiogenesis, which represents the fourth property of NCs, is mainly based on increased expression of vascular endothelial growth factor (VEGF) as well as fibroblast growth factor. Unlimited replicative potential and metastatic capacity is the fifth property. The other four properties described later (in blue) were cellular energy dysregulation; genomic instability, present in some tumours; promotion of inflammation; and the ability to evade the immune response.

In 2011, the same authors added four more features about NC. One is the genomic instability present in some tumours. Another is cellular energy deregulation and two others, which have proved to be very important in current cancer therapy, corresponding to the capacity of the NC to provoke inflammation in its environment and the capacity to evade the immune response. This shows that tumour tissue can modulate its evolution through the control of the immune system (Hanahan & Weinberg, 2011). See figure 2.

The immune system and cancer

The immune response to a tumour cell occurs through the phenomenon of immunosurveillance. In this, the immune system attempts to eliminate the initial tumour lesion, but different processes of oncogenesis may take place in the cell that favour a failure of immune protection, with the immune response succumbing to the different signals from the tumour cells and immune tolerance occurring, allowing the tumour to grow (Boussiotis, 2016; Dunn et al., 2000; Hanahan & Weinberg, 2011).

During the initial stages in the development of a neoplasm, an initial non-specific immune cell response takes place that is capable to produces the lysis of tumour cells, as well as the release of interferon-gamma (INF-γ), which acts to enhance the activity of macrophages and dendritic cells, among others (Boussiotis, 2016; Dunn et al., 2000; Hanahan & Weinberg, 2011).
After lysis, antigenic and immunogenic proteins released are taken up by antigen-presenting cells (APCs), leading to a specific immune response by CD8 and CD4 lymphocytes. This response is regulated by various agonist and antagonist molecules of the lymphocyte response so that the response is proportionate and can be inhibited by a self-cell and thus to prevent an autoimmune process if necessary (Boussiotis, 2016; Dunn et al., 2000; Hanahan & Weinberg, 2011).

In early tumour growth, the immune response is characterised by CD8 and CD4 T lymphocytes (Tumour-infiltrating lymphocytes (TILs)), regulatory T cells (Treg) and recruitment of tumour-associated macrophages (TAMs), critical components of the TME develops (Peltanova et al., 2019). These cell populations interact with each other and with tumour cells through different molecular pathways, the ones studied in this research are described below.

Tumour-associated macrophages

Macrophages are an important cellular component present in different amounts in different tumours (Mantovani et al., 2017).
It can have a very heterogeneous behaviour between different cancers and even within the same tumour or patient. This is due to the ability of macrophages to present a different phenotype that directly impact their response to a tumour (Mantovani et al., 2017).

This recruitment occurs through stimuli such as INF-γ and macrophage colony-stimulating factor (CSF-1). These recruited and activated macrophages can be either M1 or M2 (Kumar et al., 2019).

M1s are characterised by a pro-inflammatory and anti-tumour response through the production of IL-2, IL-23, INF-y. The presence of the M1-type response in the TME of lung, ovarian, colorectal neoplasms correlates with increased survival (Mantovani et al., 2017).

M1 TAMs can produce cytotoxic factors such as nitrous oxide and reactive oxygen species, which limit the viability of malignant cells. They are also capable of phagocytosing the latter and release proinflammatory cytokines that further stimulate the immune response against the tumour (Mantovani et al., 2017).

Prolonged activity of M1 TAMs over time promotes chronic inflammation driving tumour tissue to synthesise TGF¬-β, VEGF, C–C motif chemokine ligand 2 (CCL2), IL-4, IL-10 and IL-13, with the ability to repolarise TAMs towards an M2 phenotype. This also takes place if NCs release CSF1, if lactate is found in the TME and if there is increased competition for nutrients in the TME as well as hypoxia (Mantovani et al., 2017), which are characteristics of advanced tumours.
M2 TAMs favour tumour progression mainly through the secretion of TAM-derived products, such as VEGF-A, adrenomedullin and others and recruitment of endothelial cells. In addition, M2 TAMs release other growth factors such as epidermal grow factor (EGF) and promote immunosuppression by releasing many anti-inflammatory cytokines such as IL-10, TGFB1, CCL17, CCL18 and CCL22, (Feng et al., 2010; Nishikawa &

Sakaguchi, 2010) that inhibit dendritic cell maturation, also limiting antigen presentation and the recruitment of Treg (Mantovani et al., 2017).
See figure 3.

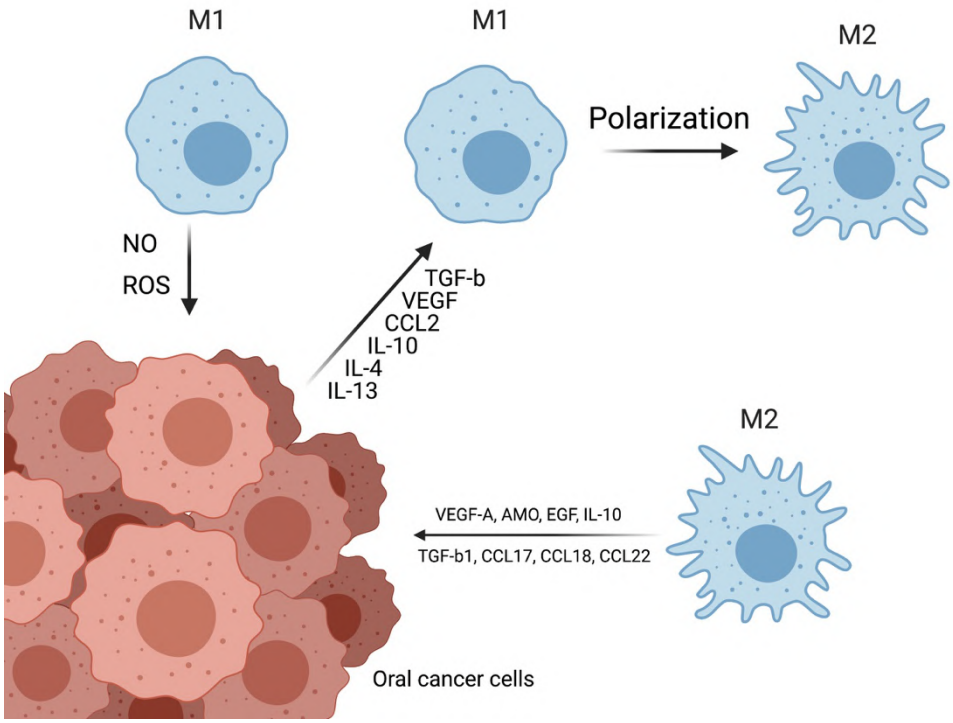


Figure 3. M1 macrophage polarisation towards M2 in the TME induced by NCs. M1 macrophages conduct an anti-tumour inflammatory response through the release of, for example, nitrous oxide (NO), reactive oxygen species (ROS) and pro-inflammatory cytokines. The response of NCs to this behaviour by the M1 macrophage is the release of TGF-b, VEGF, CCL2, IL-10, IL-4 and IL-13, leading to a polarisation of the macrophage towards the M2 phenotype, which is characterised by the release of VEGF-A, AMO, EGF, IL-10, TGF-B1, CCL17, CCL18, CCL22 favouring a pro-tumour microenvironment.

TAMs also present a surface tyrosine kinase receptor (CSF1R) that is stimulated by CSF1 ligand, secreted by tumour cells, and promotes further recruitment of circulating monocytes, survival of TAMs in the TME and macrophage polarisation towards M2 (Mantovani et al., 2017) (Figure 4).

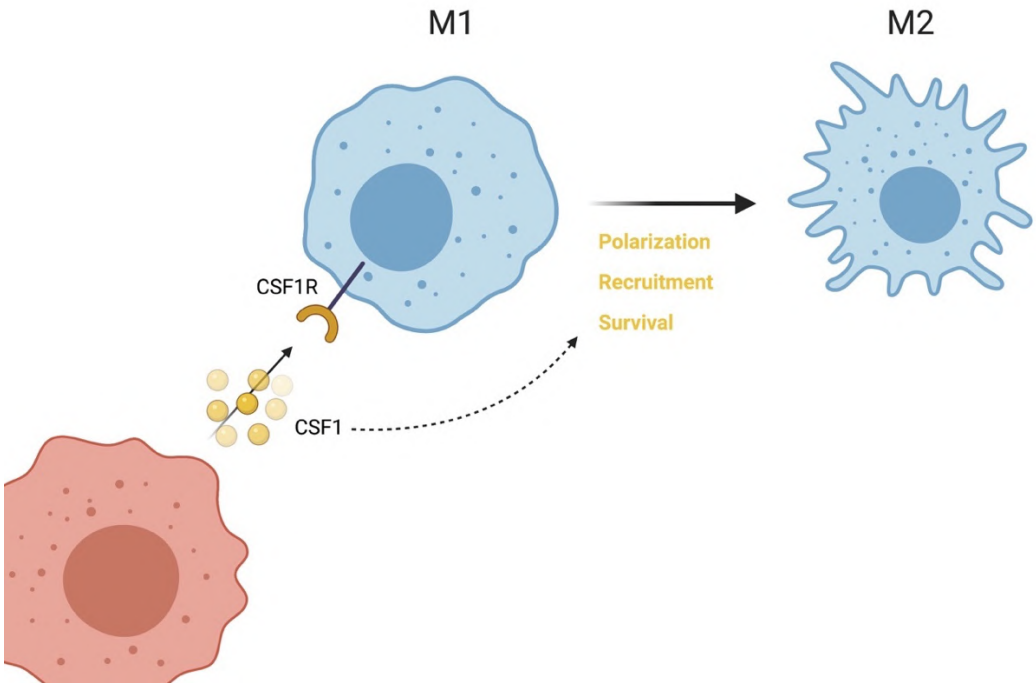


Figure 4. Illustration showing the consequences of CSF1 ligand expression by the NC, resulting in macrophage polarisation towards the M2-like phenotype, increased TAM survival and enhanced macrophage recruitment.

It should be noted that M2 TAMs also promote PD-L1 expression and thus an increase in the immunosuppressive microenvironment. On the other hand, they also prevent tumour infiltration by lymphocytes by remodelling the extracellular matrix via metalloproteinases (Mantovani et al., 2017). In addition, M2s also promote tumour progression through the recruitment of regulatory T-cell populations (Tregs), which ultimately leads to the overall promotion of tumour tissue (Mantovani et al., 2017).

Within the development of immunotherapy, there are very current therapies that target the neutralisation of M2 TAMs or their repolarisation towards M1. One very promising approach is the development of CSF1-R inhibitors that favour the presence of M1 TAMs over M2 TAMs in the TME (Mantovani et al., 2017).

Regulatory T cells

For proper control of immune tolerance and a successful response by the immune system, Tregs are of particular importance, highlighting their role in peripheral tolerance to self-antigens (Mills, 2004).

Tregs are a crucial component of the immune system, providing immune tolerance and maintaining homeostasis of immune responses (Sakaguchi et al., 2020). However, depending on their activity, they can suppress anti-tumour responses by the immune system (Vignali et al., 2008). They can suppress responses carried out by conventional T cells, B cells, APCs, natural killers (NKs) and also subsets of other CD4 T cells such as th1, th2 and th17 (Bayati et al., 2021).

CD4+ and CD25+ T cells are the subspecialised Tregs, accounting for about 5-10% of the total CD4+ lymphocyte population in the body. Expression of the transcription factor forkhead box P3 (Foxp3) and the high-affinity interleukin-2 receptor alpha chain (IL-2R α or CD25) are defining characteristics of Tregs (Bayati et al., 2021).

Tregs with specific FoxP3 expression have an important association with the development of autoimmune diseases when there is an altered or absent FoxP3 response (Sakaguchi et al., 2020).

The main mechanisms of action that Tregs exert to suppress immune cell activity are threefold:

-Anti-inflammatory cytokines production, adenosine, granzyme and perforin (Levings et al., 2002; Linterman & Vinuesa, 2010; Moore et al., 2001).

-Competition for common growth factors with other cells such as T-lymphocytes.

-The use of receptors on their surface with the ability to inhibit the action of immune cells, receptors such as cytotoxic T-Lymphocyte antigen 4 (CTLA-4), Nrp-1, galectin-1, LAG-3, TIM-3 and also through the release of the enzyme indolamine 2,3-dioxygenase (IDO) (Bayati et al., 2021; Oderup et al., 2006). See figure 5.

Research with these cells is focused on the use of immunotherapy to enhance their functions to treat autoimmune diseases or modify their functions, for example, in cancer treatment (Sakaguchi et al., 2020).

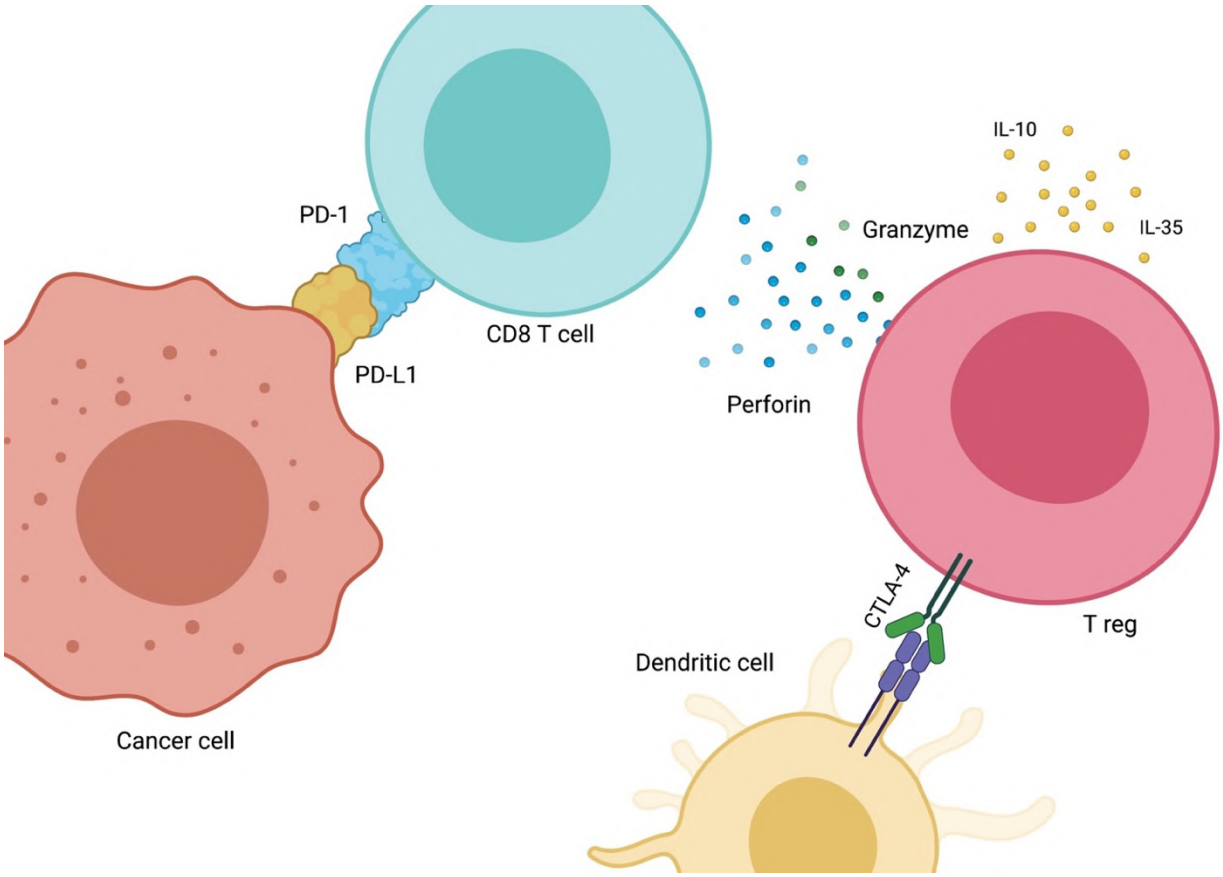


Figure 5. In addition to the interaction of PD-1 and PD-L1, this illustration showing the production of anti-inflammatory molecules such as IL-10, IL-35 by the Treg cell, the release of perforin and granzyme in the microenvironment affecting the CD8 lymphocyte and limiting its function, and the use of the CTLA-4 receptor, which can inhibit the action of the dendritic cell.

C4 & CD8 TILs

T cells are classified based on their TCR receptor and the central lineage markers CD4 and CD8. The TCR receptor gives lymphocytes the ability to recognise major histocompatibility complex (MHC) type I (on CD8 cells) and type II (on CD4 cells) molecules.

CD8 T cells are known to be cytotoxic, as they can produce high levels of anti-tumour and cytotoxic cytokines such as interferon-γ (INF-γ) tumour necrosis factor α (TNF- α), perforin and granzymes. T cells only recognise antigens presented by MHC type I expressed on the target cell or APCs. To attack the target cell, lymphocytes can release the molecules described above, or alternative mechanisms including their Fas receptor binding to the Fas ligand that is expressed on target cells (Seder & Ahmed, (2003).

CD4 T cells recognise MHC type II antigens presented by APCs. They can activate B and T lymphocytes. There are different types of CD4 T cells, mainly Th1, Th2, Th17 and follicular. (Seder & Ahmed, 2003).

Th1 cells are characterised by the secretion of mainly INF-γ (which enhances the pro-inflammatory environment, opsonisation and immunoglobulin synthesis). INF-γ promotes Th1 cell differentiation and inhibits Th2 action. Th1 differentiation occurs in response to antigen presentation by NKs and macrophages (Seder & Ahmed, 2003).

Th2 cells promote their differentiation and activation through the secretion of IL-5 and IL-6. Th2 activity is anti-inflammatory, as they also secrete IL-10, favouring the inhibition of Th1 (Seder & Ahmed, 2003).

Th17 and follicular lymphocytes are found in smaller proportions but play important roles in the immune response. Th17 are characterised by the secretion of IL-17 and IL-22, producing inflammatory responses with neutrophil activity. Follicular lymphocytes can activate B lymphocytes in lymph nodes (Seder & Ahmed, 2003).

Knowledge of these characteristics has highlighted the relevance of the immune system in the development of cancer development, leading to new approaches in cancer treatment known as immunotherapy.

Immunecheckpoint PD-1/PD-L1

The most important type of anti-tumour immune response is the acquired cytotoxic response, carried out primarily by CD8 cytotoxic T-lymphocytes.

For this response to take place, a total of seven steps must occur. The first step is the expression of tumour antigens by malignant cells; the second step is the recognition of these antigens by APCs and their presentation to lymph nodes; the third step involves the recognition of these antigens by lymphocytes, with an immune synapse known as a priming synapse taking place, leading to the activation of lymphocytes (Chen et al., 2013).

The lymphocytes then leave the lymph nodes in search of tissue expressing the antigens presented to them in the lymph nodes, which corresponds to the fourth phase; the fifth phase is related to the tumour infiltration of the lymphocytes, when they reach the tumour they carry out the effector synapse, in this synapse, which corresponds to the sixth phase, multiple activating and inhibitory molecules intervene, the sum of the action of these will determine whether the lymphocyte produces the death of the tumour cell (seventh phase) or on the contrary, the tolerance to the tumour or the lymphocyte could become anergic (Chen et al., 2013). See figure 6.

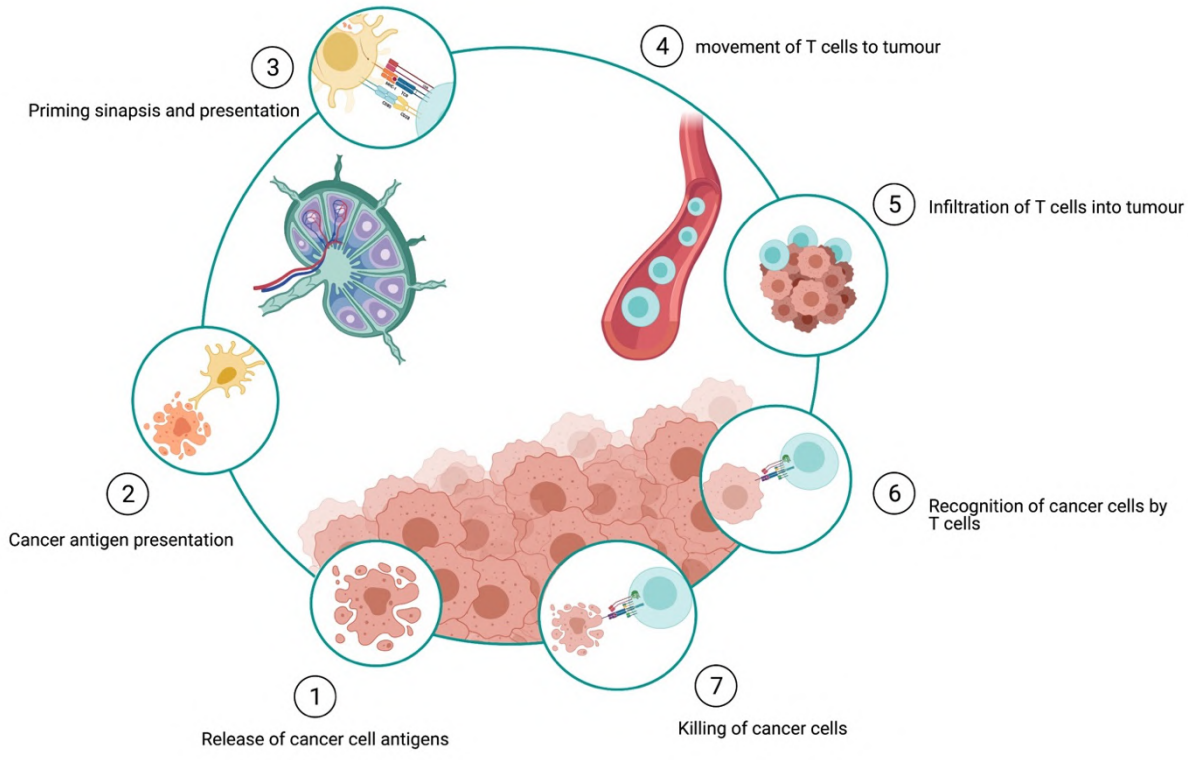


Figure 6. Illustration adapted from Chen et al., 2013 showing the different steps during the priming synapse and the effector synapse after recognition of a tumour antigen.

Advanced neoplasms have a very immunosuppressive TME (greater number of inhibitory stimuli), which causes the lymphocytes in the active phase, despite having reached the tumour, to enter a state of anergy and not attack it. The main responsibility for this situation is the expression of programmed death-ligand 1 (PD-L1) by the tumour cells and its interaction with the programmed cell death receptor 1 (PD-1) in the lymphocyte (Chen et al., 2013; Couzin-Frankel, 2013; Song et al., 2016). See figure 7 and 8.

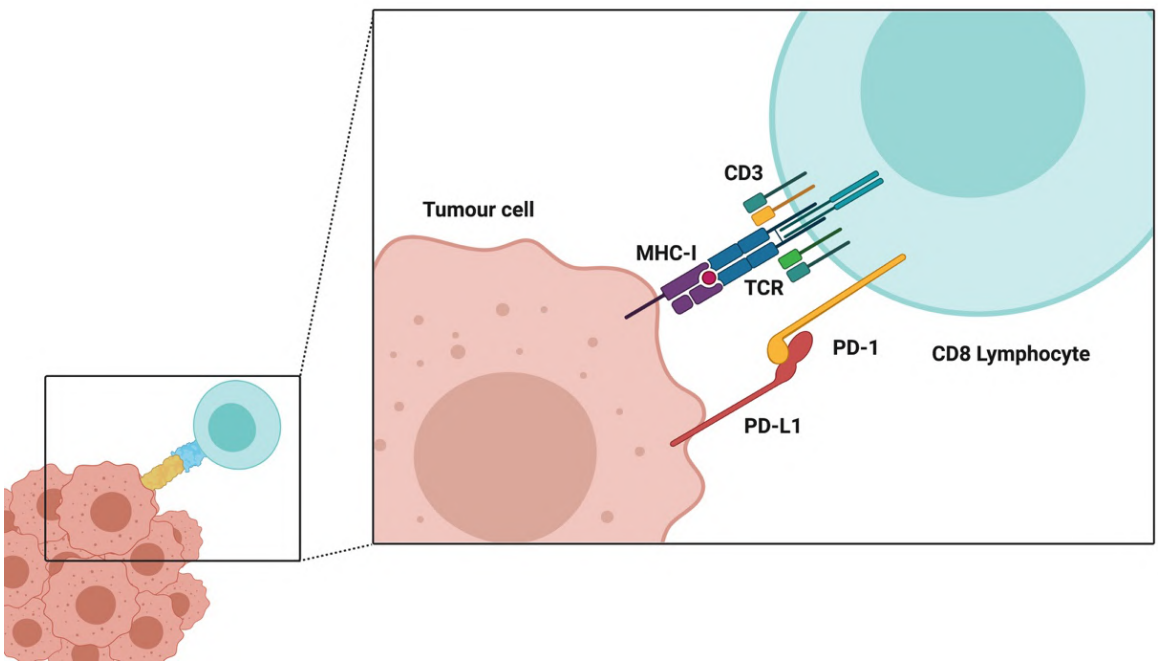


Figure 7. The interaction of a tumour cell with a CD8 T-lymphocyte can be observed. The CD8 T cell has a T-cell receptor (TCR) that is responsible for interacting with the type I histocompatibility complex (MHC) expressed at the surface of the NC. In this process, CD3 molecules responsible for stabilising the interaction of the TCR and, on the other hand, the immune tolerance receptor co-stimulatory PD-1 on the CD8 T lymphocyte also interact with its ligand on the surface of the tumour cell. When PD-1 is stimulated by PD-L1, the immune tolerance process is activated, leading to a ceasing of the immune response of the CD8 T-lymphocyte against the Tumour cell.

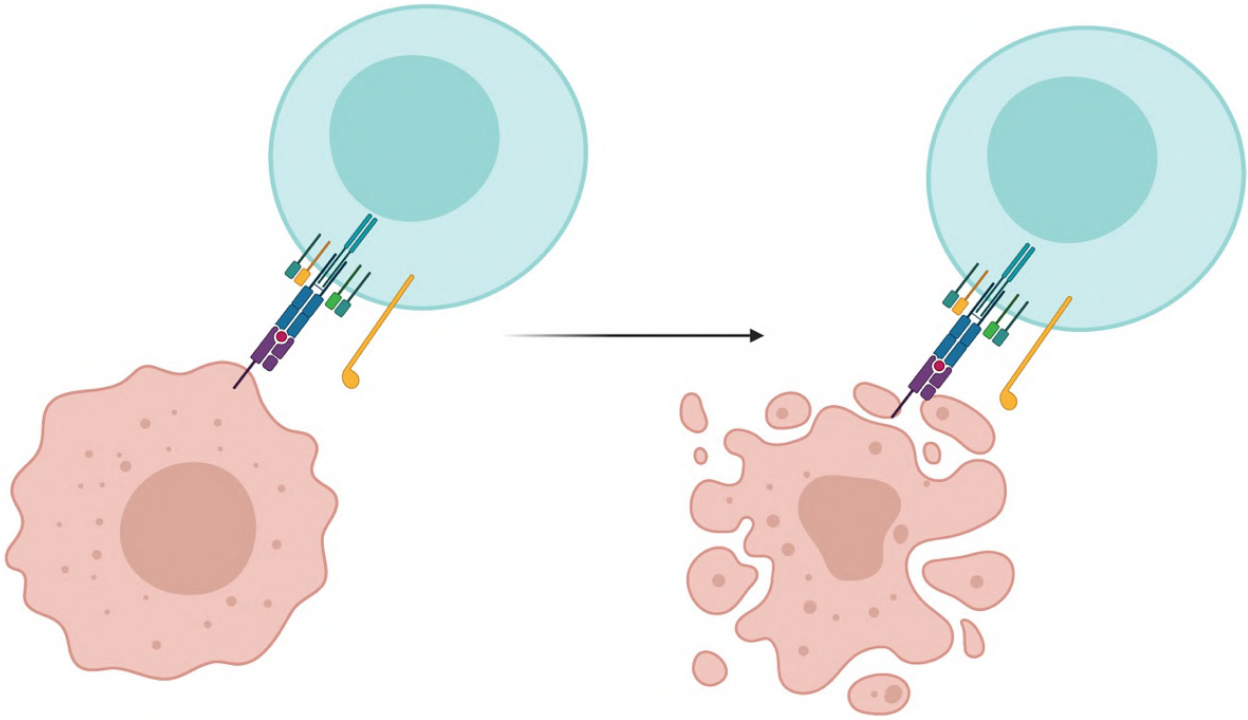


Figure 8. The function of the PD-1 receptor. When a cell, in this case, a tumour cell, does not express PD-L1, cell apoptosis is induced following MHC-I binding to the TCR of the CD8 T cell.

This mechanism occurs because activation of the PD-1 receptor on a T lymphocyte can override each of the mitogenic signals triggered following T-cell receptor activation. PD-1 activation can directly inhibit ZAP70, LAC family proteins, the PI3K-AKT-mTOR and MAPK pathways. It is, therefore one of the main immune escape mechanisms used by tumours and is responsible for tumour survival (Figure 9) (Boussiotis, 2016).

This acquired anti-tumour immune response is strongly regulated by dozens of agonistic and antagonistic mechanisms to ensure absolute control and proportionality of the immune response, as an excessive immune response can lead to autoimmune diseases and a defective response would allow the tumour to progress. Therefore, there are multiple mechanisms that can be modulated by the tumour tissue in its favour to escape the immune response. The goal of cancer immunology is to restore the immune response against the tumour, leading to the elimination of the tumour (Boussiotis, 2016; Chen et al., 2013)

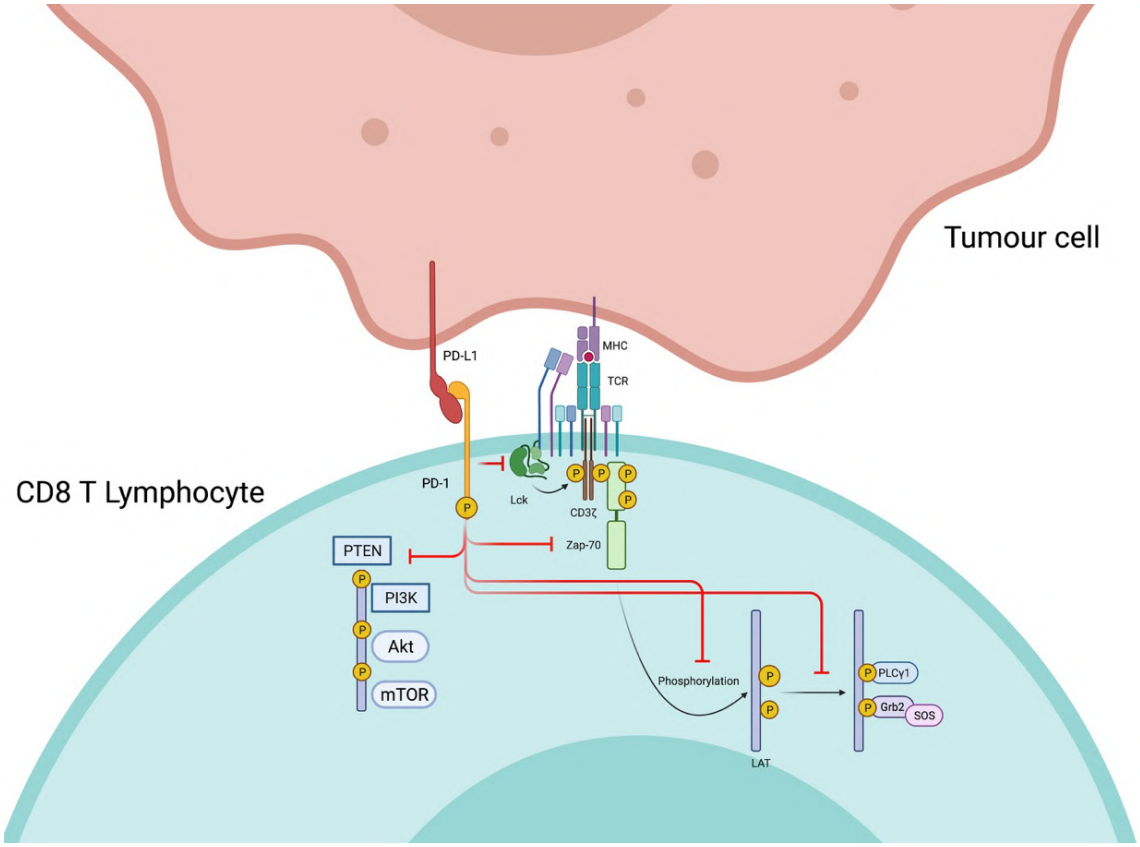


Figure 9. Following PD-1 binding to PD-L1, the tyrosines in the PD-1 carboxi-termuinus region become phosphorylated and inhibits the phosphorylation chain that should occur in signalling dowstream the TCR, including Lck, ZAP-70 and the Pi3K-Akt-mTOR pathways (Boussiotis, 2016).

Immunotherapy and PD-1/PD-L1 immune-checkpoint in OSCC.

The development of immune checkpoint blocking monoclonal antibodies as cancer therapy has brought hope in the battle against this disease. One of the most promising pathways appears to control of the PD-1/PD-L1 immune checkpoint (Zandberg & Strome, 2014).

In the evasion of the immune system by OSCC, there is a large TME involving the production of inhibitory cytokines such as IL-10, which hinders the presentation of tumour antigens by APCs and the priming and activation phase. There is also a decrease in IL-12 (and an increase in CTLA-4, PD-L1 and prostaglandins, which leads to inhibition of antigen activation and recognition by CD8 T lymphocytes at the lymphatic level (Chen et al., 2013).

Upon lymphocyte infiltration, the OSCC increases VEGF secretion, hindering the infiltration phase by immune cells. The complexity to carry out the recognition of tumour antigens increases, as OSCC presents to a great extent, mutations in genes of the major histocompatibility complex type I (HLA-I) and in the B2-microglobulin, TAP1, TAP2 and tapsin proteins. These mutations drive defects in the recognition of antigens and, therefore, induce certain tolerance of the IS (Chen et al., 2013).

Finally, there is an increased secretion of TGF-ß and PD-L1, which is a great weapon by the tumour, as it leads to CD8 T cell apoptosis (Chen et al., 2013).

Immunotherapy focused on blocking the PD-1/PD-L1 immune-checkpoint represents a paradigm shift in cancer treatment and rendered great therapeutic improvements in a variety of cancers, such as melanoma, non-small cell lung cancinoma (NSCLC), renal cell carcinoma, bladder cancer, Merkel cell carcinoma, hepatocellular carcinoma, among others (Boussiotis, 2016; Meng et al., 2015; Sharpe & Pauken, 2017).

The introduction of PD-1 monoclonal antibodies into clinical practice has improved prognosis without compromising the quality of life in patients with metastatic and recurrent HNSCC. Pembrolizumab as a sole agent or in conjunction with platinum-based chemotherapy has become a new standard in these patients and nivolumab monotherapy is also a standard treatment for the platinum-refractory disease. New immunotherapy focused on the use of checkpoint inhibitors such as PD-1/PD-L1, and their combination with CTLA-4 inhibitors represent the new future of this type of treatment. For now, the only standardised way to attempt to predict the response of PD-1/PD-L1 inhibitor drugs seems to be the combined positive score (CPS) (Yokota et al., 2020).

As immunotherapy of the PD-1 / PD-L1 immune checkpoint, the following monoclonal antibodies can be found: Nivolumab and pembrolizumab, whose can inhibit the activity of PD-1, and Durvalumab, which is capable of inhibiting PD-L1 (Raju et al., 2018). Clinical trials with other monoclonal antibodies such as durvalumab are also currently taking place in earlier stages.

Currently, there is no consensus on which immunohistochemical biomarker should be used to assess PD-1 or PD-L1 expression in head and neck cancer. Regarding these biomarkers, the most indicated at present is Dako's PD-L1 IHC 22C3 pharmDx, as it is the one indicated for pembrolizumab, approved by the Food and Drug Administration (FDA) for the treatment of HNSCC tumours and currently used in different malignancies such as bladder, breast, colorectal, oesophageal, gastric, NSCLC, melanoma, ovarian, paediatric and other solid tumours (Kwok et al., 2016).

Published data for head and neck tumours focus on platinum-resistant metastatic recurrent disease. Among the clinical trials that explore the immunotherapy approach to immune checkpoints, we can find the checkMate-141 clinical trial is a study that was conducted in platinum-resistant patients with HNSCC metastatic disease. This is a randomized phase III trial studying Nivolumab versus methotrexate, docetaxel, or cetuximab. It was carried out in 360 patients. Median survival for the nivolumab group achieved a superior survival, was 7,5 months, compared with 5,1 months for the group receiving the other therapy (Yokota et al., 2020).

Regarding pembrolizumab, we can find Keynote-012 and Keynote-055, they are a phase IB clinical trials in which the study drug is pembrolizumab. Multiple cohorts were established, demonstrating a durable objective response as monotherapy in platinum refractory HNSCC. Since this clinical trial, the FDA approved pembrolizumab to treat of platinum-refractory metastatic or recurrent HNSCC in 2016. The drug demonstrated clinically meaningful antitumour activity with an aggregate response rate of 16% with an average 8-month response duration (Yokota et al., 2020).

Besides, we also can find advanced clinical trials such as Keynote-048, a randomised phase 3 clinical trial in which participants were assigned to pembrolizumab as monotherapy, others to pembrolizumab added to platinum and 5-fluorouracil, or cetuximab added to platinum and 5-fluorouracil. In this clinical trial, the use of CPS was applied to assess PD-L1 expression instead of tumour proportion score (TPS) in patients. The results of this trial indicated that pembrolizumab significantly improved OS when PD-L1 expression was CPS $\geq$ 20 and CPS $\geq$ 1. Likewise, results of improved OS in cases treated with pembrolizumab plus chemotherapy were also found to be significantly better

than the cetuximab added to platinum and 5-fluorouracil at CPS ≥ 1 and CPS ≥ 20 (Yokota et al., 2020).

In addition to the PD-1 / PD-L1 immune checkpoint, different pathways are capable to regulate the immune response. Some function as activators of the response, and on the other hand, there are receptors that, as PD-1, have the function of promoting immune tolerance. For instance, CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) is a transmembrane protein present in lymphocytes. Its main characteristic is to produce anergy of CD4 T-lymphocytes during the priming synapse. (Panduro et al., 2016). Although CTLA-4 occurs typically at the priming synapse, it can also be expressed on TILS in the TME and can perform T reg actions.

For this immune checkpoint, monoclonal antibodies have been developed with significant results although, with high toxicity, some of these drugs are Ipilimumab and tremelimumab (Selby et al., 2013).

Another receptor with inhibitory activity is lymphocyte activation gene 3 (LAG3), expressed on Treg, dendritic cells, CD8 and CD4 T cells, which competes with the CD4 molecule (with an affinity for CHM-II) for CHM-II. In addition, LAG3 binding to CHM-II can activate intracellular pathways that inhibit TCR activation. Previous studies have shown an increase of LAG-3 in TILs, which invites further investigation.

On the other hand, increased T-cell immunoglobulin, and mucin 3 receptor (TIM-3) in HNSCC has been associated with increased resistance to treatment with PD-1 blocking monoclonal antibodies, as well as increased resistance to treatment with cetuximab. This protein is transmembrane and tends to be expressed to a greater extent in TILs, favouring them to enter a state of anergy upon continued exposure to an antigen (Banerjee & Kane, 2018; Shayan et al., 2016).
Also notable is its role in Tregs in head and neck tumours, T-cell immunoglobulin, and mucin protein-3 (TIM3+) T regs express increased levels of co-inhibitory molecules such as CTLA-4 and higher levels of FoxP3 (Panduro et al., 2016).

Biomarkers

To study the TME, different biomarkers reflecting the expression of different immune cell-associated molecules are used. The biomarkers used to understand the expression of the immune system cells assessed in the present investigation are described below.

Colony stimulating factor 1 receptor *(CSF1R)*

This monoclonal antibody for macrophage colony-stimulating factor receptor is also known as differentiation cluster 115. As indicated above, TAMs play a dominant role as orchestrators of cancer-related inflammation (Cannarile et al., 2017).

The maturation and differentiation of tissue macrophages depend on the activation of tightly regulated pathways. CSF1R is a key receptor in macrophage recruitment, whose ligand is CSF1 or IL-34, and for the survival of the mononuclear phagocyte system and macrophages in particular (Cannarile et al., 2017). The M2 phenotype is a consequence of the continued presence of growth factors such as CSF1 (Cannarile et al., 2017).

This biomarker can also be detected in other CSF1R-expressing cells such as dendritic cells, neutrophils, and myeloid-derived suppressor cells.

PD-1 & PD-L1

PD-1 and PD-L1 are transmembrane receptors. The PD-1 receptor can bind to PD-L1 and PD-L2 ligands on other cells. While PD-1 is expressed on activated T cells, B cells, NKs, macrophages and dendritic cells, PD-L1 is constitutively expressed on T cells, B cells, dendritic cells and Tregs (Kwok et al., 2016).

PD-L1 can bind to CD80 on activated T cells and APCs, thereby inhibiting both cell types (Kwok et al., 2016).

Overexpression of PD-L1 can occur due to genetic mutations carried out in NCs for example, in mediastinal B-cell lymphomas, mutations in the CHM type II and PD-L1 genes have been observed. On the other hand, in Hodgkin lymphoma, amplification of chromosome 9p23-24, where the gene for PD-L1 expression is located, has been found.

Some findings also highlight that Epstein Barr virus (EBV) causes an increased expression of PD-L1 (Kwok et al., 2016).

FOXP3

Forkhead Box P3 (FoxP3) has played a key role in the immunosuppressive functions of Tregs and as a marker for these cells (Song et al., 2016 (Schreiber et al., 2007). This has been confirmed by the finding that translation of the FoxP3 gene is sufficient to produce differentiation of native T cells into Tregs (Khattri et al., 2003).

Tregs are characterised by constitutive expression of CD25. In addition, there are other surface markers such as GITR, CTLA-4, CD39 and CD73 and high levels of folate receptor 4 (FR4) (Schmetterer et al. 2012), whose presence is related to the development and function exerted by Tregs. However, none of these markers is specific, as they are also expressed in activated T cells, so at present, the expression of the transcription factor FoxP3 is the most specific marker for their identification and study (Stasikowska-Kanicka et al., 2018). On the other hand, FoxP3 can modulate the expression of such molecules as CD25, CTLA-4 and the expression of cytokines such as IL-2, among others (Zheng et al., 2007).

P16

P16 is a marker for the p16 protein, which is derived from the suppressor gene CDKN2A, present in HPV-positive tumours and deleted in HPV-negative tumours.
Overexpression of p16 occurs when pRB is inhibited by viral E7.

One of the most important findings in the study of head and neck carcinomas is the importance of the knowledge of human papillomavirus positivity and its relationship with a better prognosis in patients. This knowledge has been critical for the treatment in patients with head and neck tumours that underwent a decrease in aggressiveness and intensity (Isayeva et al., 2012).

Immunohistochemical study of p16INK4a is commonly used clinically as an indirect biomarker, but interobserver subjectivity limits its usefulness. The immunohistochemical

criteria to consider a p16-labelled sample positive is that at least 70% of the NCs are stained at the nuclear and cytoplasmic level, an assessment that may vary depending on the observing clinician (Bishop et al., 2015).

However, a more important limitation is that overexpression of p16 protein can occur in other alterations of cell cycle regulators, such as p14, p53, CD4, EGFR and RB1 mutations, therefore, p16 positivity can occur independently of the presence of HPV, which can occur in up to 8% of head and neck tumours (Schlecht et al., 2011).

Detection of viral DNA by PCR is a simple technique with high sensitivity; however, it is not standardised for use in conjunction with p16 detection by immunohistochemistry and clinical results. Currently, one of the most effective methods to assess virus positivity in tumour cells is the PCR study of the mRNA of the oncogenic proteins E6 and E7, as this test shows high specificity and sensitivity when combined with immunohistochemical detection of p16 (Andersson et al., 2011; Jordan et al., 2012).

JUSTIFICATION AND OBJETIVES

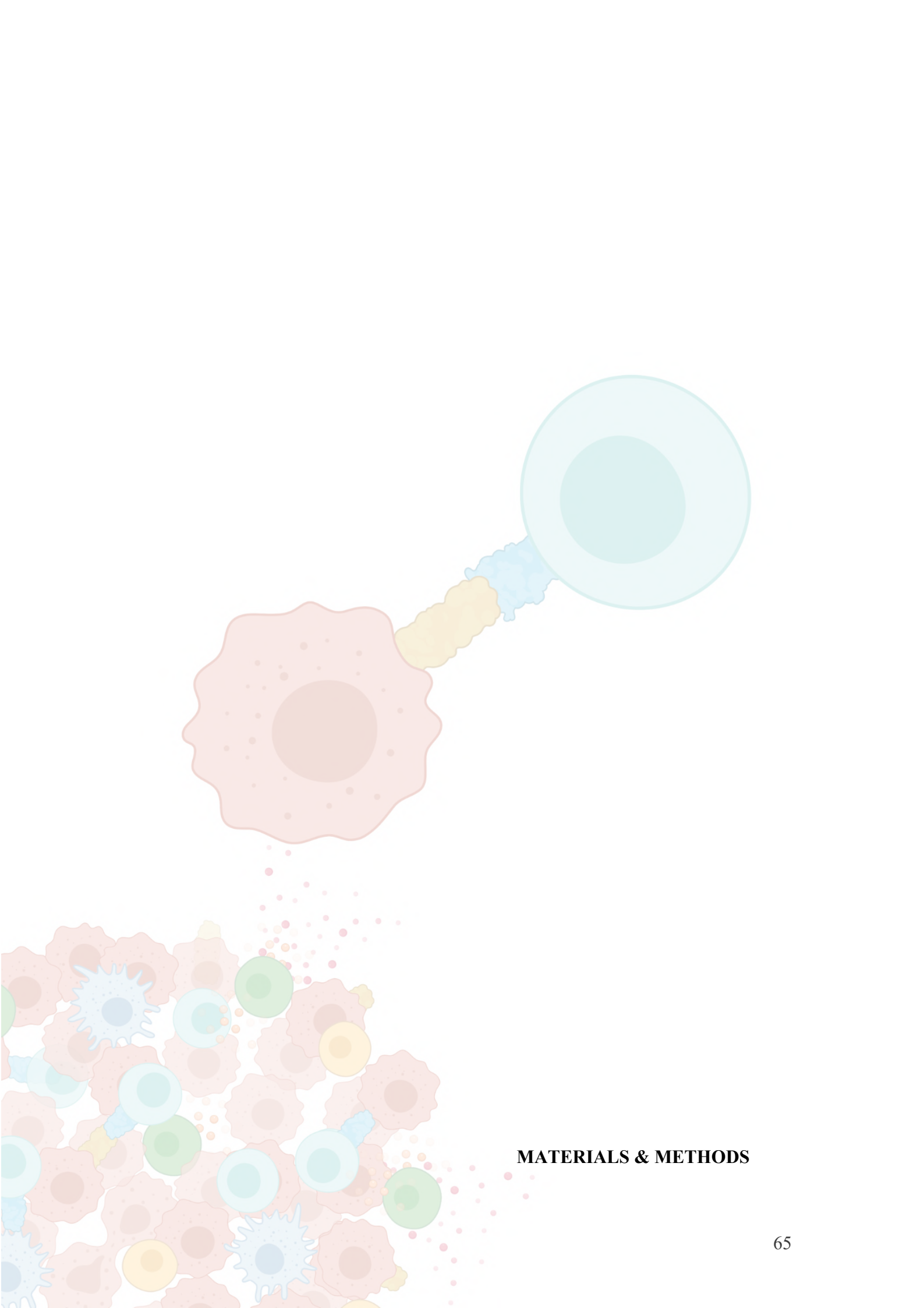
The TME that exists around OSCC has has been studied in head and neck cancer (Peltanova et al., 2019), but not yet in sufficient depth (Mohan et al., 2019). As clinical trials in recent years have evaluated drugs whose therapeutic targets are a variety of immune checkpoints (Ferris et al., 2016; Cohen et al., 2019; Mehra et al., 2018; Segal et al., 2019; Weiss et al., 2017) a better understanding of this tumour and its TME may clarify the usefulness of immunotherapy.

Objetives

1. To understand the relationship of the immune-checkpoint PD-1 / PD-L1 with the clinical evolution of OSCC.

2. To assess rates in OSCC based on TME biomarkers and histologic risk score.

3. To evaluate the clinical and histopathological relationship of OSCC with immunological TME.



MATERIALS & METHODS

MATERIALS & METHODS

This was a retrospective epidemiological study that included 65 patients diagnosed with OSCC in the anterior tongue or floor of the mouth, treated at the Oral and Maxillofacial Surgery Department and the Pathological Anatomy Department of La Paz University Hospital (HULP) in Madrid between 2010 and 2015.

Collection of clinical data

Inclusion criteria: Patients with an anatomopathological diagnosis of primary OSCC after surgical resection in the anterior tongue (C02.0, C02.01) and/or floor of the mouth (C04) between 2010 and 2015.

Exclusion criteria: 1. Patients who had been treated with oncological therapy, either pharmacological or radiotherapeutic before surgical removal of the tumour. 2. Patients whose diagnosis was an oral carcinoma with microinvasion. 3. Patients with missing relevant information or with insufficient histological material to be able to perform the histopathological analysis.

The clinical variables sex, age, smoking habit (never, current, former), drinking habit (never, current, former), the primary location of the tumour, the dates of diagnosis and treatment (surgery, radiotherapy, chemotherapy), the presence of recurrences (local, regional, distant) and the presence of oral potentially malignant disorders (OPMD) were collected based on the latest classification of these lesions (Warnakulasuriya et al., 2020). The characteristics of the neoplasia, such as tumour size and the presence of regional or distant metastases, were recorded according to the latest tumour–node–metastasis (TNM) classification of the head and neck region of the AJCC (Amin et al., 2017).

Preliminary anatomopathological analysis and selection of histological blocks

The tissue samples came from the Pathology Department of the HULP. Each sample was analyzed through a multiview light microscope at the same time by three independent observers. Prior to the evaluation of the samples, the three observers came to a consensus on how to determine the histological features of each sample and on a comparison for

immunohistochemical interpretation. Samples were studied in random order and blinded to the clinical information of the patient.

Based on the histopathological characteristics, the most representative paraffin blocks of each case were selected. We had previously assessed all the histological samples, confirming that it was an objective and representative sample of the tumour.

Histological features were evaluated using the histologic risk assessment model (Brandwein- Gensler et al., 2005; Brandwein-Gensler et al., 2010). Following the definitions laid out for this histologic risk model, the lymphocytic host response (LHR), the worst pattern of invasion (WPOI), and PNI were considered. After categorization according to this classification, the tissues were divided into three groups (risk 0-1, risk 2-3, risk 4-7) to facilitate statistical analysis. See figures 10-15.

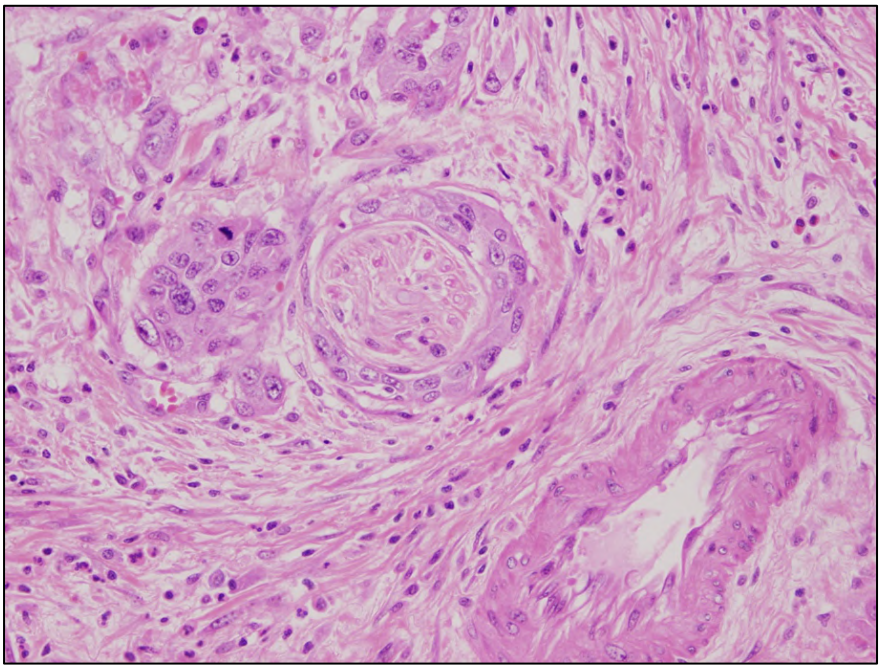


Figure 10.
Perineural invasion >1 mm in a poorly differentiated tumour, with a sparse lymphoplasmacytic, infiltrate, whose DOI was 1.1 mm, with a WPOI type 2 and therefore a risk score of 6. Sample of a 40x magnification.

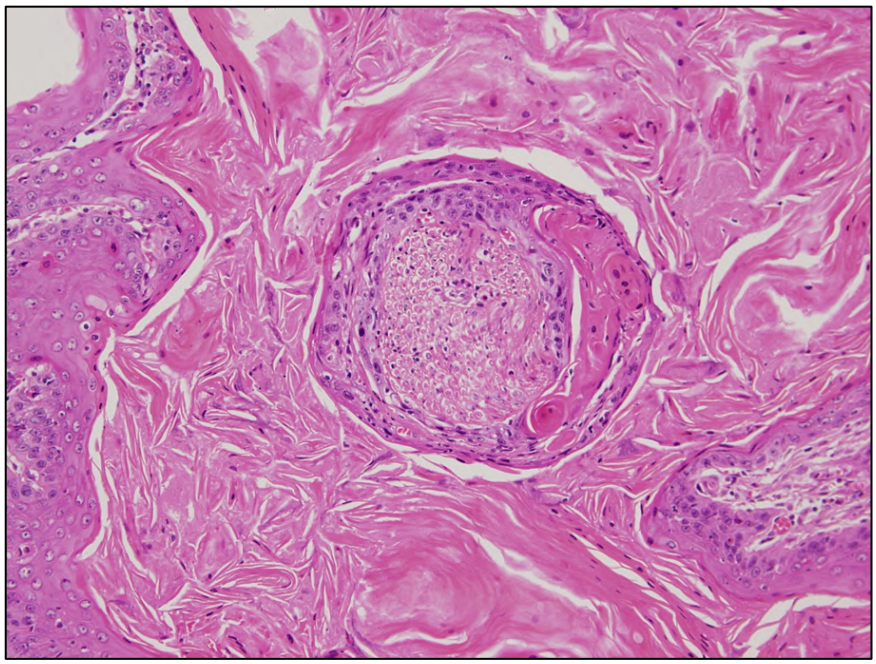


Figure 11.
Perineural invasion recorded by microphotography. It occurred in a moderately differentiated OSCC.

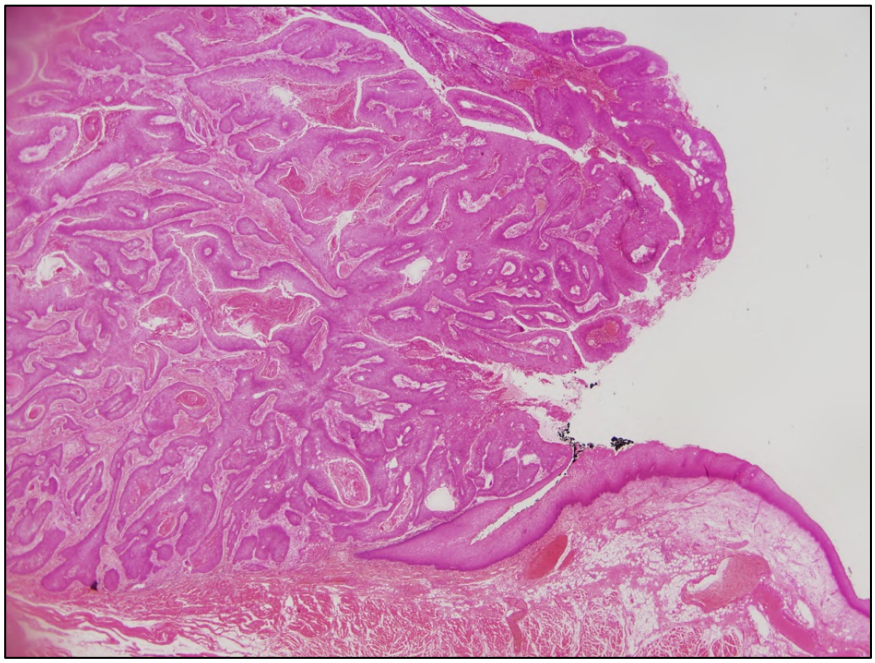


Figure 12.
Microphotograph (10x magnification) of a well-differentiated OSCC with exophytic growth, sparse lymphoplasmacytic infiltrate and

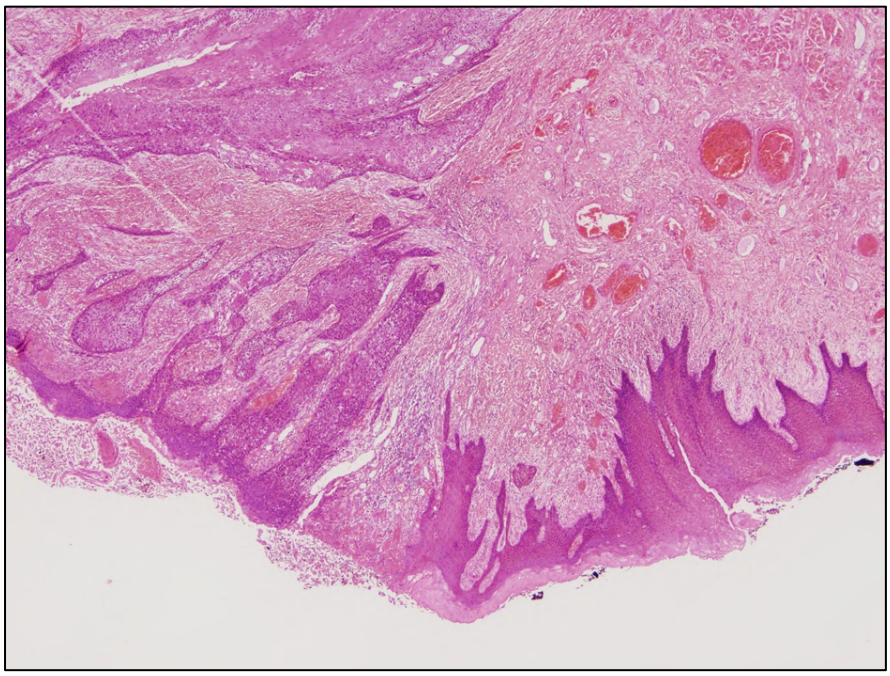


Figure 13.
An ulcerated, moderately differentiated tumour is observed, with a scarce inflammatory infiltrate, with a WPOI type 3.

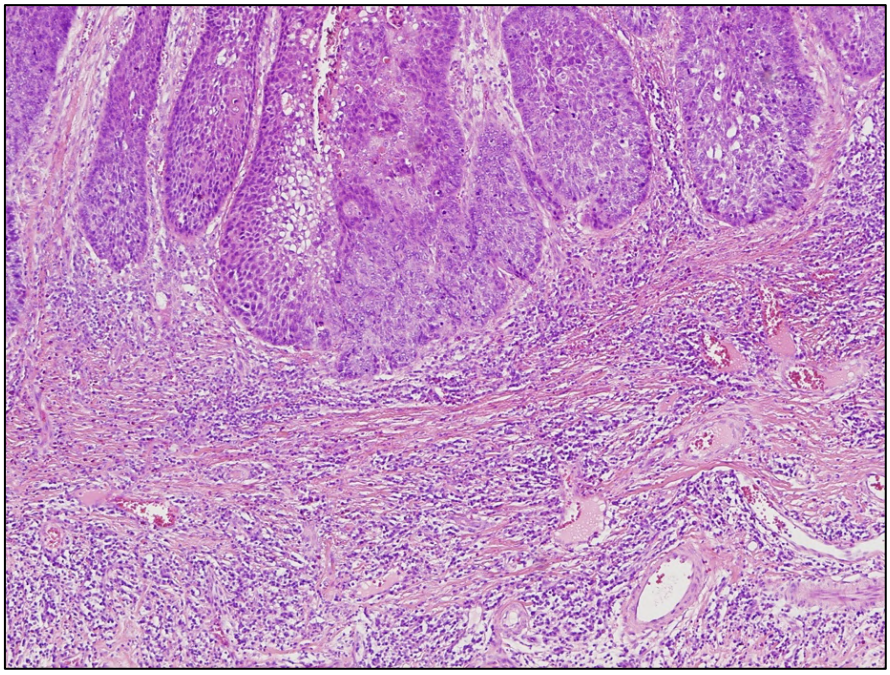


Figure 14.
Moderately differentiated OSCC with expansive growth (WPOI 1). Moderate lymphoplasmacytic infiltration can be seen. The DOI recorded is 5 mm. Risk score type 11. 20x magnification

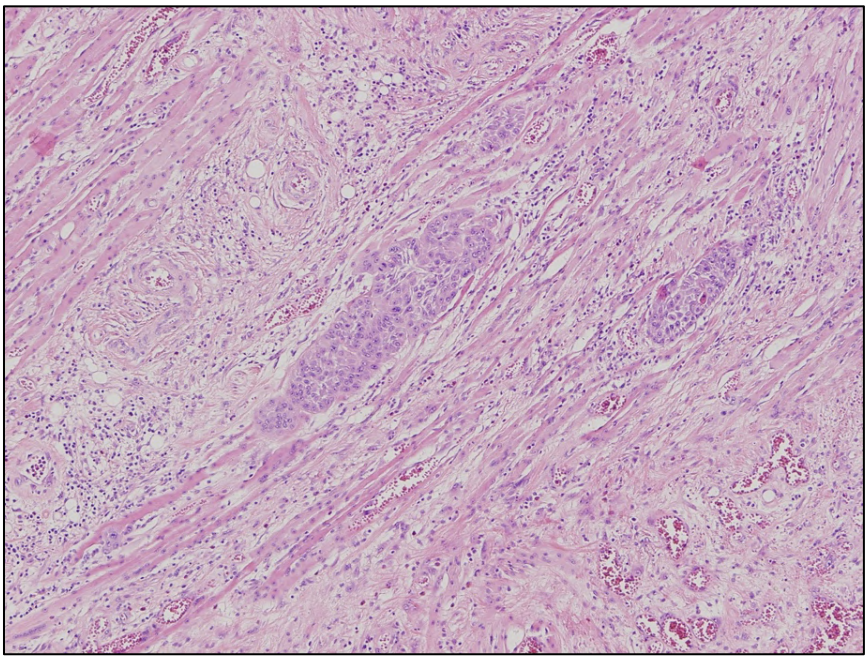


Figure 15. Photomicrograph of moderately differentiated OSCC with little lymphoplasmacytic infiltration. The specimen had affected margins with DOI >10mm. The present case had a WPOI 5 and perineural invasion.

The histological grade of the tumour was also recorded, classifying it as poorly (PD), moderately (MD), or well differentiated (WD) according to the criteria of the World Health Organization (WHO) (see figures 10-15), as well as whether the tumour presented vascular and/or lymphatic invasion (Thomson, 2006; El-Naggar et al., 2017). See figure 16.

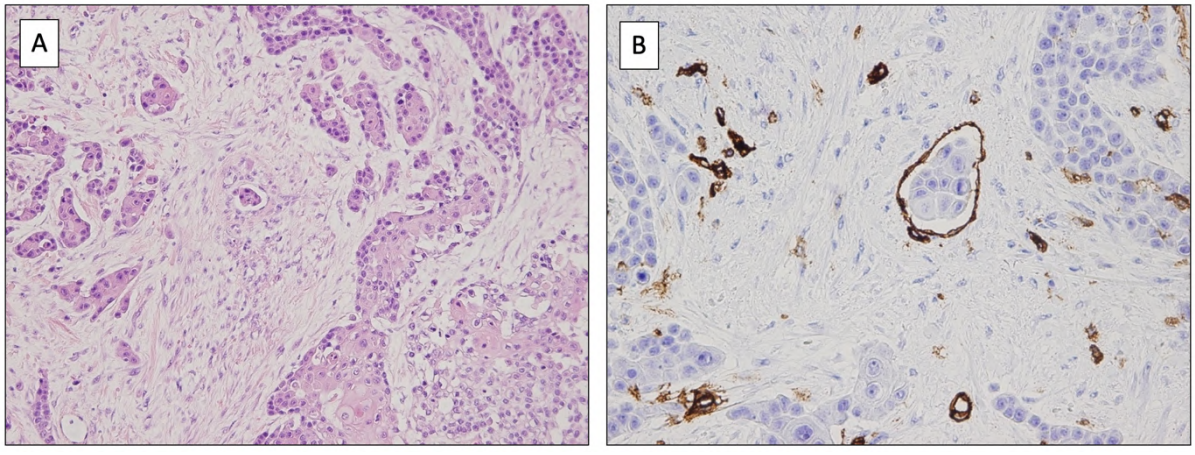


Figure 16. A. Histological microphotograph (20x magnification) of a histological slice in hematoxylin-eosin of a moderately differentiated OSCC showing a lymphovascular invasion. B. The same sample subjected to the CD31 immunohistochemical marker in which this invasion is confirmed (40x magnification).

To record the DOI, we first determined whether the lesion was exophytic or ulcerated. Then a horizontal line was drawn delimiting the basal membrane and a vertical line ("plumb line") from this to the invasion front of the tumour, to classify it as a mild (≤ 5 mm), moderate (> 5 mm and ≤ 10 mm), or deep invasive lesion (> 10 mm) (Lydiatt et al., 2017). See figure 17.

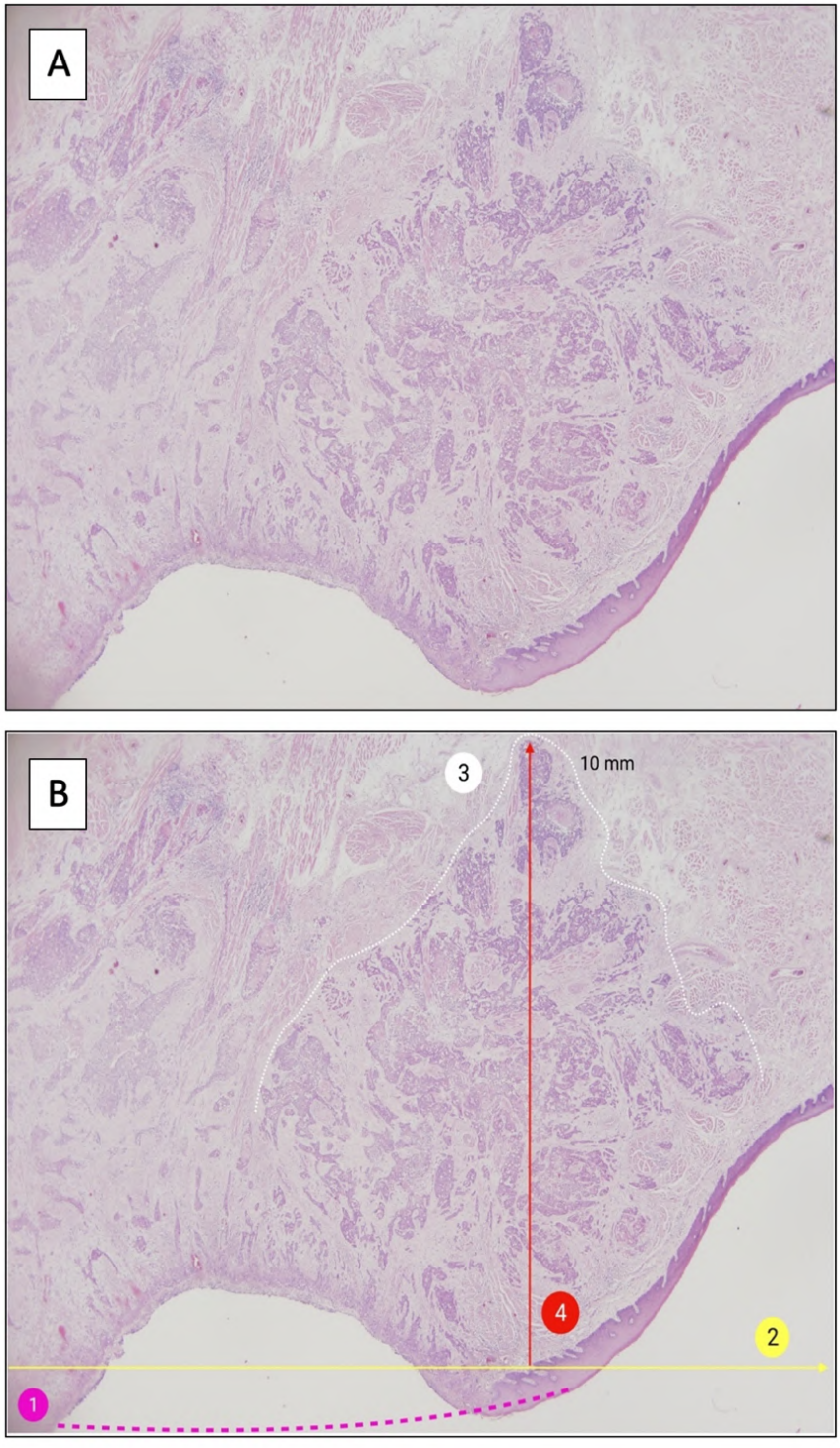


Figure 17.
Methodology for the DOI analysis in a sample of OSCC (10x magnification). Firstly, it must be determined whether the tumour is exophytic or has presented ulceration to draw a true trajectory of the tumour perimeter (in pink), that avoids over- or under-estimates of the length of the DOI. Secondly, the position of the epithelial basement membrane (yellow) is determined by drawing a first horizontal line. Thirdly, the sample is analysed to determine the exact tumour invasion (white front). Finally, a vertical plumb line is drawn from the horizontal line of the basement membrane to the tumour invasion front, the latter being the one to be measured and giving the result in millimeters. In the present case, the DOI corresponded to 10 mm.

Immunohistochemistry

Immunohistochemical staining was performed as follows: Two-metre-thick sections were prepared from formalin-fixed, paraffin-embedded tissue blocks. After this, all sections were oven-dried overnight at 60° C. The sections were placed in a Bond Max Automated machine.

Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Wetzlar, Germany) according to the following procedure. First, the tissues were deparaffinised and pre-treated with Epitope Retrieval Solution 2 (EDTA buffer pH 8.8) at 100°C for 20 minutes. After washing, for a period of 10 minutes, a peroxidase blocking was carried out with the Bond DC9800 polymer detection kit (Leica Microsystems GmbH).
The tissues were washed again and then incubated with the primary antibodies for 30min. Tissues were incubated with the polymer for 15 min and then with DAB-Chromogen for 10 min. In parallel, positive and negative controls of human tonsils were performed. As controls for the technique, incubations omitting the specific antibody or containing unrelated antibodies were used.

Primary antibodies were used following this technique: PD-1 (antibody type: mouse monoclonal; clone name: NAT105, dilution: 1:4 supernatant; source: CNIO), FoxP3 (antibody type: mouse monoclonal; clone name: 236A, dilution: prediluted; source: CNIO), CD4 (antibody type: mouse monoclonal; clone name: 4B12, dilution: prediluted; source: DAKO), CD8 (antibody type: rat monoclonal; clone name: NOR132H, dilution: 1:5 supernatant; source: CNIO), CSF1R (antibody type: mouse monoclonal; clone name: FER216, dilution: 1:20 supernatant; source: CNIO).

The immunohistochemistry for PD-L1 (antibody type: mouse monoclonal; clone name: 22C3, prediluted, source: DAKO) was performed on 3-µm tissue sections that had been deparaffinised in an oven at 60 °C for 20 minutes and unmasked in a buffer of low pH at 97 °C for 20 minutes, all of which took place in the Autostainer Link 48 system with EnVision FLEX reagents (K8002) (DAKO). The tissue was incubated with primary antibody for 30 minutes, endogenous peroxidase inhibitor for 10 minutes, a secondary antibody for 30 minutes, diaminobenzidine for 10 minutes, and hematoxylin for 7 minutes, followed by buffer, distilled water, an ascending series of alcohol, and xylol.

For the p16 biomarker (antibody type: mouse monoclonal; clone name: E6H4; source: Roche), histological sections were made at 3 µm and then deparaffinised in an oven at 70 °C for 15 minutes, followed by histological unmasking in pH buffer high at 95 °C for 20 minutes. The section was incubated in the primary antibody for 30 minutes, followed by washing in buffer. Then, endogenous peroxidase was inhibited for 10 minutes, and theminutes. After this came another buffer wash, diaminobenzidine for 10 minutes, hematoxylin for 7 minutes, buffer, distilled water, ascending alcohol, and xylol. Staining was carried out using an automated OMNIS system (DAKO).

The biomarkers PD-L1 and p16 were also detected with positive and negative controls in the study samples. Table 3 summarise all the biomarkers used in this research. See figure 18.

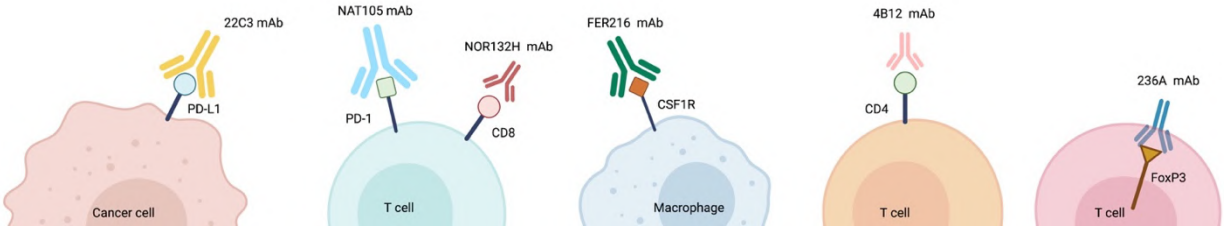


Figure 18. Molecular targets of the different biomarkers used in the study.

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Immunohistochemical observation

The immunohistochemical interpretation was performed by the three observers with the same initial criteria. The area studied was the peripheral intratumoral component. Nuclear staining for FoxP3 was considered to identify Tregs. CSF1R in the membrane and cytoplasm was considered to identify tumour-infiltrating macrophages (TAMs). CD4 and CD8 at the membrane level were considered to study tumour-infiltrating lymphocyte (TILs). PD-1 and PD-L1 were considered positive expressed when the staining was only at the membrane level.

For the objective scoring of the expression of the biomarkers, the samples were observed under a light microscope at magnifications of 10×, 20×, and 40× for discernment in cases of weak staining. We calculated the percentage of expression of the biomarkers except for PD-L1, for which the following three scoring systems were used:

1. TPS: defined as the percentage of viable tumour cells showing partial or complete membrane staining for PD-L1 relative to all viable tumour cells present in the sample (positive and negative). Cytoplasmic staining, as well as infiltrating immune cells, normal cells, necrotic cells, and debris, do not go into the score. See figure 19.

2. CPS: defined as the number of PD-L1-positive cells (tumour cells, lymphocytes, macrophages) divided by the total number of viable tumour cells, multiplied by 100. Although the result of this calculation can exceed 100, the maximum score is defined as 100. CPS was stratified into three groups: <1, ≥1, and ≥20. See figure 19.

3. Intensity: recorded with the classical classifications of negative, weak, moderate, and strong.

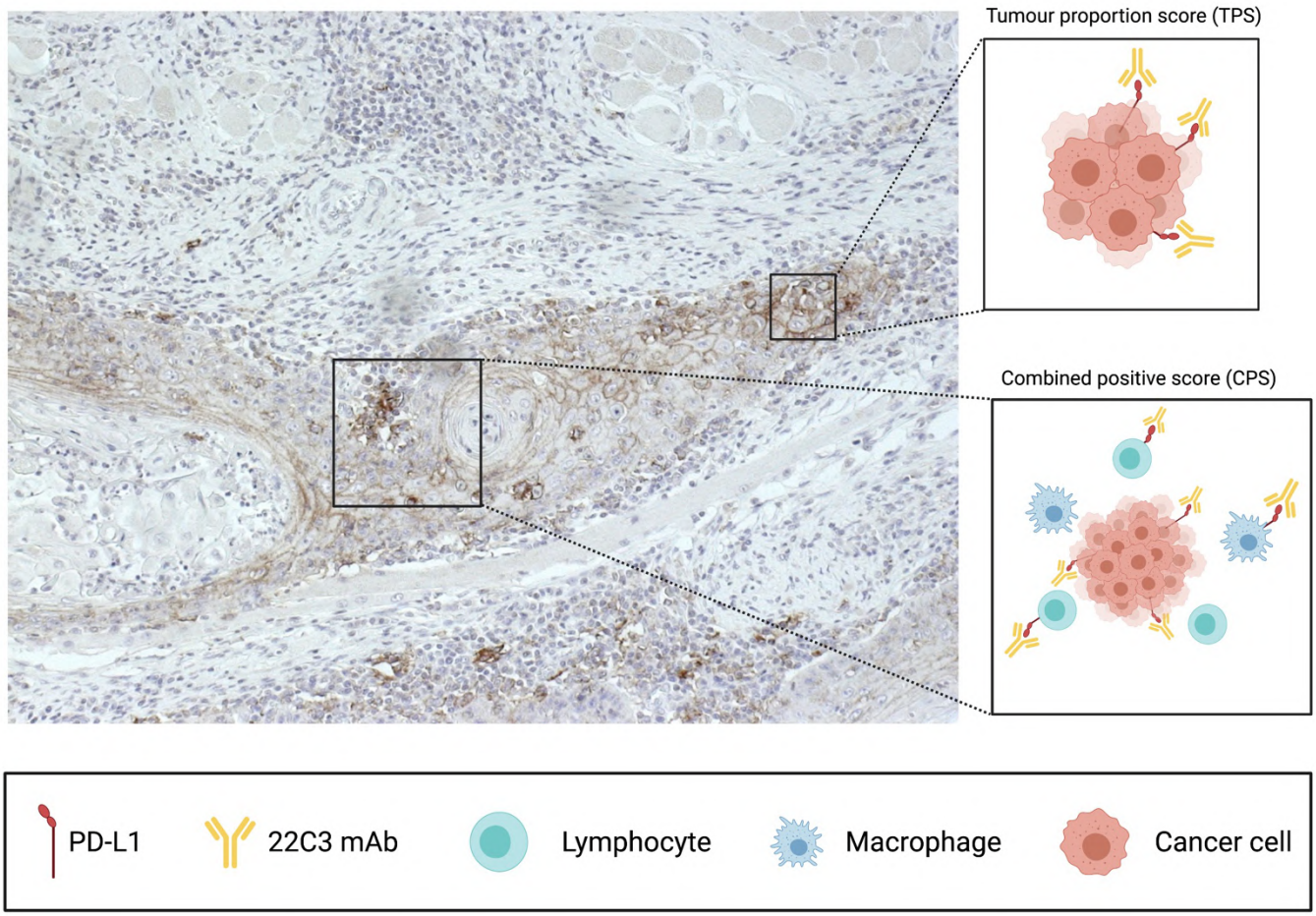


Figure 19. This image shows a histological sample of OSCC, the definition of SCP and TPS. TPS comprises only tumour cells whereas, in the case of SCP, the immune cell component (macrophages and lymphocytes) is also considered. Therefore, to analyse a sample, PD-L1 expression could be recorded using these two recording methods.

For PD-1, tumours with an expression percentage > 0% were considered positive. Tumours with CPS > 1 and TPS ≥ 5% were considered positive for PD-L1. See figure 20 and 21.

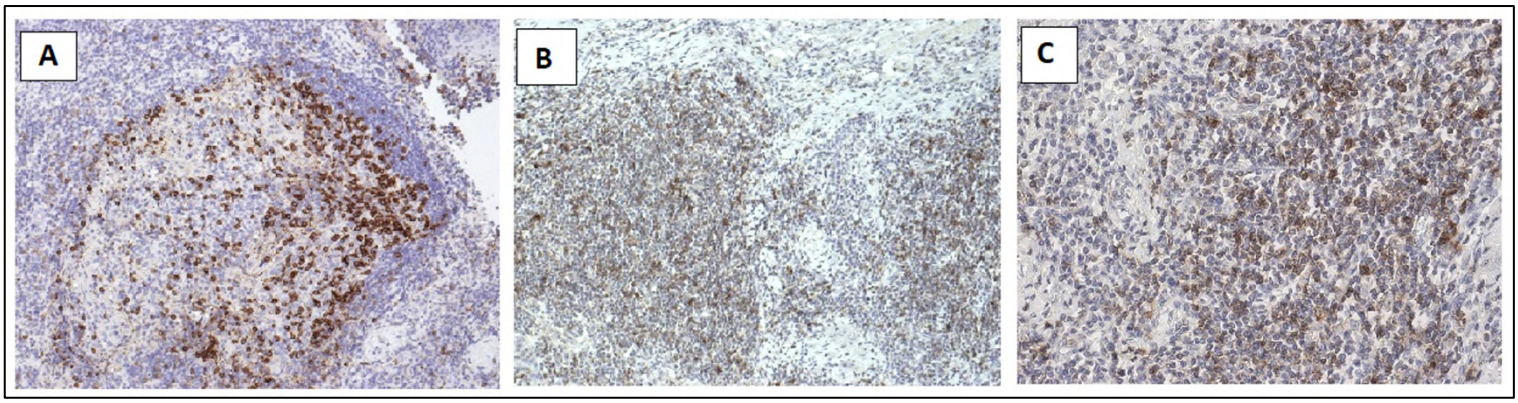


Figure 20. Positive control of PD-1 with 10x magnification (A), B and C: histological section with an expression of 7% PD-, magnification was 10x (B) and 20x (C).

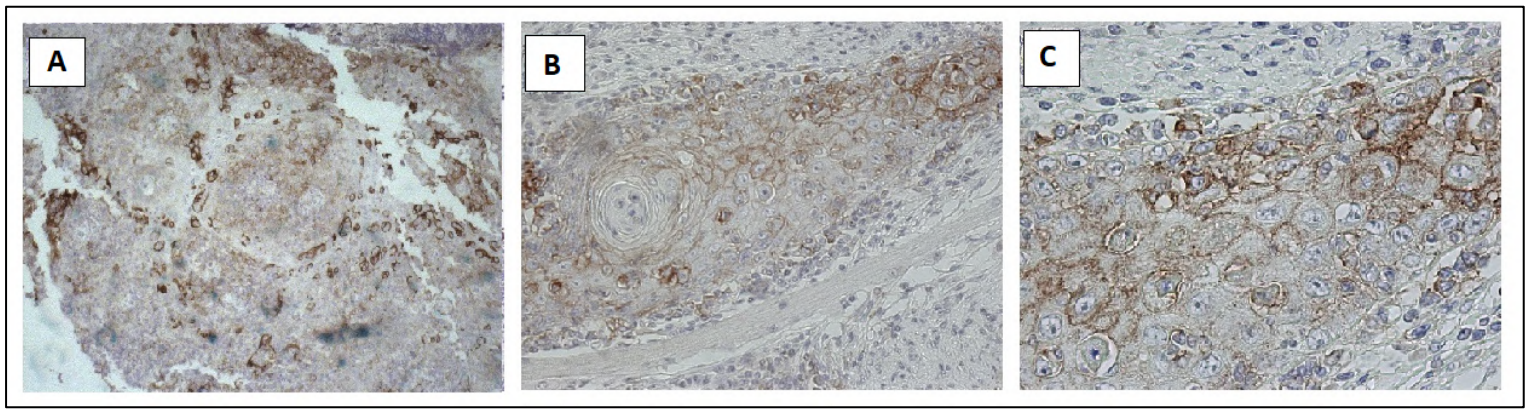


Figure 21. Positive control of PD-L1 with 10x magnification (A), E and F. Microphotographs showing 75% (TPS) PD-L1 expression (CPS: 85) at 20x (B) and 40x magnification (C).

To assess the positivity of human papillomavirus (HPV) through p16, the criteria of the College of American Pathologists were used. Positive cases were considered those whose tumour tissue showed nuclear and cytoplasmic immunoreactivity in ≥ 70% of the cells (Lewis et al., 2018). After the immunohistochemical study, positive cases were re-evaluated by PCR. $p16^{+}$ cases by both immunohistochemistry and PCR were considered positive. See figure 22.

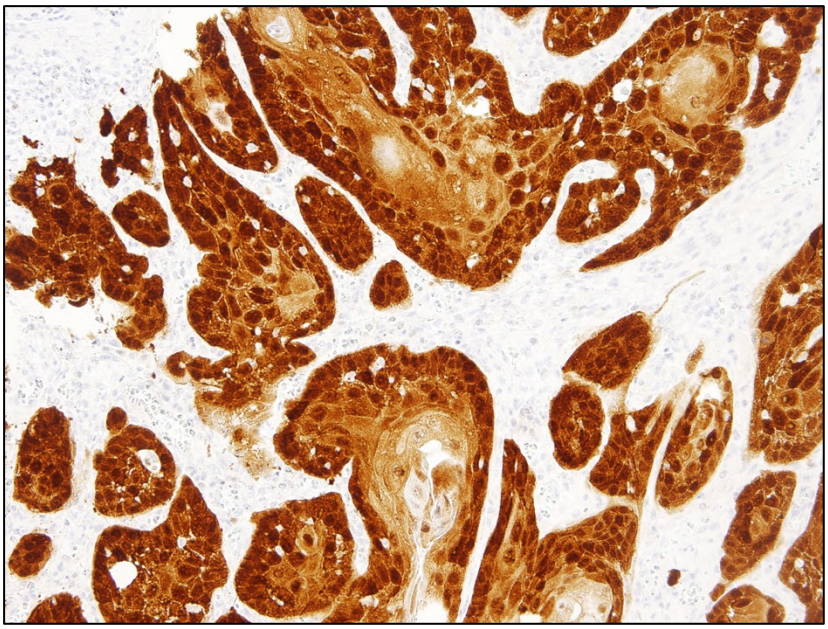


Figure 22.
Well-differentiated tumour, with moderate inflammatory infiltrate, WPOI type 3 and with a DOI of 0.8 mm, being risk score 1. The present case was p16 positive. 40 x magnification photomicrograph microphotograpy

The other biomarkers were categorised for statistical analysis as follows: CD8 (expression of 0-10% (mild), 10-50% (moderate), ≥50% (severe)); CD4 in three groups (tertiles): 5-25%, 25-35% and 35-50%; FoxP3 in four groups: 1-5%, 5-10%, 10-20%, and 20-100%; and CSF1R according to the cut-off median in two groups: 0-8% and 8%-100%. See figure 23-26.

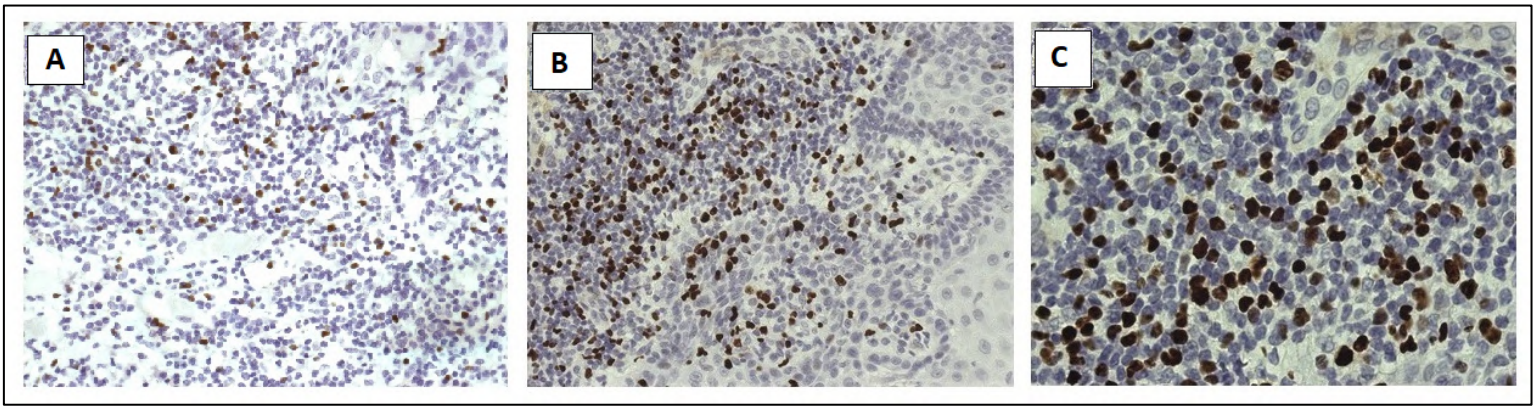


Figure 23. A.Positive control of FoxP3 with 10x magnification, B and C. Image by light microscopy (20× and 40×) of histological sections of a sample whose total expression of FoxP3 was 6%.

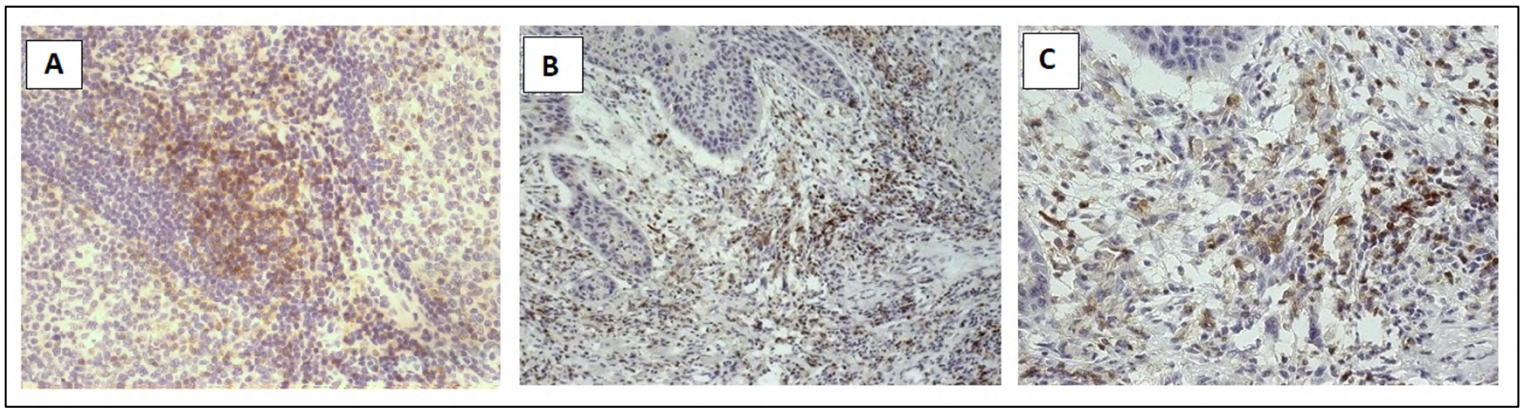


Figure 24. A. Positive control of CD4 with 10x magnification. B and C. 10× and 20× images of the CD4 biomarker in a sample with a total expression of 35%.

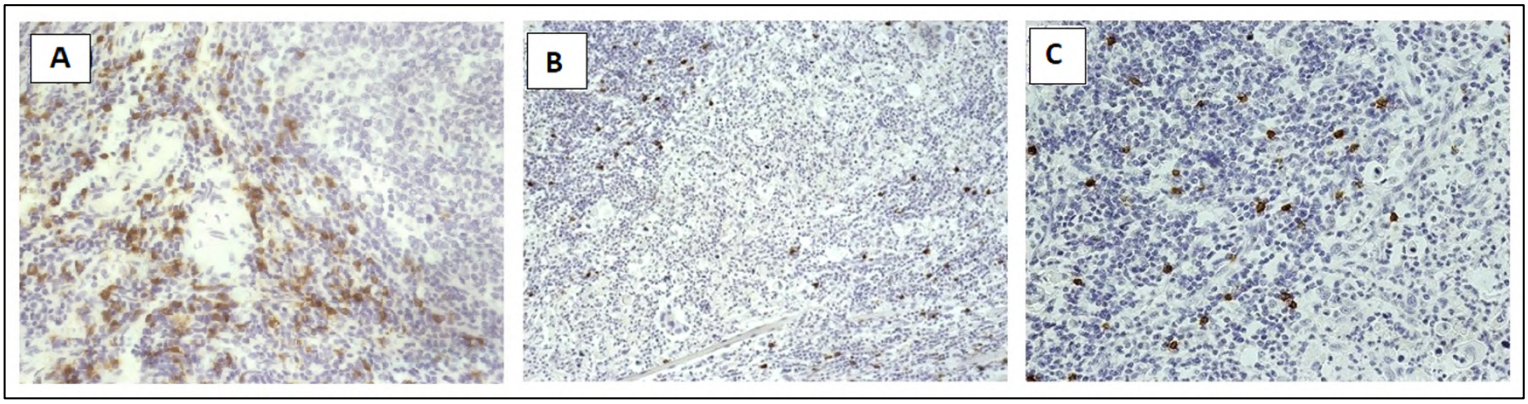


Figure 25. A. Positive control of CD8 with 10x magnification. B and C. Histological section of the expression of 5% CD8 with 5× and 20× magnification.

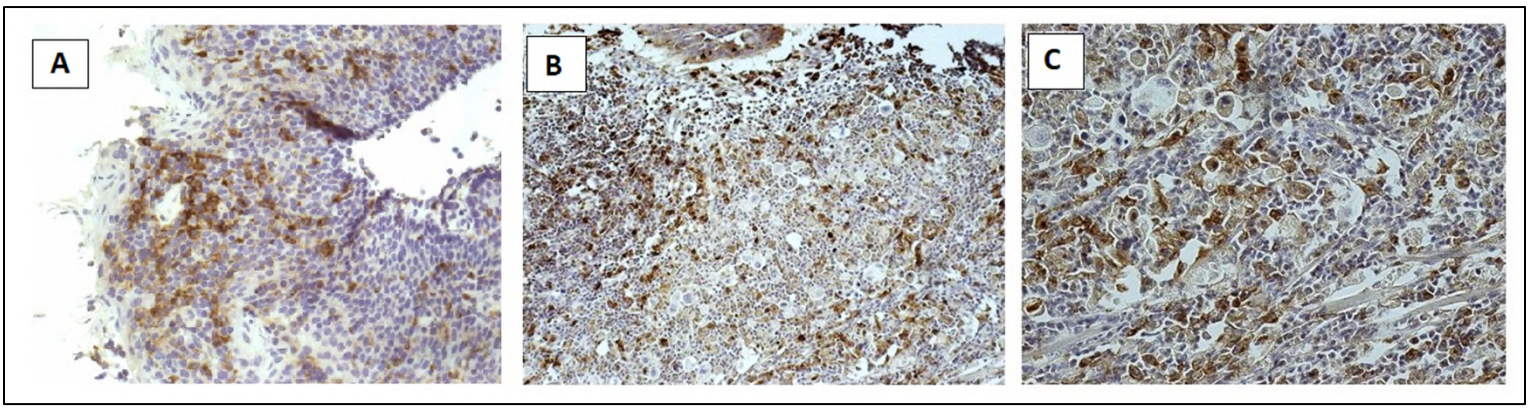


Figure 26. A. Positive control of CSF1R with 10x magnification. B and C. Microphotographs showing 20% CSF1R.

Survival

Patient survival data were collected from clinical records. The follow-up period was from the date of diagnosis to the closest month to the results. Outcomes were defined as follows: death from OSCC, death from other causes, recurrence (local, regional, distant), and alive without recurrence. According to these results, we considered three survival definitions: disease-specific survival (DSS), where only death from OSCC was considered an event; disease-free survival (DFS), where either recurrence (any type) or death from OSCC (but not death from another cause) was considered an event; and overall survival (OS), where events were defined as death by any reason.

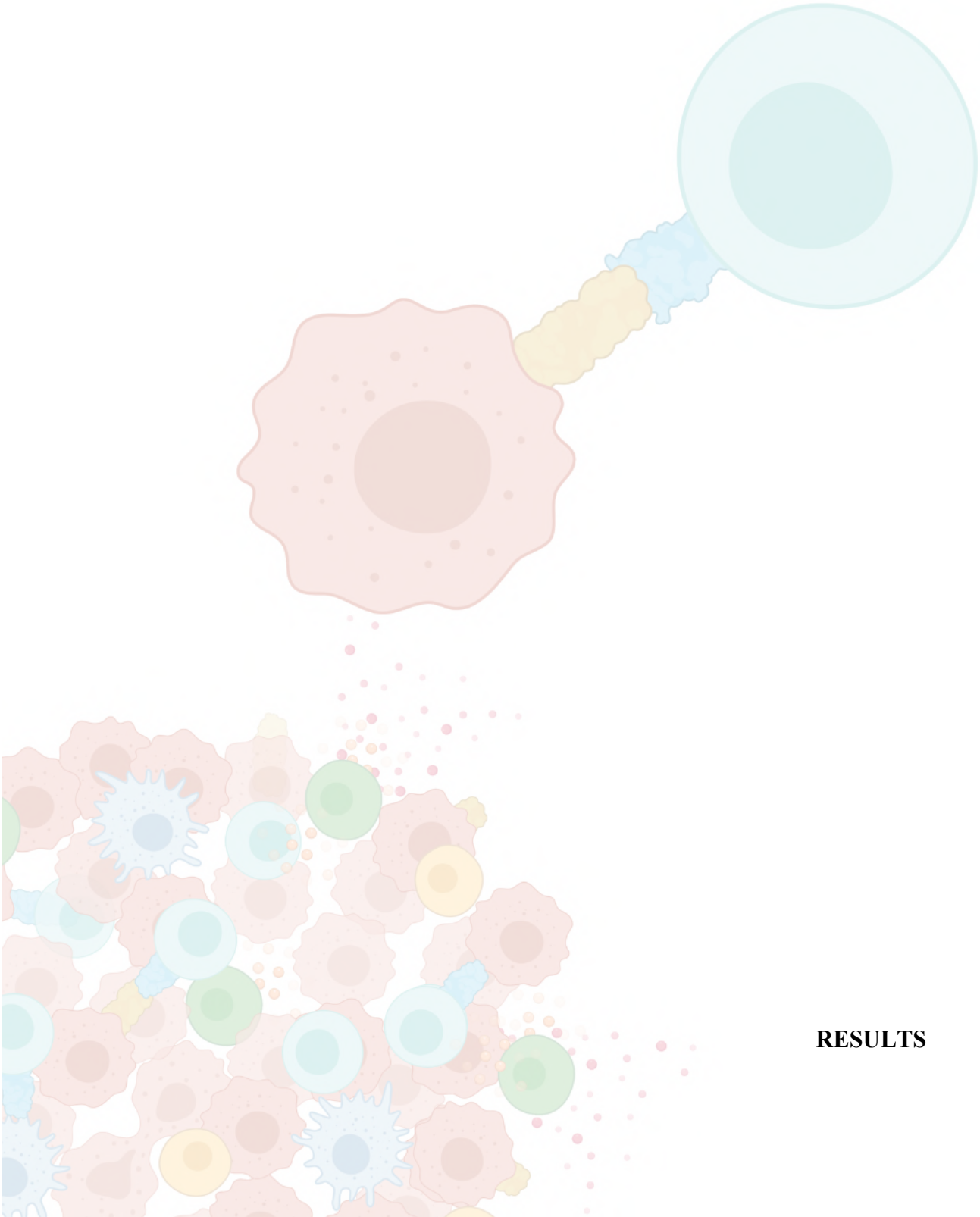
Statistical analysis

Normally distributed continuous variables were described as mean and standard deviation; nonnormally distributed continuous variables were described using the median and interquartile interval. Discrete variables are presented as count and percentage. Differences in continuous variables were tested using Student´s *t-test* if normality assumption was met or the Wilcoxon Rank Sum if not. Homogeneity between proportions was tested using Pearson's chi-squared or Fisher's exact test, as appropriate.

Kaplan-Meier survival functions were plotted using the same cut-off and were compared using the Wilcoxon-Breslow-Gehan test. Incidence rates for the three different survival definitions (DSS, DFS, OS) were estimated as the number of cases divided by the number of person-years of follow-up. The associations between demographic and clinical factors and events for each event definition were analysed using univariate and multivariate Cox proportional hazards models. Multivariate models included those variables associated with a new event in univariate analysis ($p<0.15$) and those considered relevant regardless of the p-value. Proportional hazard assumption was tested using the Grambsch-Terneau test. All models fulfilled the proportionality assumption.

Bivariate correlation between biomarkers was estimated using non-parametric Spearman's rank-order correlation, with p-values adjusted for multiple testing with Holm´s method.

Survival analysis was performed using the R *survival* package (version 6.0-1).Kaplan-Meier curves were plotted using the *ggsurvplot* function from the *survminner* package (version: 0.4.8). Incidence rates and 95% confidence intervals were estimated using SurvRate function from the *biostat3* package (version 0.1.59). All analyses were performed in R (version 4.0.3, GNU GPL-3) via RStudio (version 1.3.959, GNU GPL-3).



RESULTS

RESULTS

Sample selection

The initial sample consisted of 84 patients with a primary diagnosis of OSCC in the floor of the mouth and/or mobile tongue after consulting the database of the Oral and Maxillofacial Surgery Service between 2010 and 2015. In the final selection, 19 patients were excluded (three with non-OSCC neoplasms, three without enough histological material for the study of histopathological features, six with oral carcinoma with microinvasion, and seven with OSCC not in the floor of the mouth or anterior tongue region), leaving a total of 65 to be studied here. See table 4.

Clinical and histopathological characteristics

The clinical and histopathological characteristics of the patient sample are reflected in table 5. The study finally included 40 men (62%) and 25 women (38%) with a mean of 65 years. The mean age was 6 years higher in women (95%CI -2.6;10.6, p-value=0.23). Forty-eight percent had never smoked, and this proportion was higher in women than in men (76% vs. 29%, p=.001). The proportion of nondrinking women was also higher (96% vs. 50%, p<0.001). The most frequent primary location was the tongue (65%), followed by the floor of the mouth (26%). In 9.2% of cases, the tumour was present in both, so 74% of patients had a tumour on the tongue and 35% on the floor of the mouth. Sixty-six percent of tumours were stage III or IV, with a similar distribution in both sexes. A total of 15% of the patients (n=10) presented OPMD before the diagnosis of OSCC, with six cases of lichen planus, four cases of leukoplakia and one case of chronic actinic cheilitis, see table 6 and figure 27.

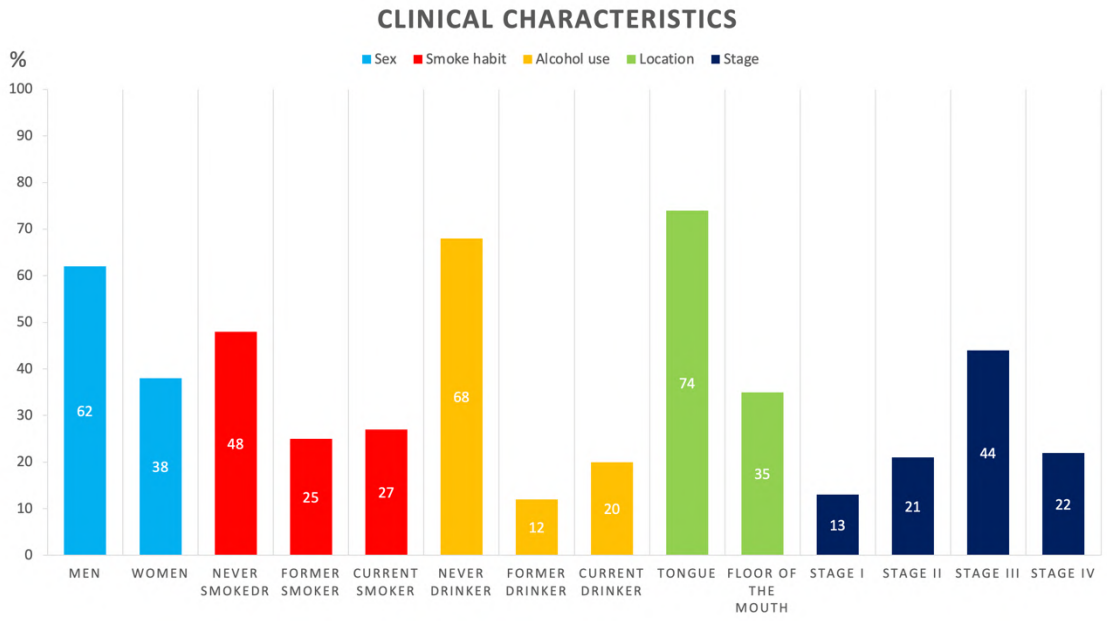


Figure 27. Clinical characteristics of the studied sample. It is represented in a bar graph with the value of the percentages of each variable. Men, non-smokers, non-drinkers, with a main location in the tongue and in stage III were the most common patients in the sample.

From the histopathological point of view, 91% of the tumours were well or moderately differentiated, without differences by sex, and the evaluation of the pattern of WPOI showed that 89% of these were in types 1 to 4. PNI was present in 40% of patients (28% women, 48% men, p=0.118), and vascular invasion was present in 5 (7.7%). Lymphocytic infiltration was complete in five cases (7.7%), moderate or intermediate in 25 (38%), low in 33 cases (51%), and null in 2 (3.1%).

The median DOI was 9 mm. A total of 35% of the patients had mild (≤5 mm), 29% moderate (6-10 mm), and 37% deep (≥10 mm). Four cases had affected margins. The distribution was not different according to sex. See figure 28.

Regarding the risk score, larger tumours (T3 + T4) and a drinking habit were associated with higher (6 and 7) risk histologic scores.

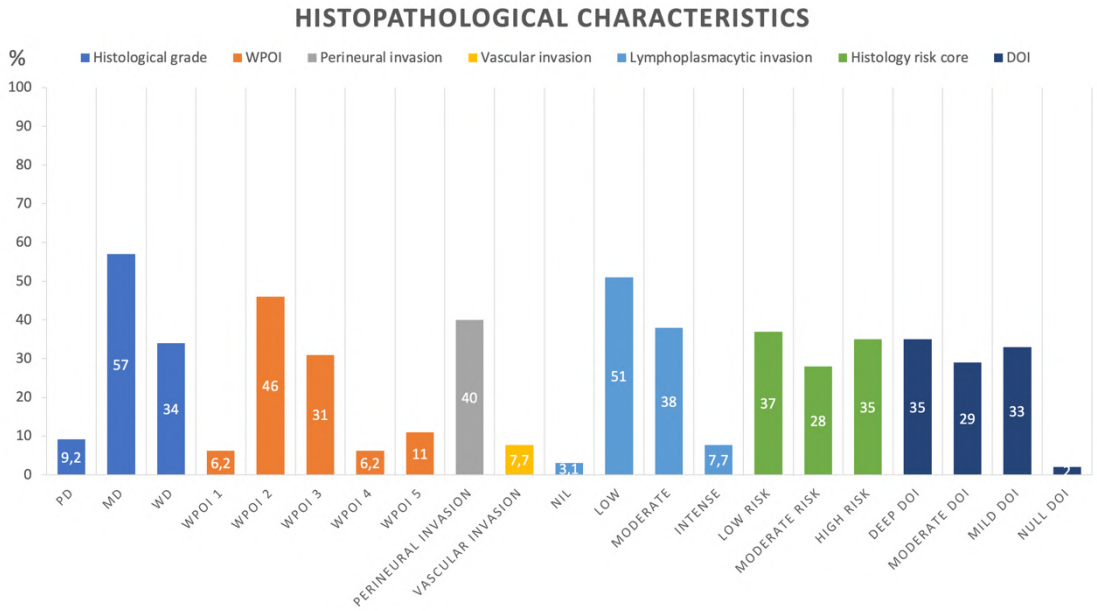


Figure 28. Histopathological characteristics of the studied sample. It is represented in a bar graph with the value of the percentages of each variable. Histologically, the samples showed a moderate degree of histological differentiation, a type 2 WPOI, the degree of lymphoplasmacytic invasion was mainly low or moderate. The perineural invasion was present in 40% of the cases, the vascular one was only 7% of the cases. The risk score had a similar distribution between low, moderate, and high as well as the DOI, which also had a similar number of cases in the deeply, moderately and mildly groups.

Biomarkers

The percentage of expression of each biomarker in the whole sample and in each sex is shown in table 7. The distribution was only normal for CD8, so the correlations were analysed using the Spearman rho coefficient (Table 8). Although none of the correlations was significant in our analysis, the correlation between FoxP3 and CD4 (rho=0.34, p=0.114) was noteworthy. PD-L1 was also positively correlated with PD-1 (rho=0.25, p=0.737) and with CSF1R (rho=0.28, p=0.452), but the correlations were not significant after p-value correction for multiple testing.

PD-1 & PD-L1

The median expression of PD-1 was 1%, and 80% of the tumours analysed (75% in men, 88% in women, p=0.34) were positive (>0%).

PD-L1 expression was evaluated using three scoring systems: CPS, TPS, and staining intensity. The first two had higher medians among women. After categorisation according to the previously suggested cut-offs for each scoring system (CPS>1, TPS≥5%), CPS classified as positive to the 54%, while only 34% of the samples were over >5% for TPS, with 23% of discordant pairs (CPS>1 and TPS<5%, Mcnemar´s test, p-value 0.0037).

The proportion of subjects above the cut-off was slightly higher in women in both cases (CPS>1: 64% vs. 48%, p=0.194; TPS≥5%: 48% vs. 25%, p=0.057). The evaluation of staining intensity using the classical criterion (four categories) revealed a slightly higher proportion of women in the moderate and strong categories (p=0.25). See table 7 and 9.

PD-1 expression

PD-1 positivity was associated with a lower DOI (median DOI 8 vs. 12, p=0.017) (Table 7 and 9). Therefore, PD-1-positive tumours were more often in the less invasive category (WPOI I and II) (39% vs. 17%, p=0.087) and more often were small (67% vs. 33% in category T1 or T2, p=0.073). See figure 29.

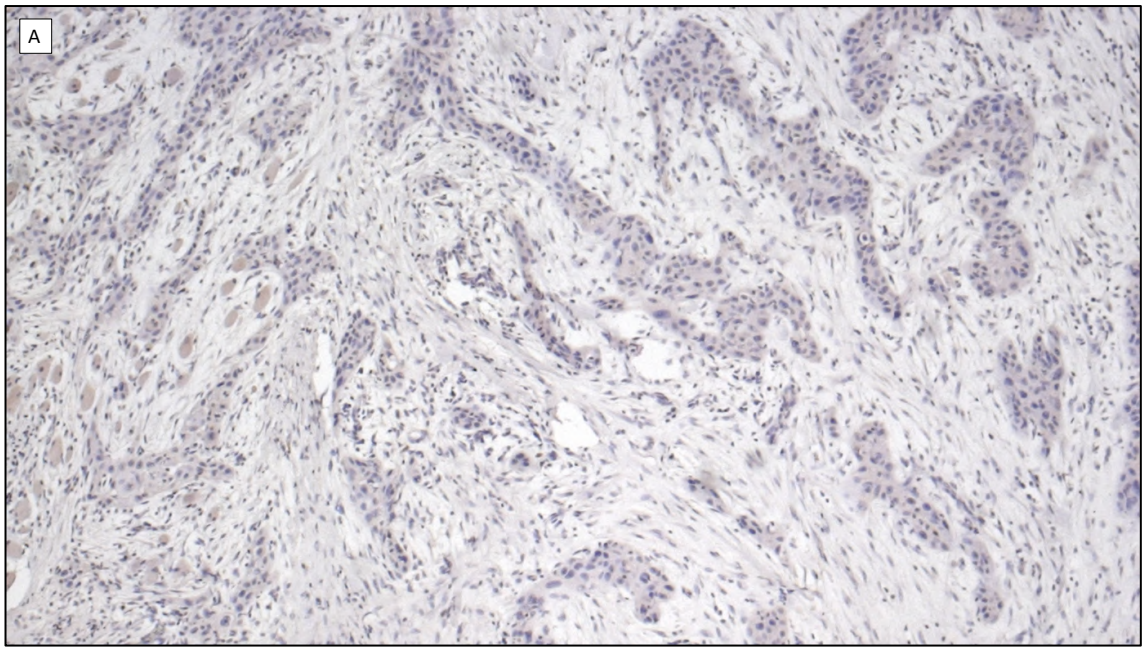


Figure 29A. 10× histological section of a moderately differentiated tongue OSCC sample. Its expression of the PD-1 biomarker was null, making it negative for PD-1 (<0%).

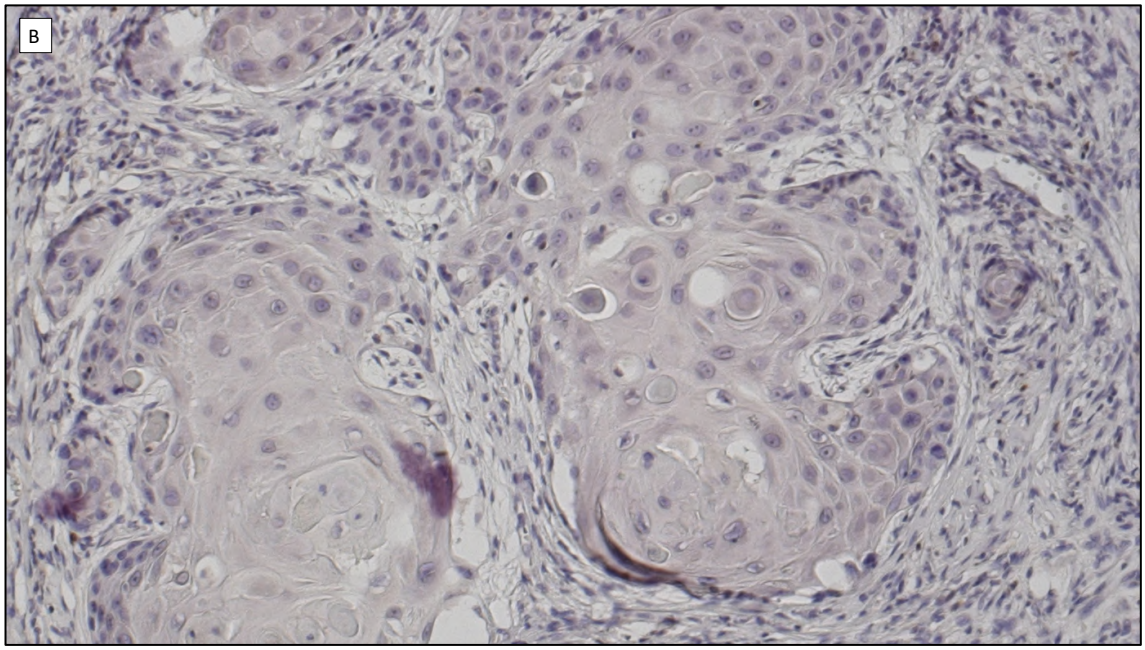


Figure 29B. 40× histological section of a moderately differentiated tongue OSCC sample. Its expression of the PD-1 biomarker was null, making it negative for PD-1 (<0%).

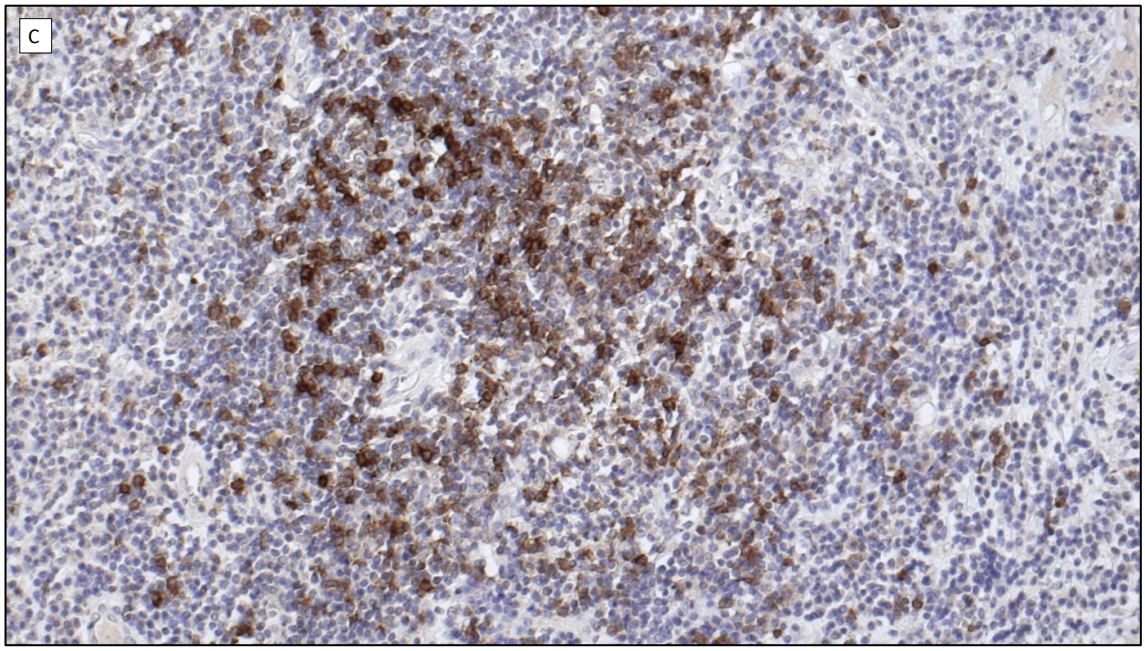


Figure 29C. 10× histological section of a moderately differentiated tongue OSCC sample was observed, with scarce lymphocytic infiltration and a PD-1 expression of 15%, therefore being positive (> 0%).

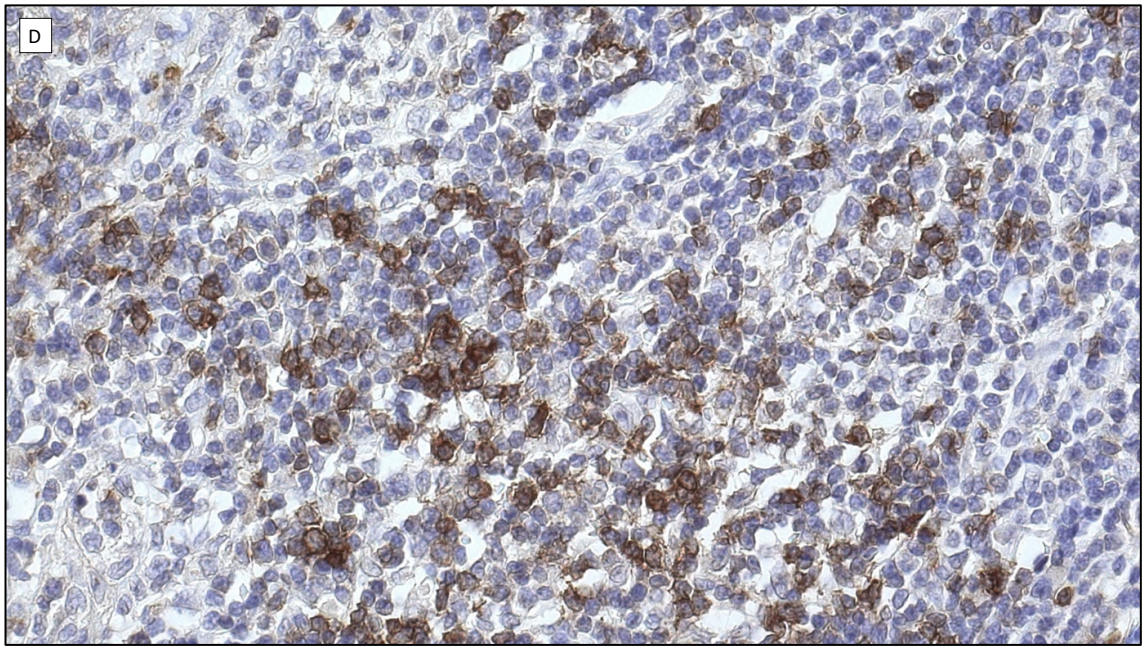


Figure 29D. A 40× histological section of a moderately differentiated tongue OSCC sample was observed, with scarce lymphocytic infiltration and a PD-1 expression of 15%, therefore being positive (> 0%). It is possible to observer the membrane staining of the biomarker.

PD-L1 expression

High PD-L1 expression values (TPS≥5%) were associated with a greater median DOI (10 mm vs. 7 mm, p=0.17) but with a more favourable WPOI (WPOI-5: 0% vs. 16%, p=0.085). When the criterion used was CPS>1, a similar relationship was observed in the WPOI (2.9% vs. 20%, p=0.043). CPS>1 was also correlated with a lower histological risk score (CPS ≤1 risk score 4-7: 47% vs CPS>1 risk score 26%, p-valor 0.043) and a higher proportion of local recurrences (40% vs. 17%, p=0.039) (Table 7 and 9). See figure 30 and 31.

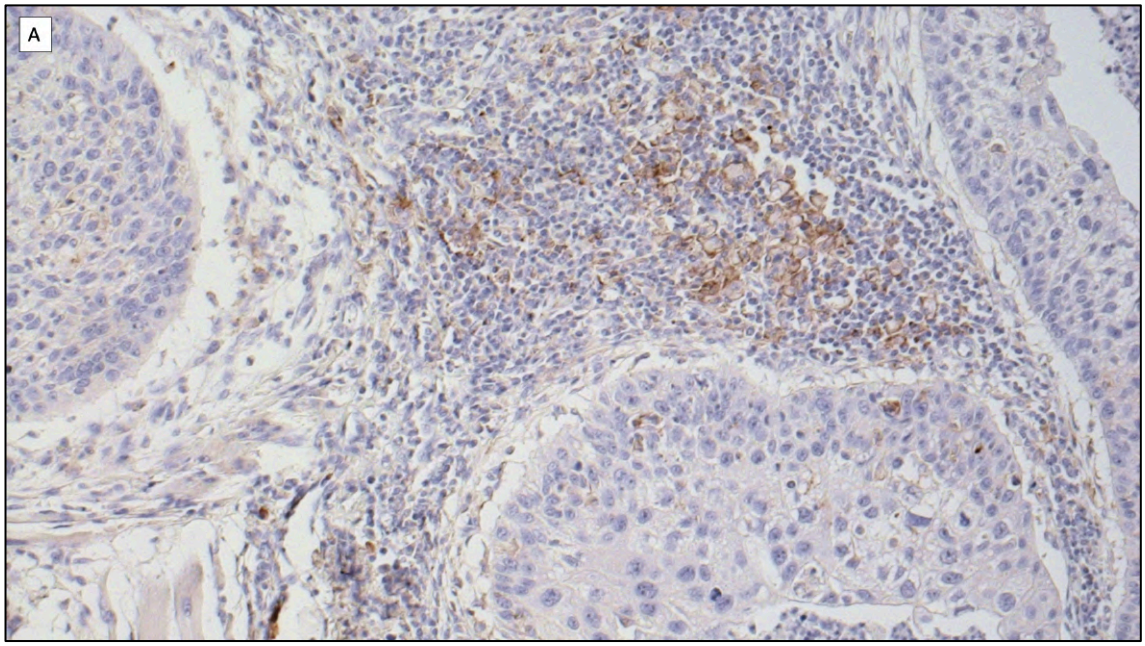


Figure 30A. 10x magnification of an OSCC in the tongue, moderately differentiated and with intense lymphocytic infiltration and a PD-L1 expression <1%.

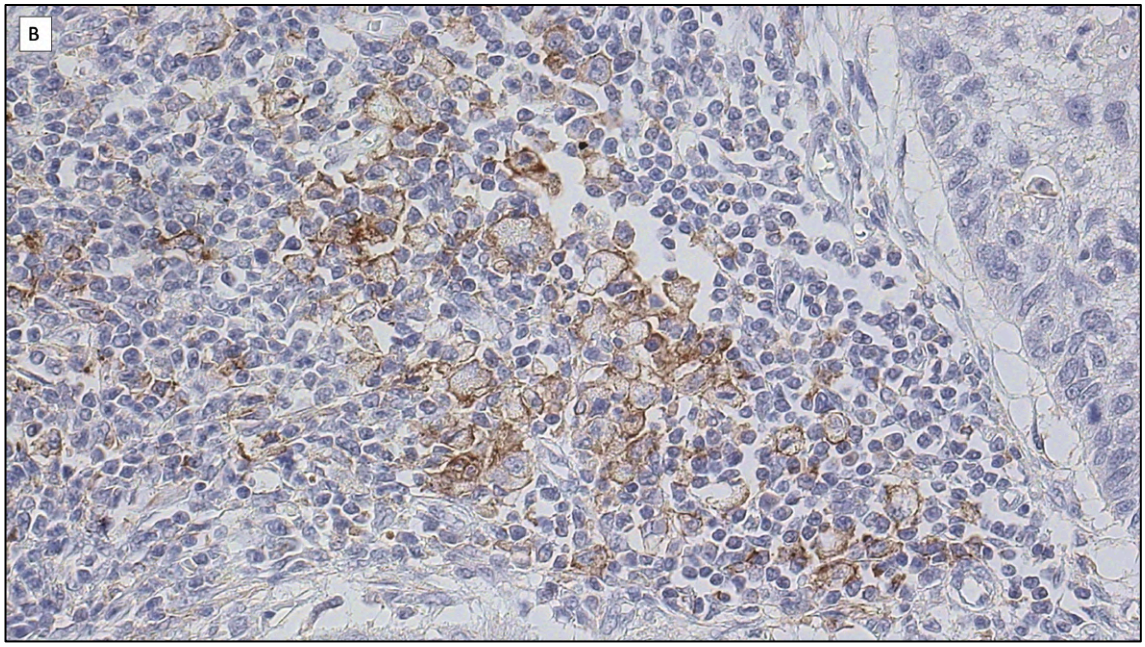


Figure 30B. 20x magnification of the sample indicated above.

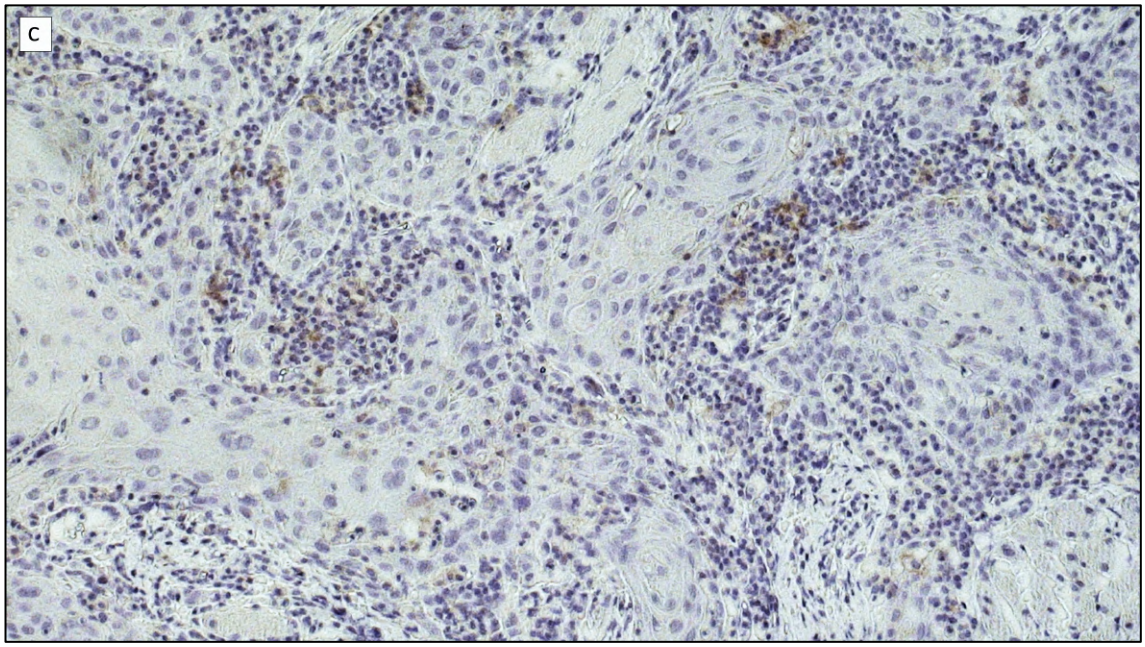


Figure 30C. 10× magnification. Moderately differentiated case with intense lymphocytic infiltration whose PD-L1 expression was >1%.

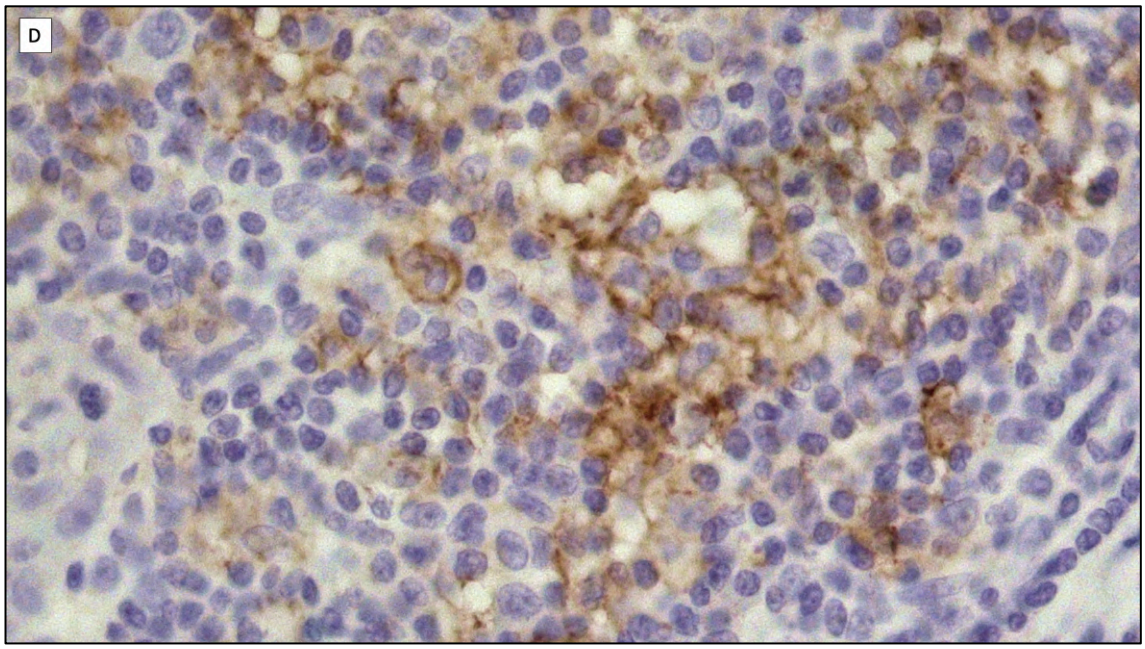


Figure 30D. 20x magnification of the sample indicated above.

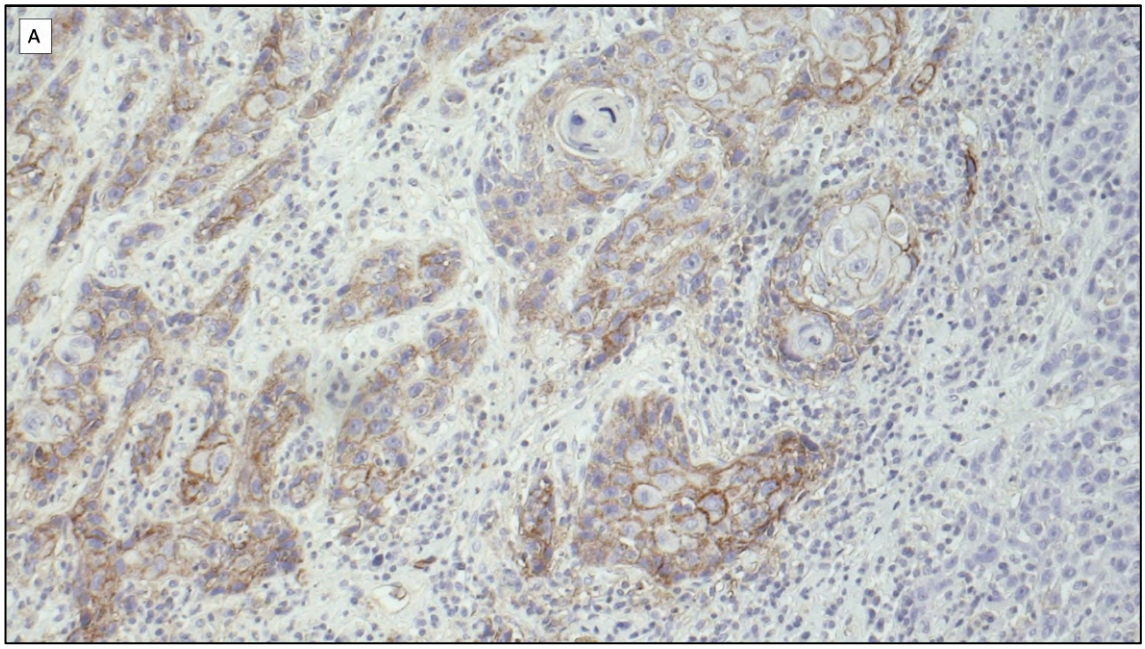


Figure 31A. Case of PD-L1 <5% at 10×. The expression in this case was 2%.

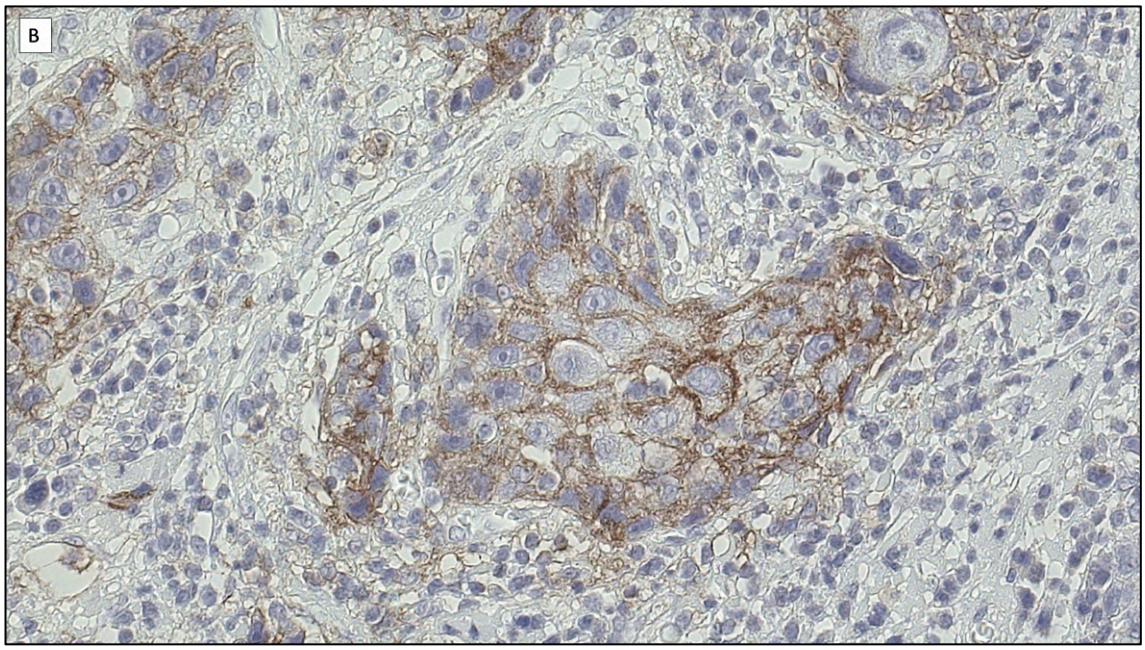


Figure 31B. 20x magnification of the sample indicated above.

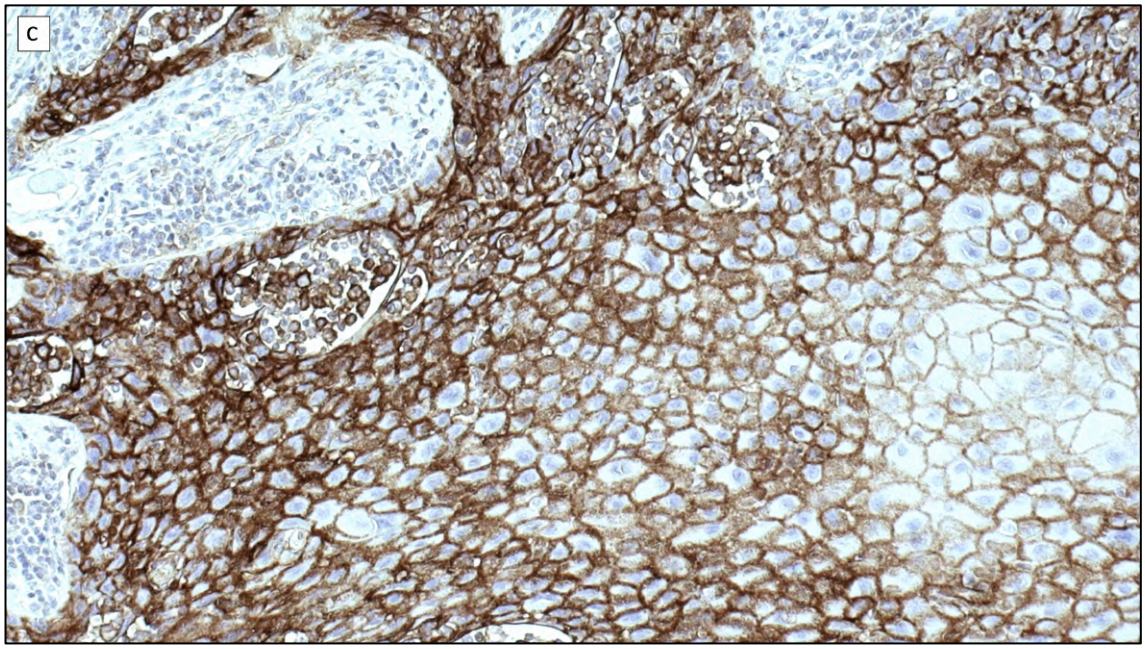


Figure 31C. In this sample, PD-L1> 5% is observed at 10×. The expression of PD-L1 in this case was 80%, and a granular linear membrane staining pattern was exhibited.

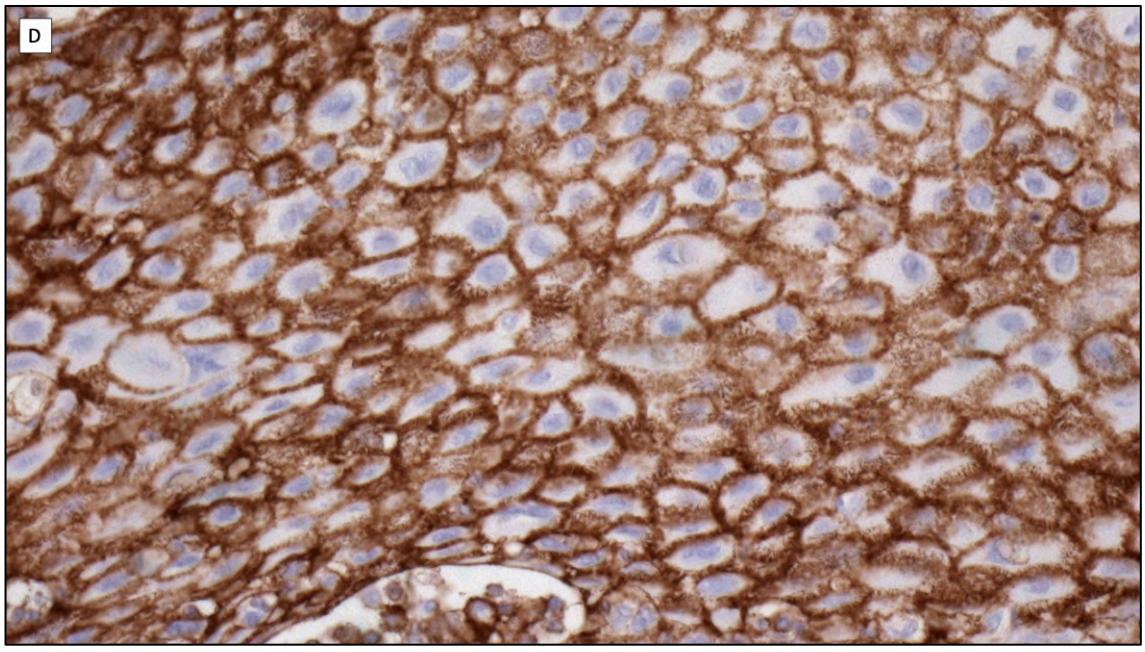


Figure 31D. The case above of PD-L1> 5% observed at 20×.

FoxP3 expression

FoxP3 expression above 10% occurred mostly in T2 tumours (p=0.066). There was a trend towards higher DOI the lower the FoxP3 expression (p=0.056). See table 10.

CD4 expression

There is a trend towards lower WPOI with lower CD4 expression as 86% of WPOI 3 had CD4 expression between 5-35%, while in WPOI 5, the sample had CD4 expression percentages greater than 35% in the majority way. See table 11.

CD8 expression

A greater distribution of CD8 mild cases was observed in N0 (80%), and as the proportion of CD8 expression increased, nodal involvement increased, with 67% of N2 and N3 cases in the severe category (p=0.089).

Thus, CD8 lymphocyte infiltration correlated with severity of the tumour and lymph node involvement and was higher in men in our sample. See table 12.

CSF1R expression

There was a higher expression of this biomarker in cases with a poorly differentiated histological grade (p=0.064) although a large proportion of cases with CSF1R expression had no metastases (>0.999). See table 13.

Table 7 shows the descriptive statistics for each biomarker according to sex. We found a higher expression in FoxP3 among women (median 15% vs. 10%, p=0.073). Fifty-five percent of the sample expressed FoxP3 in more than 10% of the tumour cells (45% men, 72% women, p=0.061). p16 was found in three samples, but only two were positive for HPV on PCR, while CSF1R was found in 42% of the samples, with a similar distribution in both sexes. CD4 expression was slightly higher among women (median 30% vs 20%, p=0.223), and 32% of the analysed samples showed expression higher than 35% (25% men, 44% women, p=0.126). CD8 was expressed similarly in men and women (median 30%). See figure 32.

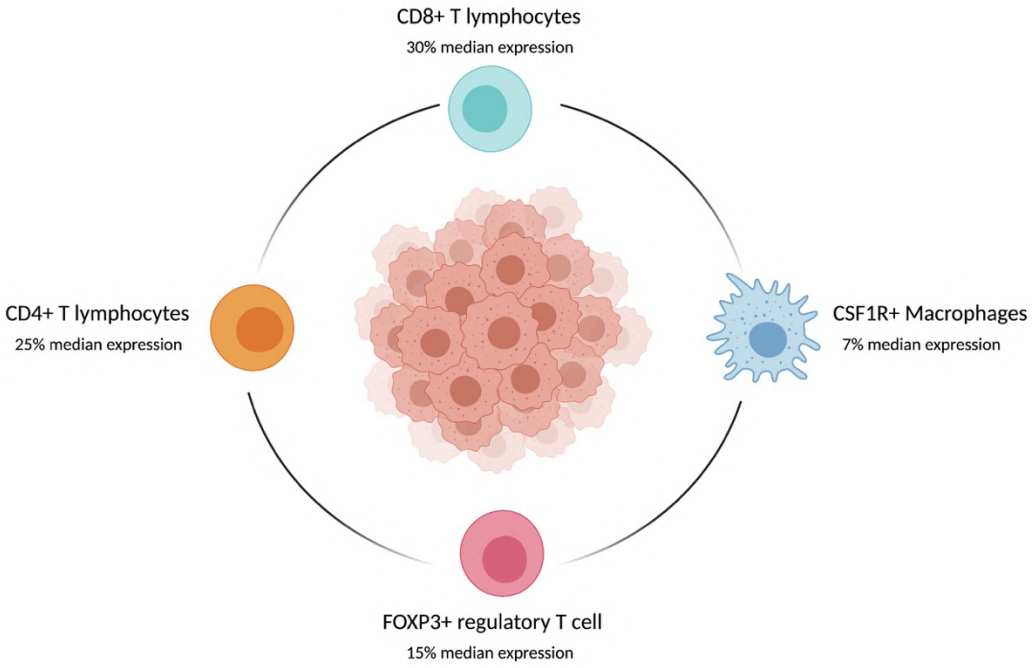


Figure 32. *The illustration reflects the median expression of the cells studied in the TME of the samples and reflects that the majority cellular component is lymphocytes.*

Survival analysis

During a median follow-up (OS) of 73 months (p25-p75: 45-96), 32 deaths (49%) by any cause (42% by OSCC) were observed. None were lost to follow-up. The incidence rate was 8.4 events per 100 person-years (95% CI: 5.7,11.8) for the whole group, and it was significantly higher among men (11.2 [7.2,16.7] events/100 persons-years compared to woman (4.7 [2.03-9.29] events/100 persons-years) , p=0.032).

Kaplan-Meier analysis showed an increased OS hazard for those who were PD-1 negative and for those who were PD-L1 negative whether TPS≥5% or CPS>1 was used (Figure 2). TPS-negative but not CPS-negative patients also had a higher cumulative hazard for DSS. See figures 33-36.

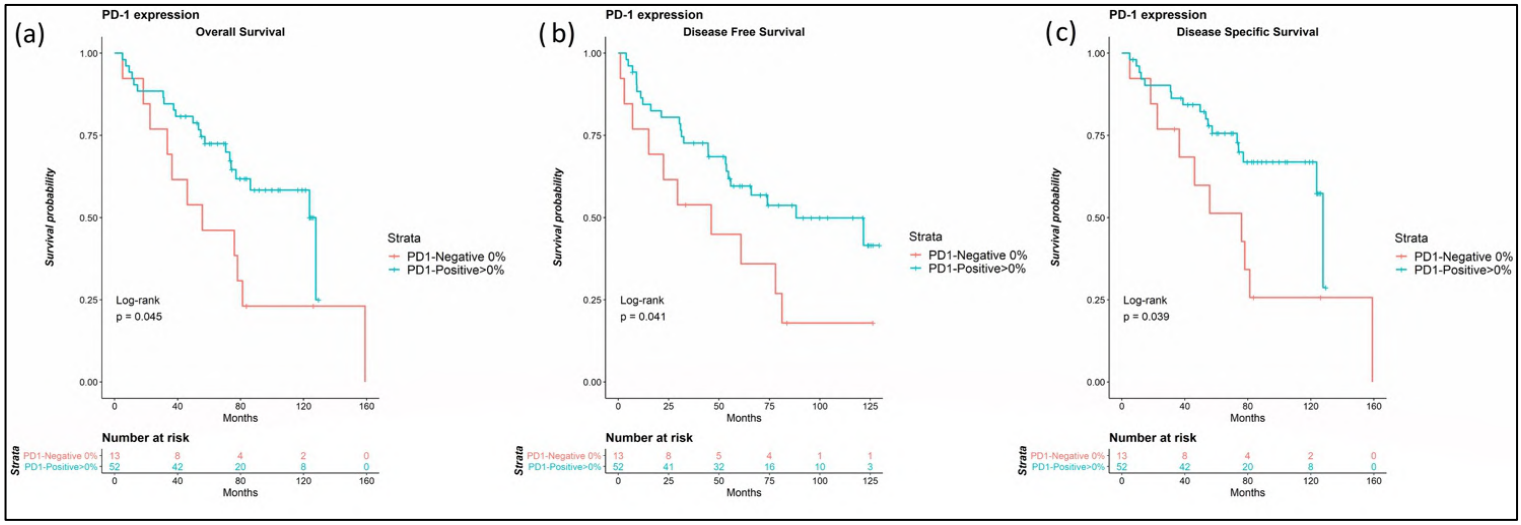


Figure 33. Kaplan-Meier curves. A, B, and C show the survival curves of the PD-1 negative and positive patients. Up to 120 months PD-1 positive patients show greater OS, DFS and DSS.

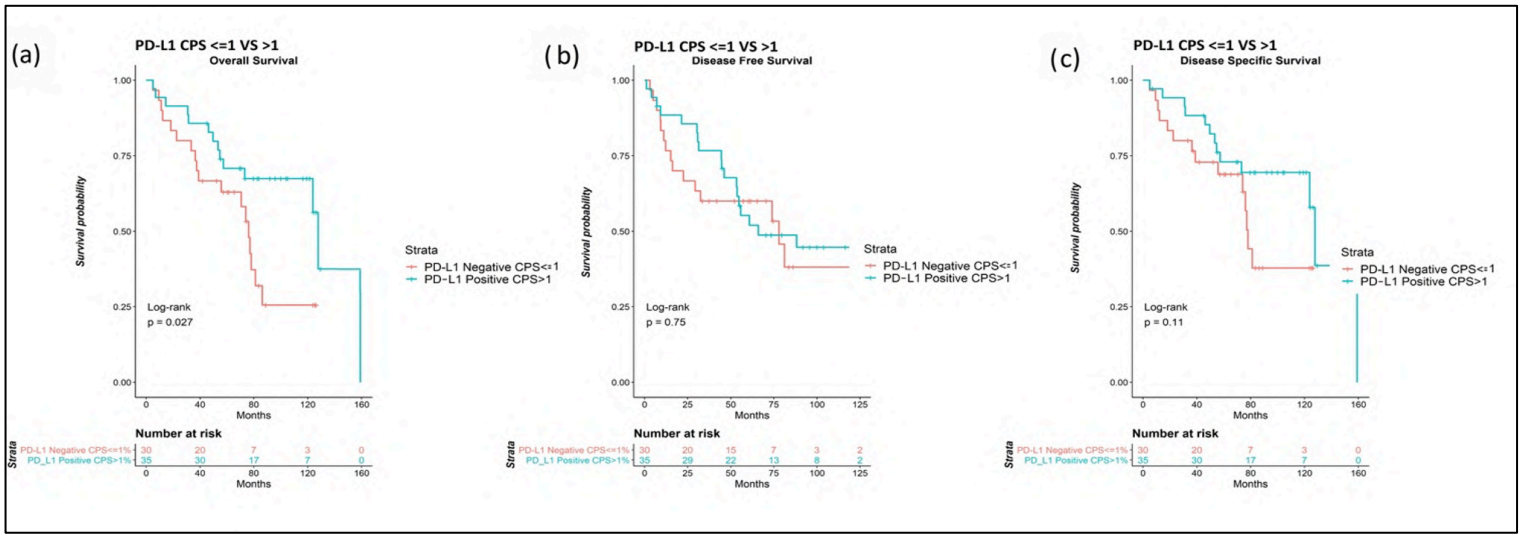


Figure 34. Expression of PD-L1with categorisation by CPS <=1 vs. >1.

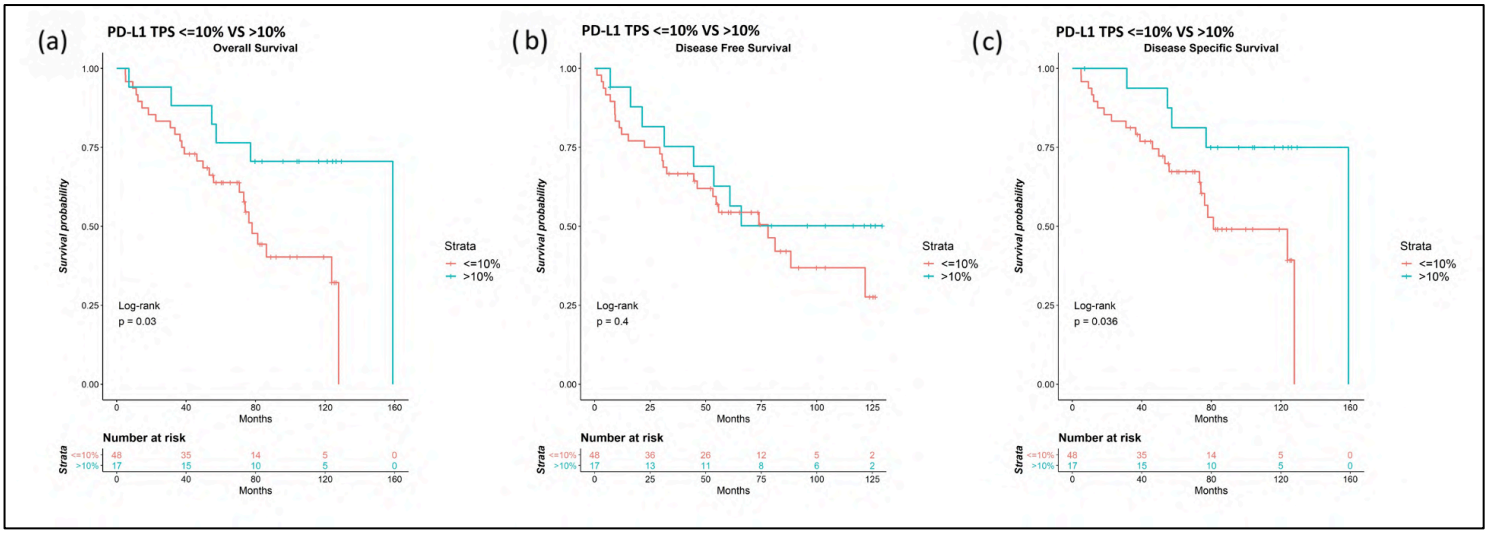


Figure 35. The present figure reflects categorisation by TPS<10% vs. >10 in Kaplan-Meier curves analysis of OS, DFS and DSS.

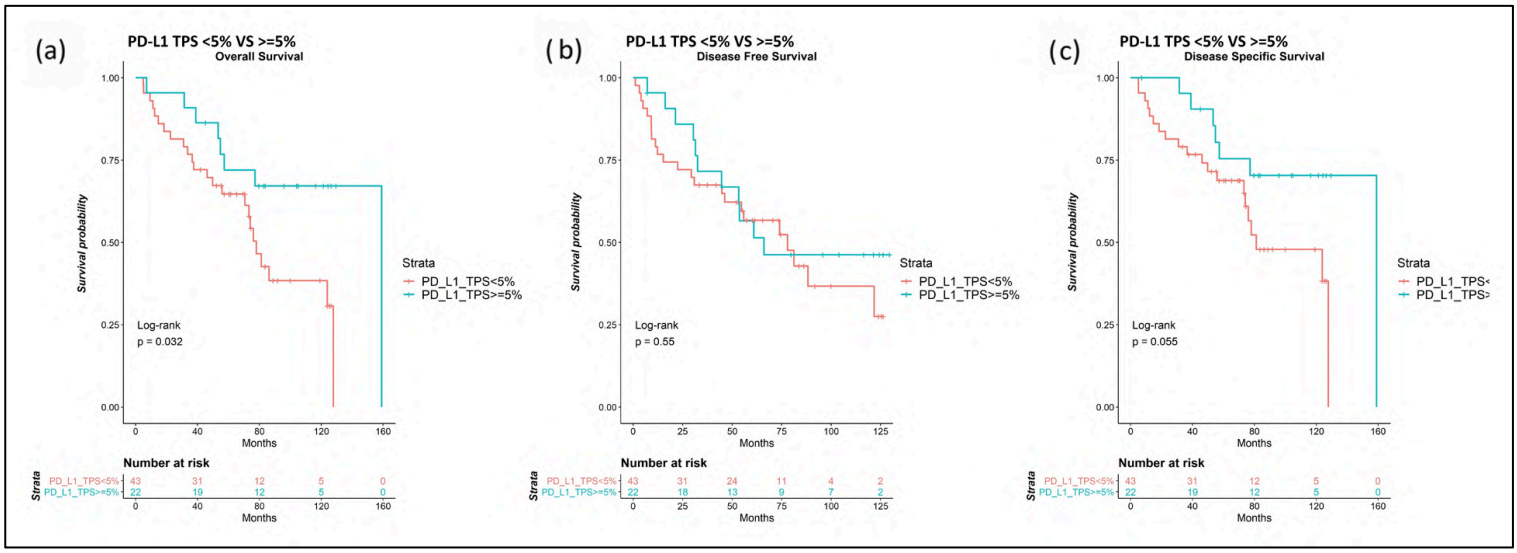


Figure 36. Categorisation by TPS<5% vs. >5% in Kaplan-Meier analysis of OS, DFS and DSS.

Univariate Cox models (table 14) showed that smoking, metastasis, a poorly differentiated tumour, and WPOI score 4-5 were associated with hazard of death (OS and DSS models), while women had a lower hazard (HR 0.356 [0.14,0.89], p=0.028) compared to men. The Kaplan Meier curves confirm these data and can be seen in figures 37-44.

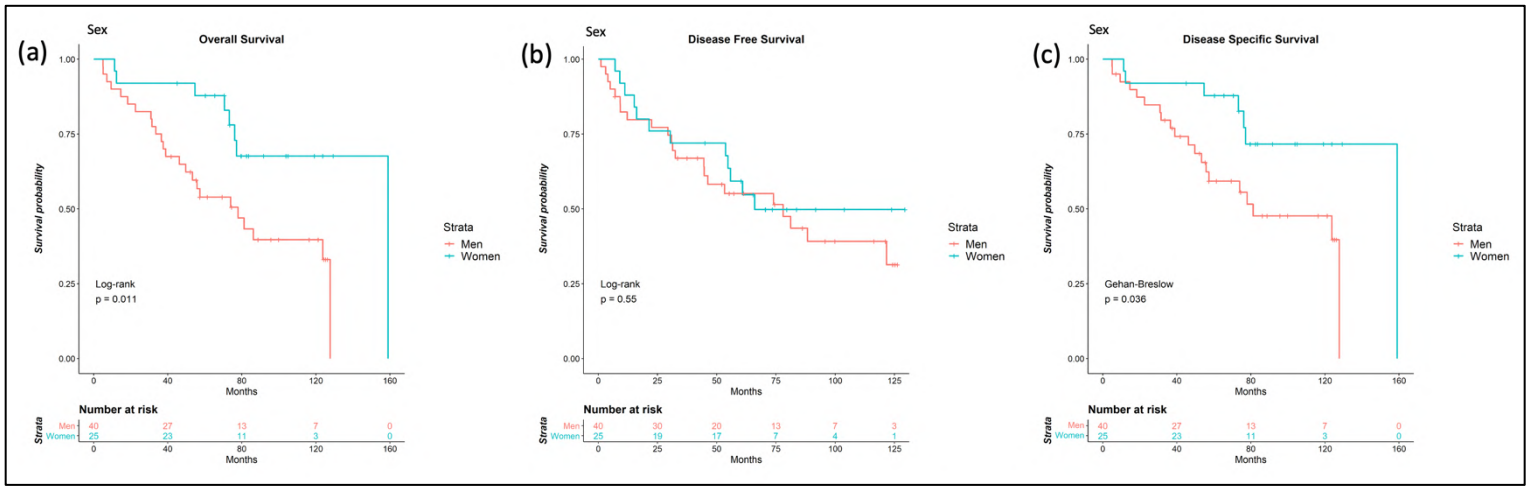


Figure 37. Kaplan-Meier analysis of survival. OS and DSS is lower in men than in women. For DFS sex differences were not apparent until 80 months

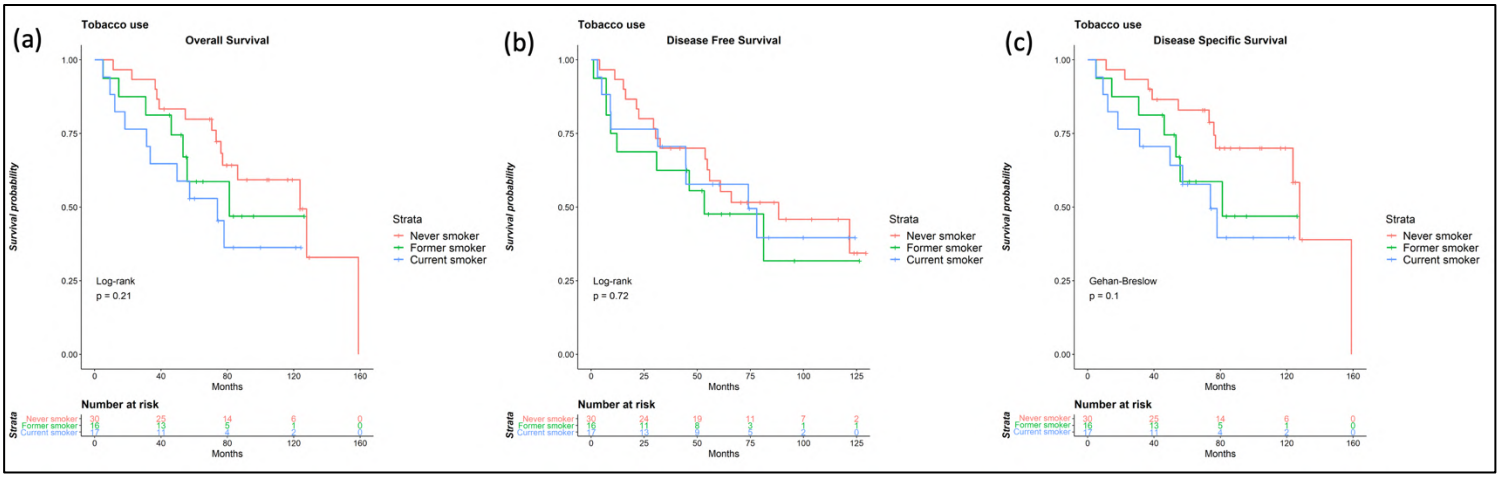


Figure 38. Kaplan-Meier analysis to show that smoking is associated with worse survival. Cases that are former smoker have a better prognosis, but patients who do not have this habit have the longest survival.

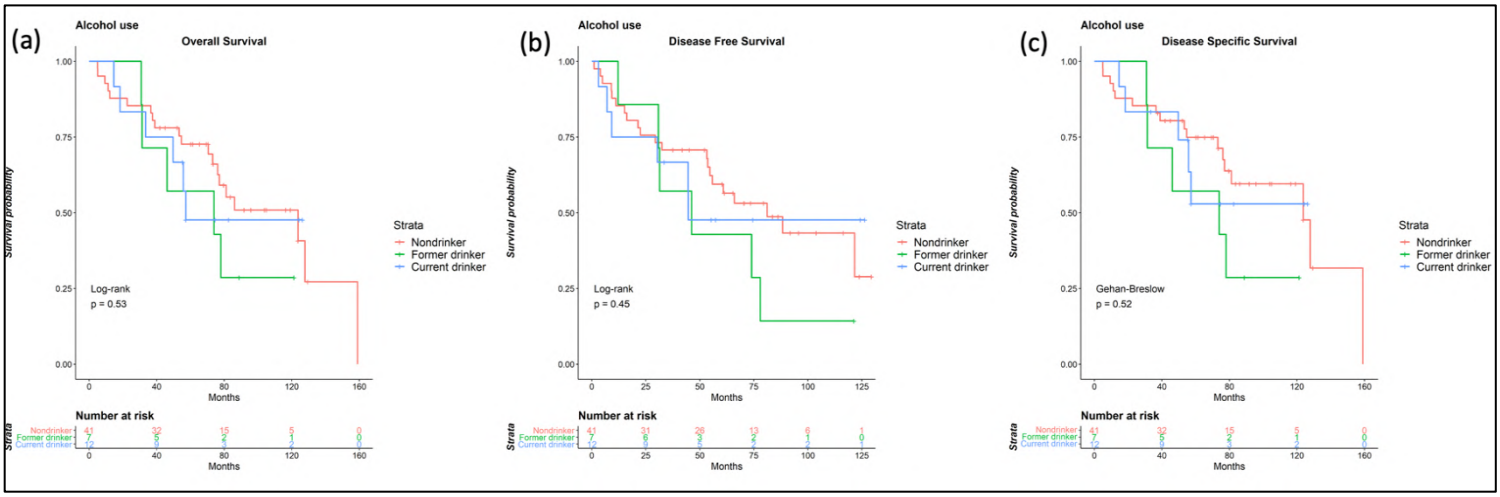


Figure 39. Kaplan Meier analysis show that patients who never drink alcohol have a better prognosis, although with very low significance.

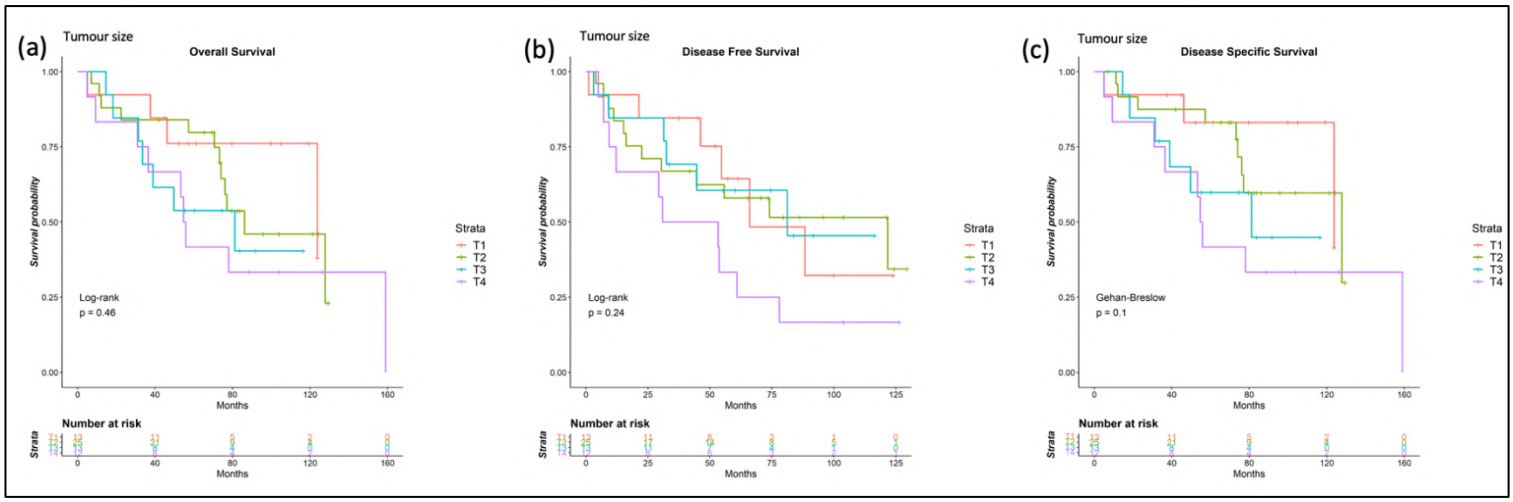


Figure 40. Kaplan Meier analysis show that larger tumours have poorer survival.

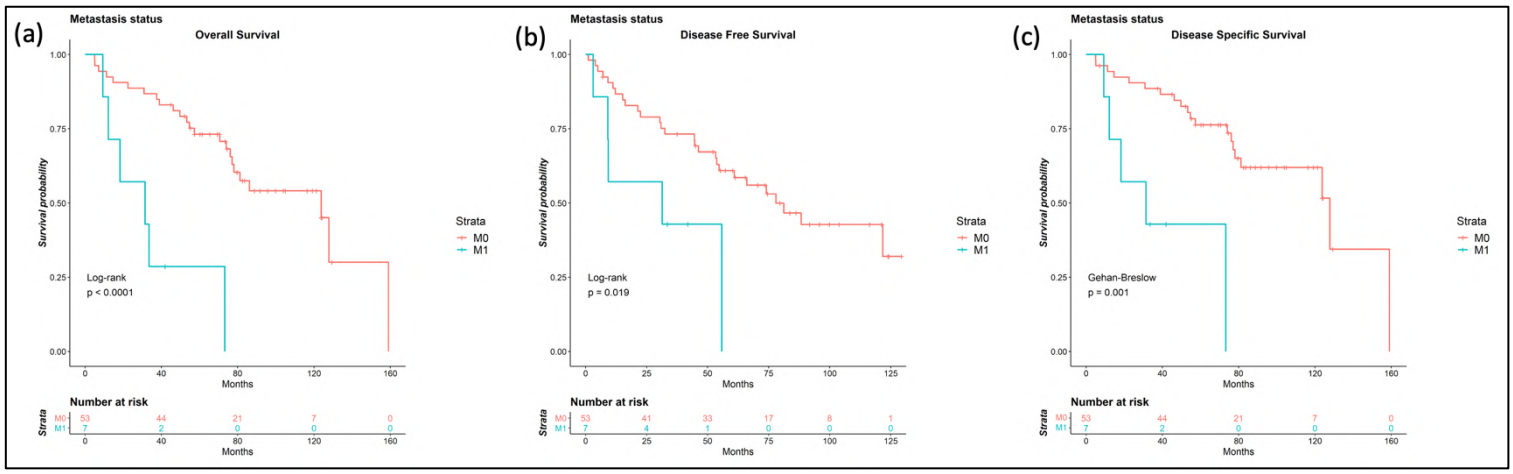


Figure 41. Kaplan Meier analysis show that cases of metastasis are associated with worse survival.

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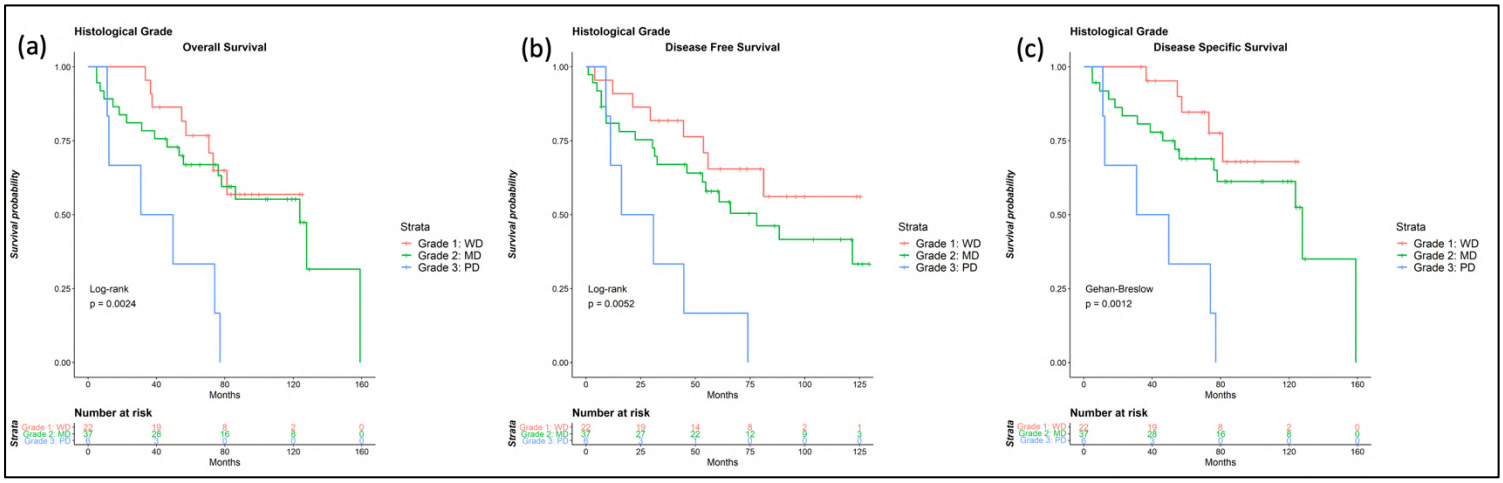


Figure 42. The poorly differentiated grade histologic cases have a considerably worse prognosis in all survival models.

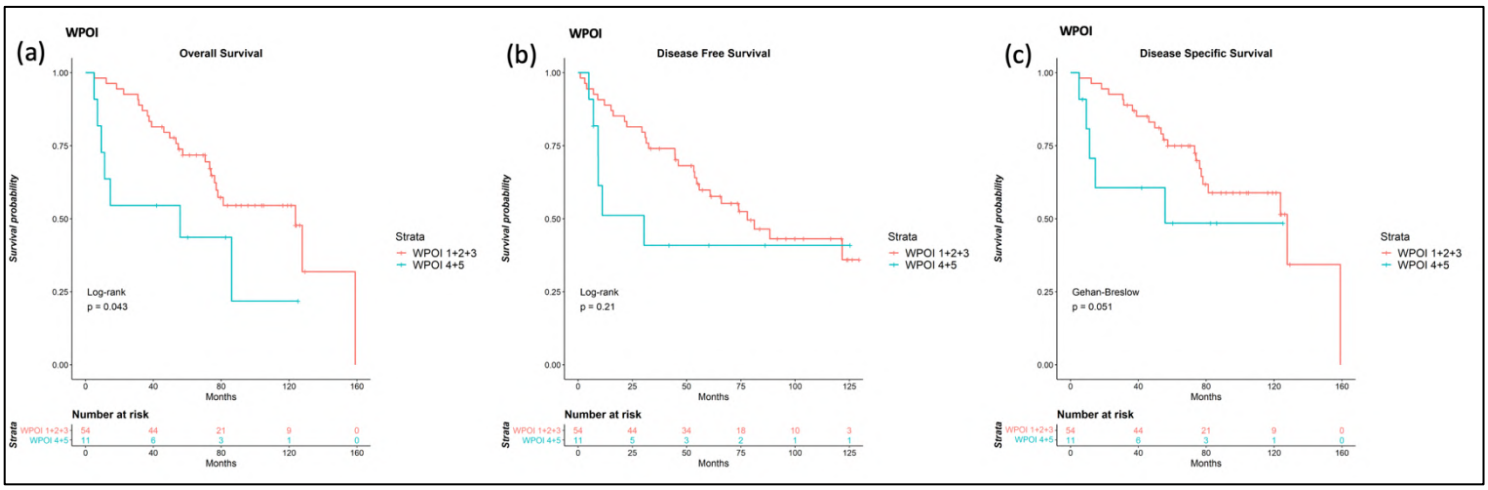


Figure 43. Kaplan Meier analysis show that that the WPOI 4 + 5 group has a significantly worse prognosis than the WPOI 1 + 2 + 3.

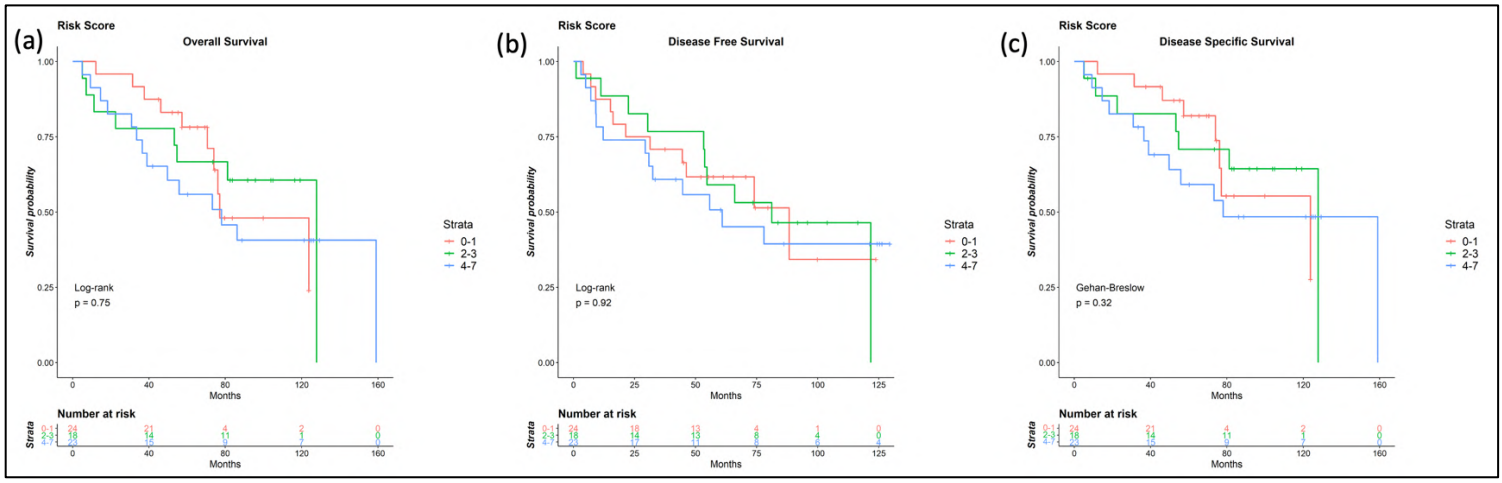


Figure 44. Kaplan Meier analysis show the inverse correlation between Risk Score and survival.

Among biomarkers (Table 15), PD-1 positivity was associated with a protective effect (DSS model, HR 0.43 [0.19,0.98], p=0.044; OS model, HR 0.47 [0.22,1.02], p=0.05; DFS model, HR 0.47 [0.22-0.99], p=0.047). PD-L1 positivity (TPS≥5%) was also associated with a better prognosis (DSS model, HR 0.42 [0.17,1.05], p=0.063; OS model, HR 0.41 [0.17,0.95], p=0.038). The results were similar when positivity based on CPS (>1%) was used instead (DSS model, HR 0.53 [0.24,1.17], p=0.12; OS model, HR 0.44 [0.213,0.927], p=0.031).

The expression of CD4, CD8 and CSF1R markers was not related to significant changes in survival in the univariate analysis or the Kaplan Meier curves. However, the FoxP3 group with an expression greater than 20% indicates a greater survival, although due to the sample size this association cannot be confirmed. (See figures 45 -48).

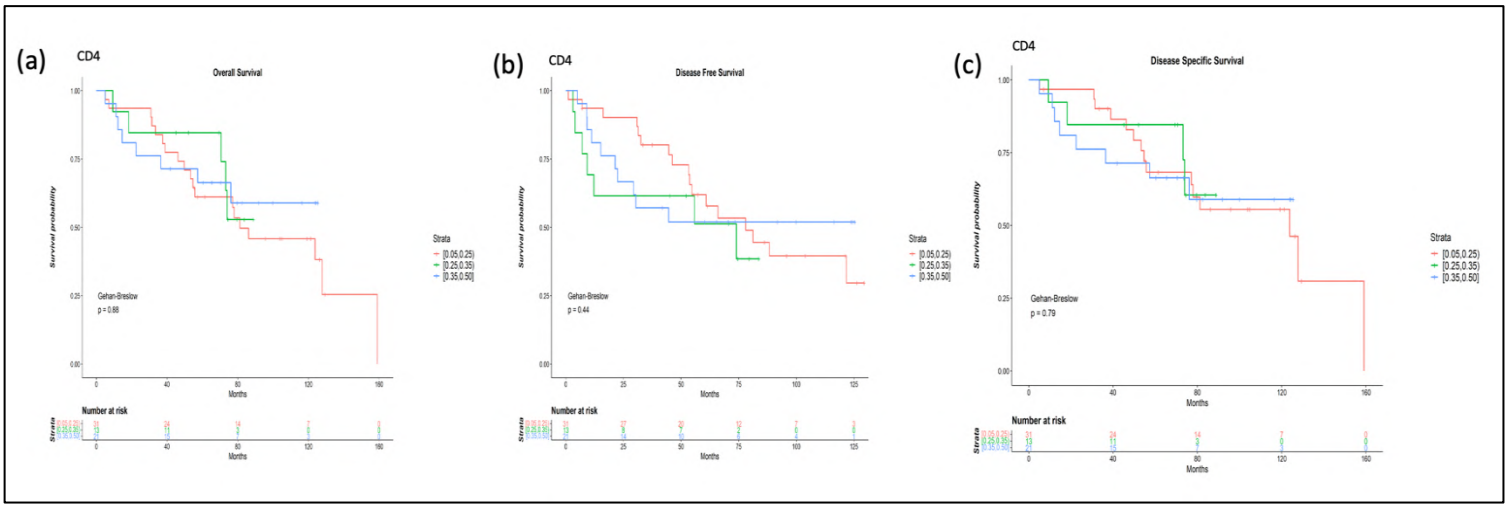


Figure 45. Kaplan-Meier plot of the CD4 biomarker. No survival differences are observed in this analysis.

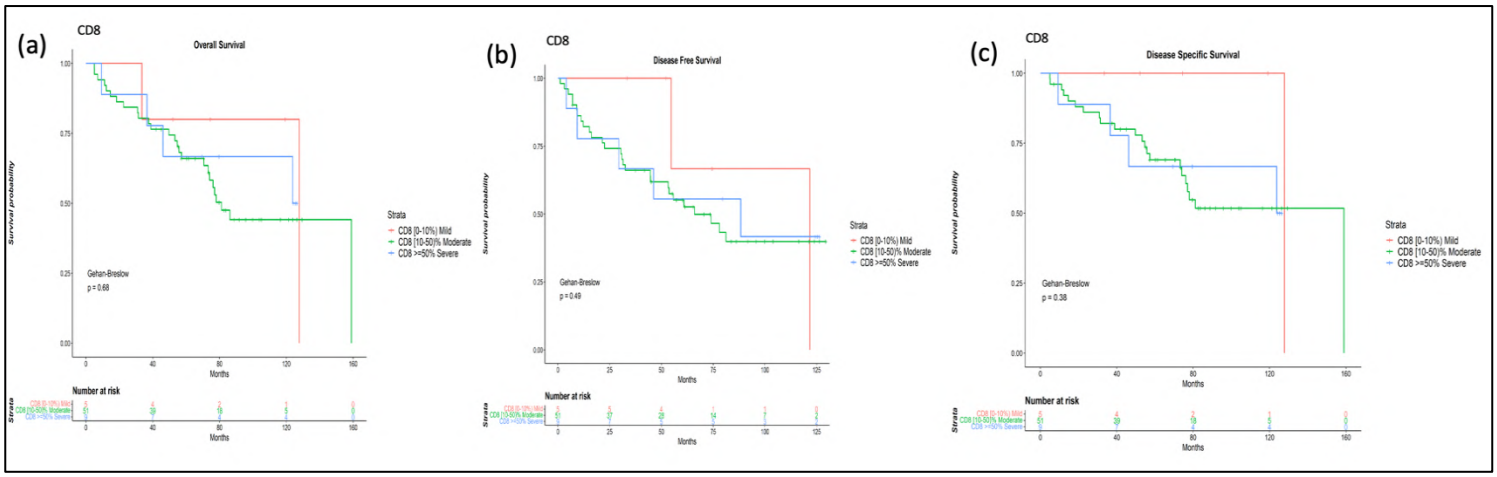


Figure 46. Kaplan-Meier plot of the CD8 biomarker. Higher survival is shown in cases with a lower number of CD8 lymphocytes, however, the number of events is too limited to affirm this trend.

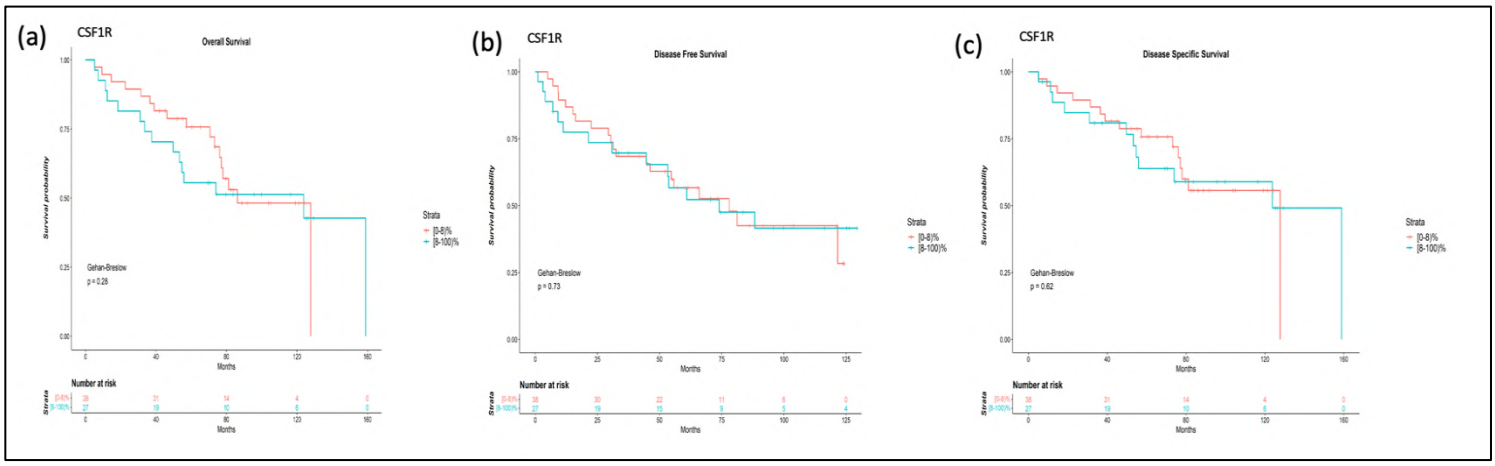


Figure 47. Kaplan-Meier plot of the CSF1R biomarker. No survival differences are observed in this analysis.

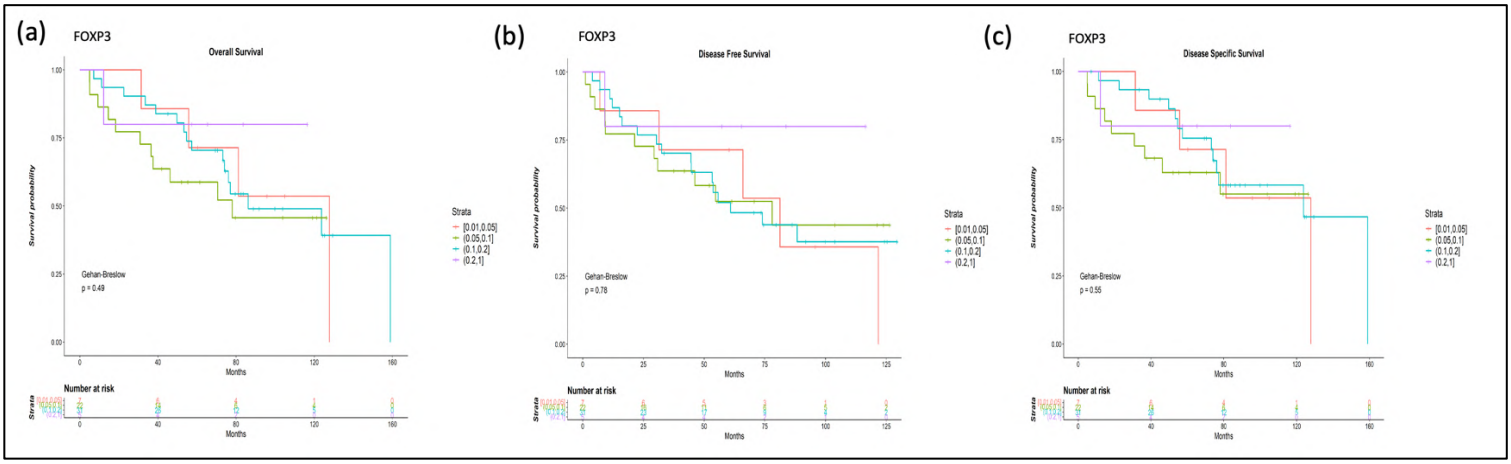


Figure 48. Kaplan-Meier plot of the FOXP3 biomarker.

On the other hand, only the presence of metastasis and a poorly differentiated tumour were associated with higher risk of recurrence (any type) or death from any cause (DFS model). In this model, only PD-1 positivity showed a protective role (HR:0.47 [0.23,0.99], p=0.047). Stratified analysis showed that the protective role of PD-1 positivity was not modified by the PD-L1 status in any model, regardless of the cut-off definition (TPS>5% or CPS>1). See figures 49-51.

Regarding biomarkers, only p16 positivity was associated with a poor prognosis in the univariate OS model (HR 4.45 [1.038,19.1], p=0.044), but only two patients were HPV positive. Although it looks very promising, more patients need to be recruited in order to confirm this tendency.

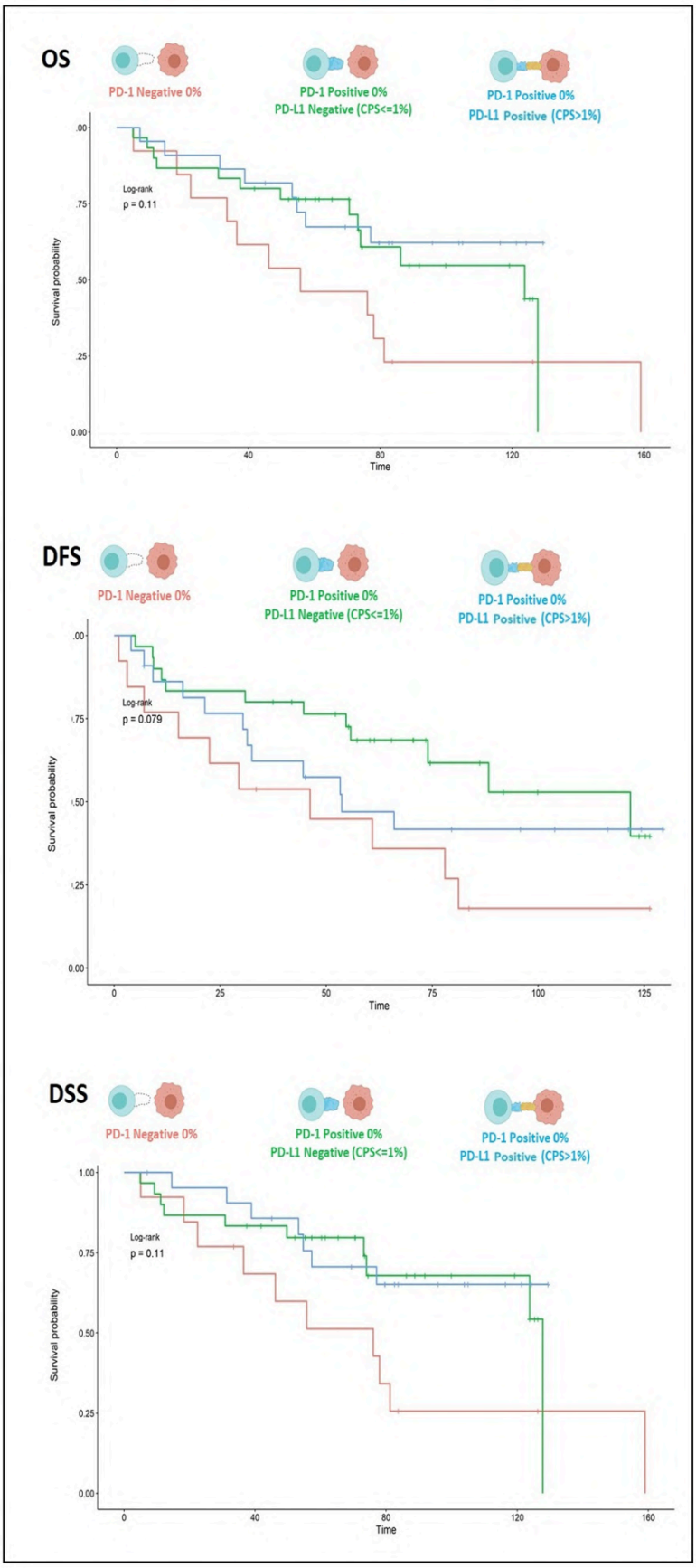


Figure 49. Kaplan-Meier analysis of PD-1 and PD-L1 association with survival. PD-1-positive cases (>0%) and PD-L1 cases with greater positivity cuts of 1% show a better prognosis than cases that are PD-1 negative (<0%). This phenomenon is observed in OS and DSS.

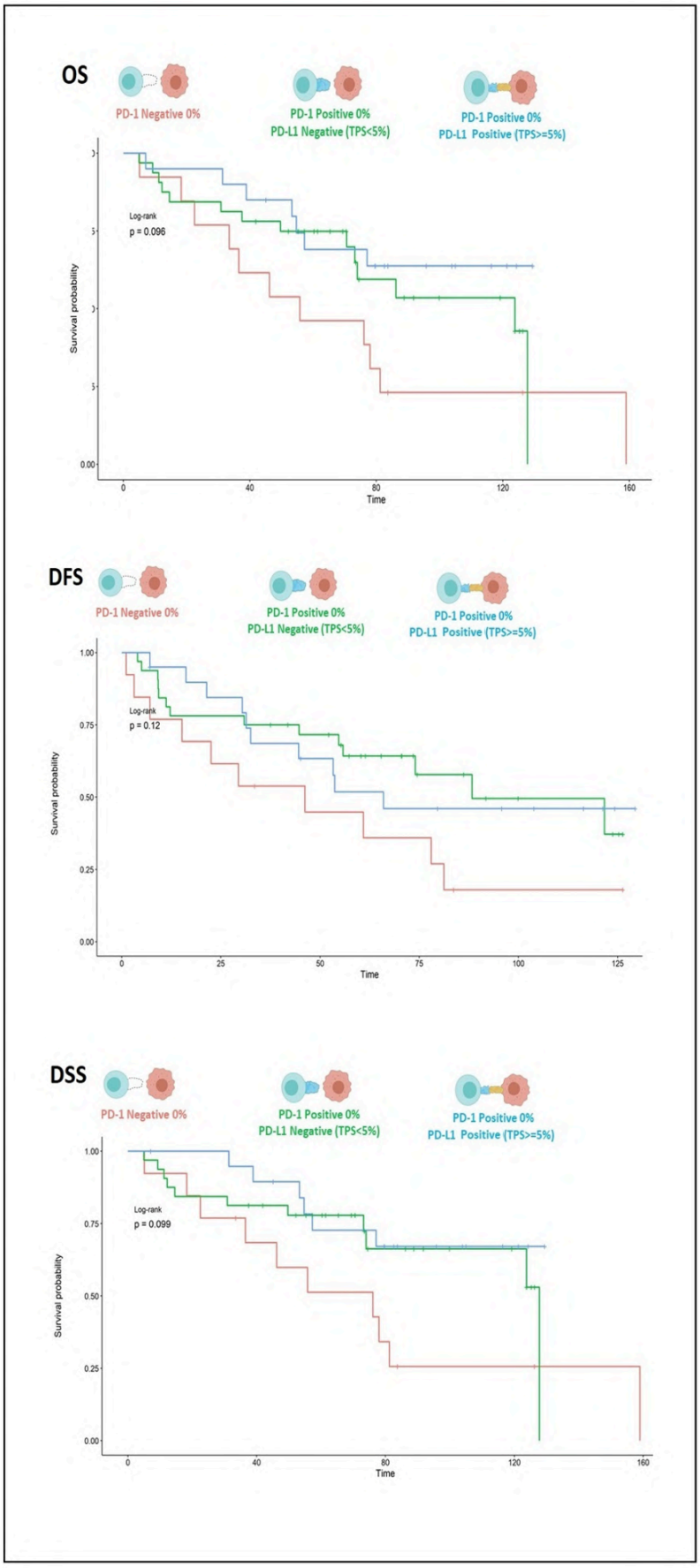


Figure 50. Kaplan-Meier curves showing that PD-1-positive cases (>0%) and PD-L1 cases with greater and lesser positivity cuts of 5% show a better prognosis than cases that are PD-1 negative (<0%). This phenomenon is observed in all three types of survival studied (OS, DFS, DSS) and again, as was the case when considering PD-L1 positivity at 1%, DFS has this effect to a lesser extent.

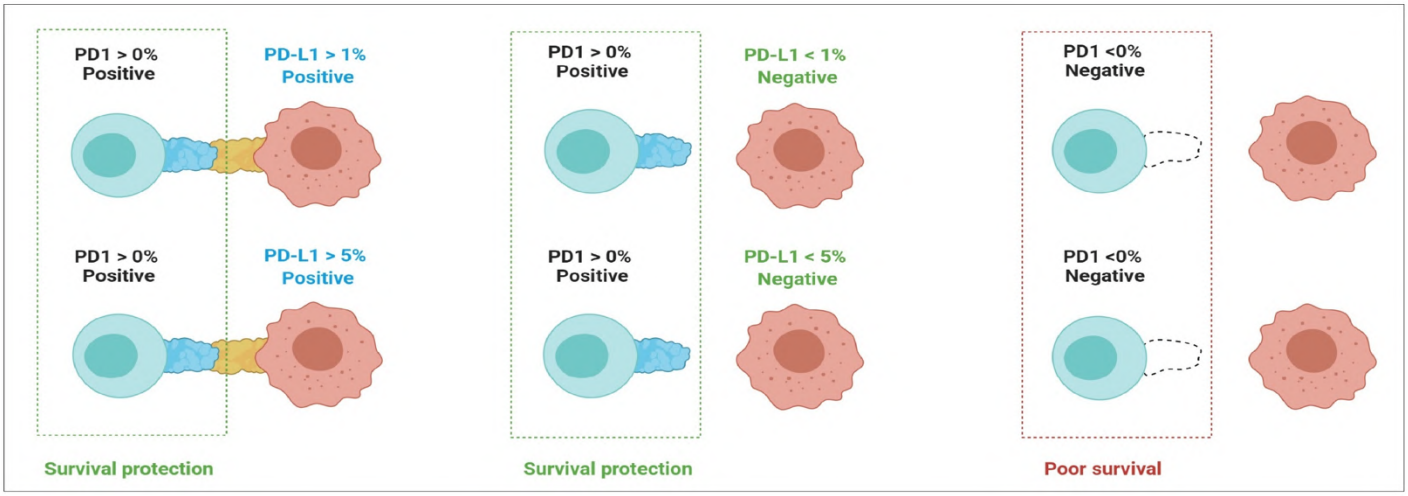
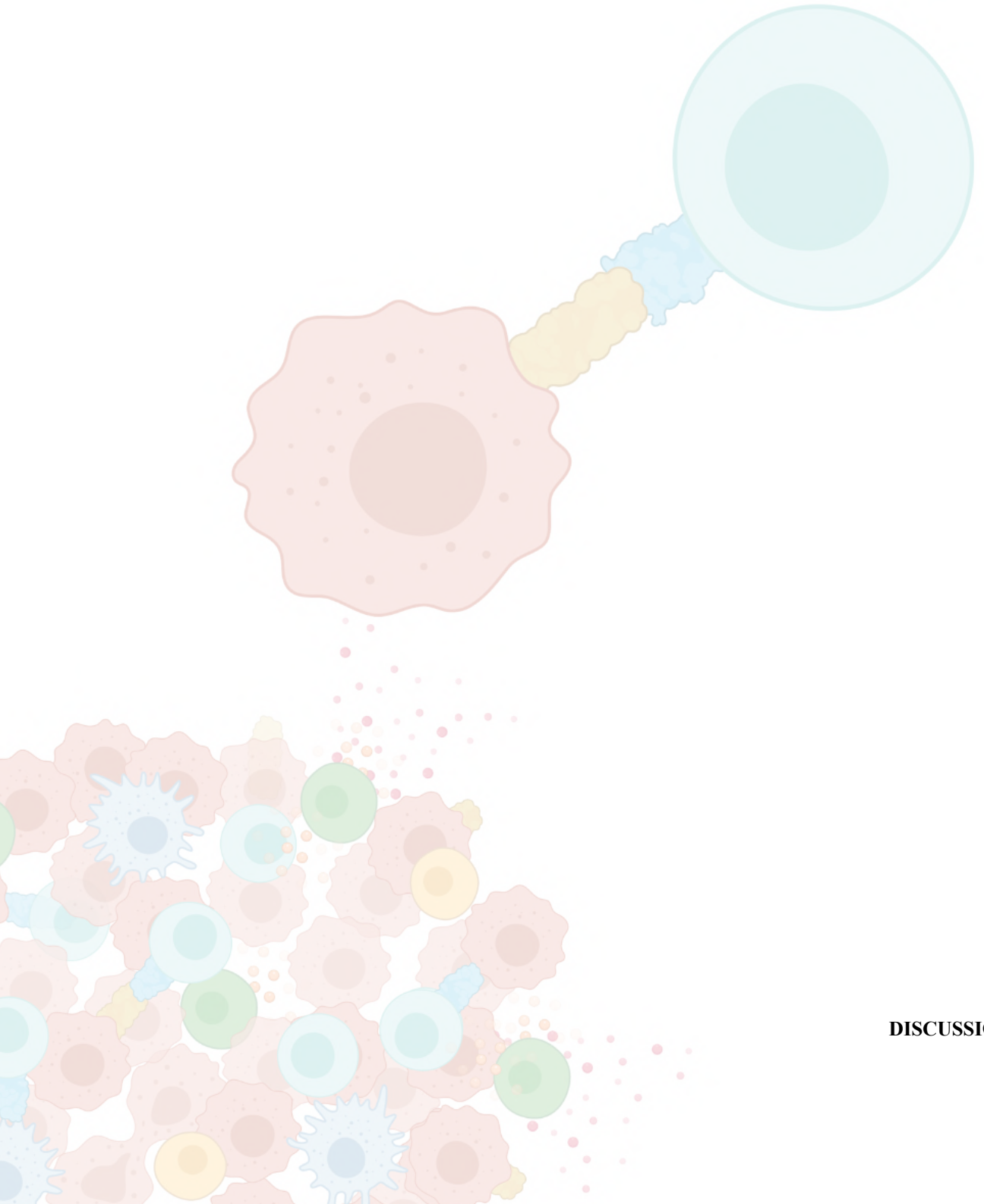


Figure 51. Illustration showing the protective role of survival by the positive presence of PD-1 expressed in the lymphocyte membrane. Said survival protection remains independent of the values of expression and the degree of positivity of PD-L1 expressed by the tumour cell. The worst prognosis for survival is seen when PD-1 expression is negative. The DFS also considers recurrences as an event, therefore, the protective effect of PD-1, independently of PD-L1 expression, is mainly related to survivals whose event is death (OS, DSS).

Multivariate analysis

After multivariate analysis (Table 16), the presence of metastasis and a moderate or poorly differentiated tumour were associated with poor prognosis in all the survival models. A worse type of WPOI also increased the hazards for all the outcomes, while PD-1 positivity was a protective factor, even after the adjustment by sex and PD-L1, and especially in the DFS model (HR 0.36 [0.14,0.93], p=0.034).



DISCUSSION

Our research indicates that the expression of PD-1 in the TME has an independent protective role in all survival models evaluated. PD-L1 positivity also exhibits protective effects on OS and DSS by univariate analysis.
PD-L1 expression values above 10%, were associated with protective effect on DSS (p=0.047) and OS (p=0.037). The significance of the PD-L1 protective effect was increased for CPS values between 20 and 100, on both DSS (p=0.024) and OS (p=0.019) (Table 7).

In support of our data, Kogashiwa et al. (2017) also found a significant association between PD-L1 positive tumours, (those expressing greater than 5%), and greater DFS and OS (Kogashiwa et al., 2017). Additionally, Hanna et al., studied a population of young patients with OSCC, and found that PD-L1 expression ≥10% was associated with greater survival and a lower risk of recurrence in women (Hanna et al., 2018). Moreover, Ahn et al., found that higher expression of PD-L1 was associated with a favorable OS prognostic factor (Ahn et al., 2017).

A higher expression of PD-L1 in females and a lower rate of recurrence in females with higher PD-L1 has been found in other studies (Ahmadi et al., 2019; Hanna et al., 2018; Kogashiwa et al., 2017; Lenouvel et al., 2021). Our data is in agreement, since we found higher median expression of PD-L1 in women. This result is also confirmed in a recent meta-analysis (Lenouvel et al., 2020). Concordantly, we also found that female sex was a protective factor (DSS p=0.028, OS p=0.015) in univariate analysis. But the strength of this association weakened (more than 30% change in the coefficients) with the entry of PD-L1 TPS>10% in the model. However, the protection associated to female sex did not weaken with PD-1 positivity, smoking or drinking (all of them more frequent in women). The data supports an independent protective role for the expression of PD-L1 in this group.

A very relevant finding, the protective role of PD-1 for survival, regardless of the value of PD-L1, an effect that is maintained after multivariate adjustment (HR: 0.36 [0.14,0.93], p=0.034 (Tables 9, 10, and figure 3) highlighting its importance. In support of our data, Kikuchi et al. found a similar association of PD-1 in tumour-infiltrating cells with a better

prognosis (HR: 0.2, p=0.02) (Kikuchi et al., 2021). In our study, the patients who were PD-1 negative and PD-L1 negative also showed a worse survival prognosis by Kaplan-Meier analysis, supporting the protective role of PD-1 positivity (Figure 3). The protective effect of PD-1 is mainly observed in OS and DSS. However, in DFS the protective effect is decreased perhaps due to the fact that tumour recurrence is considered an event in DFS and positive expression of PD-1 and PD-L1 has been related to increased local recurrences by some authors (Naruse et al., 2019).

The prognostic roles of PD-1 and PD-L1 in OSCC is controversial. Heterogeneity in the results from different studies may be due to the use of different biomarkers to study the PD-1/PD-L1 immune checkpoint and the positivity cut-offs for these biomarkers. Cut-off points at 5% are the most frequently used for PD-L1 (Chen et al., 2015; Hanna et al., 2018; Kogashiwa et al., 2017; Lenouvel et al., 2021; Maruse et al., 2018; Oliveira-Costa et al., 2015; Satgunaseelan et al., 2016; Straub et al., 2016; Troeltzsch et al., 2017), although some authors use 1% (Ahmadi et al., 2019; de Vicente et al., 2018; Kikuchi et al., 2021; Mattox et al., 2017) and, with less frequently, 10% (de Vicente et al., 2018; Hirai et al., 2017; Wirsing et al., 2018). The choice of cut-off is mainly based on statistical criteria or on the cut-off points of previous studies.
Cut-off points for PD-1 analysis are more variable with some authors that estimate PD-1 positivity when it is > 30% in the sample (Mauruse et al., 2018), while others when it is >1% (de Vicente et al., 2018). We estimated PD-1 positivity when >0%, and although an overestimation can be assumed, our mean PD-1 expression percentages (80%) are not very different from other studies such as Mauruse et al. (2018) which was 61.1%, or Kouketsu et al. (2019) which was 68.9%.

The present investigation advocates the division of the PD-L1 positivity study into three groups based on the CPS classification used in clinical trials and taking into account the expression of >1 CPS, as this has been associated in the long term with better prognosis with the use of immunotherapy (Miranda-Galvis et al., 2020).

The clinical advantages of the use of pembrolizumab demonstrated by different clinical trials and the establishment of CPS as a measure to record the expression of PD-L1 with a clinical approach (Cohen et al., 2019) prompted us to analyze the clinical therapeutic

possibilities by recording the CPS and the TPS for comparison. In addition, we also recorded the staining intensity which has been assessed less often. This should allow better comparison of our results with those of other studies.

In addition, we used the monoclonal antibody PD-L1 22C3 to study the expression of PD-L1, as it is used in pembrolizumab assays in OSCC. Few studies have recorded the expression with this biomarker, such as (Lenouvel et al., 2021; de Vicente et al., 2018) but both studies recorded only TPS and not CPS. One study, (Miranda-Galvis et al., 2020) using both TPS and CPS, found no relationship between PD-L1 and survival. However, the PD-L1 expression pattern showed a relationship with survival, despite the use of a biomarker different from the 22C3.

As indicated above, the 22C3 is the biomarker used in clinical trials to evaluate pembrolizumab for cancers. Alternative biomarkers to study PD-L1 exist, for example E1LN3. Differences in PD-L1 detection between 22C3 and E1LN3 were reported by De Vicente et al. (2018). In a clinical trial, which compared the use of PD-L1 22C3 (Dako), 28-8 (Dako), Ventana SP163 and Ventana SP142, the staining was similar for all biomarkers except for SP142 which was lower (Hirsch et al., 2017). However, in another study, 22C3 was less sensitive in tumour and immunological cells. These results suggest that assay results are not interchangeable and clearly indicate the need to systematize the assays (Xu et al., 2017).

The intensity of the PD-L1 staining was also recorded in this study. Although there is no standard protocol for this, different studies have performed this type of recording and indicated that higher intensity is mainly associated with worse survival and the presence of regional metastases (Moratin et al., 2019; Cho et al., 2011). On the contrary, in our univariate analysis, moderate intensity of PD-L1 expression was associated with better survival ($p=0.063$) than no expression.

To understand this discrepancy, we studied the whole histological block since the expression of biomarkers of the TME, such as PD-L1, presents a great variability within the same tumour (Rasmussen et al., 2019; Botti et al., 2016). In fact, a previous finding that we have verified is the most evident expression of PD-L1 in the tumour invasion front (Botti et al., 2016). Many studies with results different from ours have been based

on the study of samples using tissue microarrays (TMAs) (Lin et al., 2015; Satgunaseelan et al., 2016; Straub et al., 2016; Mattox et al., 2017; Moratin et al., 2019; Yoshida et al., 2018; Tsai et al., 2019), which have great limitations These biomarkers have heterogeneous expression in different regions of the tumour tissue, and often leave out, for example, the invasion front. TMAs are inadequate to reproduce the clinicopathological correlations that exist in an analysis of complete sections (Rasmussen et al., 2019; Botti et al., 2016). Thus, studying the entire sample to correctly record the tumour area and its microenvironment (Khouja et al., 2010), as we did bring some advantages.

In addition, differences in methodology may account for different results. Variations in tissue fixation times, the thickness of histological sections, application of biomarkers, timing from sample collection, etc affect the sensitivity and specificity of the antibodies used (Rizk et al., 2019). One study concluded that cell membrane biomarkers (as PD-1 and PD-L1) were more sensitive to antigenic degradation over the years and estimated that histological blocks stored beyond 15 years possessed worse quality for sample study (Grillo et al., 2017). The samples from our present study are more recent and its results could be compared with similar studies.

PD-1 expression was also associated with a lower median DOI ($p=0.017$). Higher values of DOI have been associated with higher risks of regional recurrence and metastasis in OSCC (Faisal et al., 2018; Shim et al., 2010). We did not find the aforementioned association between DOI and survival, and although PD-1-positive cases have been associated with a lower median DOI, the multivariate model supports the independent role of PD-1 on survival. This model cannot explain the lower risk of regional recurrence present in positive PD-1 cases due to a lower DOI. On the other hand, the positive expression of PD-L1 was associated with an increased risk of local recurrence ($p=0.074$), yet unrelated to DOI.

In the relationship of biomarkers with the histologic risk assessment score, positivity for PD-1 tended to be associated with a lower score (42% vs. 15% with risk score 0-1, $p=0.17$), although the difference was not significant. The risk score was also not correlated with the rest of the biomarkers analyzed or to survival.

Positive PD-1 and positive PD-L1 showed some tendency toward more favourable WPOI, but the differences were not significant. In the case of the WPOI, type 5 was associated with a worse prognosis in DSS (p=0.075), and for OS, WPOI 4-5 yielded a negative prognosis (p=0.05) when compared with the other categories. The point estimate did not change much in the multivariate models, so we cannot rule out that it has some prognostic relevance, although our study does not demonstrate this due to lack of statistical power.

One of the histological aspects that played a major role as a prognostic factor for survival was the histological grade. In this study, poorly differentiated cases were strongly associated with a worse prognosis in all survival analysis (DSS p=0.001, DFS p=0.003, OS p=0.003) and, although this was confirmed in multivariate analysis (p=0.000001), moderate differentiated cases were also associated with poor prognosis (p=0.0377).

The present investigation did not find differences in cell population in TME according to histological grade. However, other authors showed that well-differentiated OSCCs had had low proportion of Tregs through FoxP3. Other authors found that poorly differentiated OSCCs have a higher presence of TILs (Al-Qahtani et al., 2011).

TILs have been considered both a prognostic biomarker of disease and predictive of immunotherapy treatment (Rizk et al., 2019). The study of TILs through intratumoral CD4 and CD8 did not reveal a relationship with survival. Previous studies have reported a direct relationship between the expression of CD4 and PD-L1 (de Vicente et al., 2018; Hirai et al., 2017; Kogashiwa et al., 2017, Lenouvel et al., 2020), which is associated with longer survival (Kogashiwa et al., 2017), and smaller tumours (Hirai et al., 2017). Our study had an average CD4 biomarker expression of 12%, and its expression was not correlated with survival or PD-L1 expression; however, it was directly but not significantly correlated with CD8 and FoxP3 (rho=0.34). Perhaps a more homogeneous sample in terms of tumour stage is needed for these correlations as TIL infiltration is a dynamic phenomenon that changes through tumour evolution.

An important feature to take into account is that although in the present study there is no evidence of a relationship between TILs and survival, there are studies that do establish

such a relationship, especially when studying their presence in different areas of the tumour. One study showed that CD8 lymphocytes located in the center of the tumour are associated with better OS (Zhou et al., 2018), while another study showed better OS and DSS survival in tumours with CD8s located in the margin or in the peripheral stromal region (Shimizu et al., 2019).

FoxP3 plays a key role in the immunosuppressive functions of Tregs and is a marker of these cells (Song et al., 2016). Its presence in different malignancies has been associated with a worse prognosis and an increased risk of progression (Song et al., 2016; Schreiber et al., 2007). However, other studies have linked it to a better prognosis. These contradictory findings may be related to the localisation of FoxP3 at the cytoplasmic versus nuclear level (Weed et al., 2013) and call for its standardisation. In the present study, the FoxP3 biomarker was recorded at the nuclear level, and its expression was not related to OSCC prognosis in univariate and multivariate analyses, however, Kaplan Meier analysis suggests a higher survival in the group with greater than 20% FoxP3 expression, and therefore a higher presence of T regs, although this association is not clear due to the low number of events in the sample.

Furthermore, as with CD8 lymphocytes, one study has highlighted that FoxP3 expression in different areas of the tumour may play a different role. They found a better prognosis in OS, DSS and recurrence-free survival in cases with FoxP3 expression at the parenchymal invasive front. Likewise, the presence of other CTLA-4+ cells close to FoxP3 may influence their role at the invasion front and favour antitumour immunity, linking these cases to lower metastasis-free and recurrence-free survival (Koike et al., 2020). Therefore, not only the the nuclear or cytoplasmic location of the marker may be influencing the results, but also the relationships with cells in the microenvironment and the intratumoral localisation.

The presence of TAMs has been correlated with lower OS (Cannarile et al., 2017). CSF1R had a median expression of approximately 7% in the OSCC samples of the present study. We found a weak direct correlation between the expression of CSF1R and PD-L1 (Spearman's rho p=0.28). These results show some similarity with the findings of Suárez-

Sánchez et al. (2020) which reflects that TAMs, identified by the expression of CD68 and CD163 were abundant in those cases with PD-L1 expression (Suárez-Sánchez et al., 2020).

Previous studies have reported a relationship between TAMs and worse survival in other tumours such as pancreatic, breast, ovarian, thyroid, gastric and bladder (Zhang et al., 2012). On the other hand, the presence of CD68+ and CD163+ macrophages are related to a worse prognosis (Evrard et al., 2019). In contrast, a meta-analysis relates high CD8 expression to a better prognosis. However, our research did not find a relationship in any of the models analysed.

As noted above, we found a higher degree of PD-L1 expression at the tumour invasion front. This is an aspect shared by other studies and should be explored. One study has observed and analysed that PD-L1 expression by tumour cells can be patchy and diffuse (Miranda-Galvis et al., 2020) and another study indicates that PD-L1 expression at the tumour front is due to increased expression of INF-γ by the tumour and T-cell activation (Ribas & Hu-Lieskovan, 2016).

We found p16 positivity in only two cases (3.1%), a prevalence that coincides with the published literature (Lingen et al., 2013). Although meta-analyses on the coexpression of PD-1 and HPV in head and neck tumours have shown an increase in HPV positivity (Tang et al., 2020; Hong et al., 2019), we cannot establish such a relationship in OSCC due to its low HPV prevalence. A meta-analysis has shown increased PD-1 expression in HPV positive head and neck cancer (Tang et al., 2020; Hong et al., 2019). Our study does not allow to study such a relationship due to the low HPV prevalence.

Even though OSCC is not related to a high HPV prevalence, in oropharyngeal cancer this prevalence is high, and the studies have demonstrated that this cancer presents a better prognosis, and it could be related to a higher immunity infiltration in the TME, the basal immunogenicity and a high PD-L1 expression (Oliva et al., 2019)

Studies such as Kelly et al. show a significant change in PD-L1 expression in 50% of patients with advanced esophageal adenocarcinoma after chemoradiation. Therefore, to avoid this limitation in the present investigation, only biopsies of patients before cancer

treatments were included and patients with previous chemotherapy or radiotherapy treatment were discarded (Kelly et al., 2018).

One of the biomarkers studied, CSF1R in TAMs can up-regulate PD-L1 expression and increase CD8 T-cell infiltration, which eliminates anti-PD-1/PD-L1 resistance (Kumagai et al., 2020).

Tumours with high mutational load are more likely to generate neoantigens, as they are more immunogenic and can induce CD8 T-cell reactivity and increased tumour infiltration by CD8 T cells (Lei et al., 2020). Conversely, tumours with low mutational loads are less likely to respond to anti-PD-1 therapy as there is less infiltration by CD8 lymphocytes due to the absence of immunogenic neoantigens (Lei et al., 2020). Thus CD8 lymphocyte density might be positively related to response to anti-PD-1 therapy (Kumagai et al., 2020).

In studies in NSCLC that expressed a high level of PD-1, the response to anti-PD-1 therapies was unexpectedly poor. Some *in vivo* studies have shown that Tregs are responsible for resistance to anti-PD-1 therapies (Lei et al., 2020). This might be due to the blockade of PD-1 in Tregs which allows Tregs to exert their immunosuppressive function (Kumagai et al., 2020). This may explain results indicating that PD-1 expression in lymphocytes is a sign of exhaustion and immune tolerance and is associated with a worse prognosis (Ehrhan et al., 2021).

Although relevant results have been found in this study regarding survival in the PD-1 and PD-L1 biomarkers, these results could be better clarified with a larger sample size. Given the low prevalence of OSCC the sample size decreases the statistical power of comparisons, so some relationships may not have been detected. Protocols and biomarker standardization may allow comparisons to overcome this difficulty. However, this study does report an important series of cases from a single hospital with its own database, avoiding some of the limitations that arise during the collection of relevant variables and improving internal validity. Furthermore, the sample shares epidemiological data with published studies concerning sex, age, habits, and the other clinical variables.

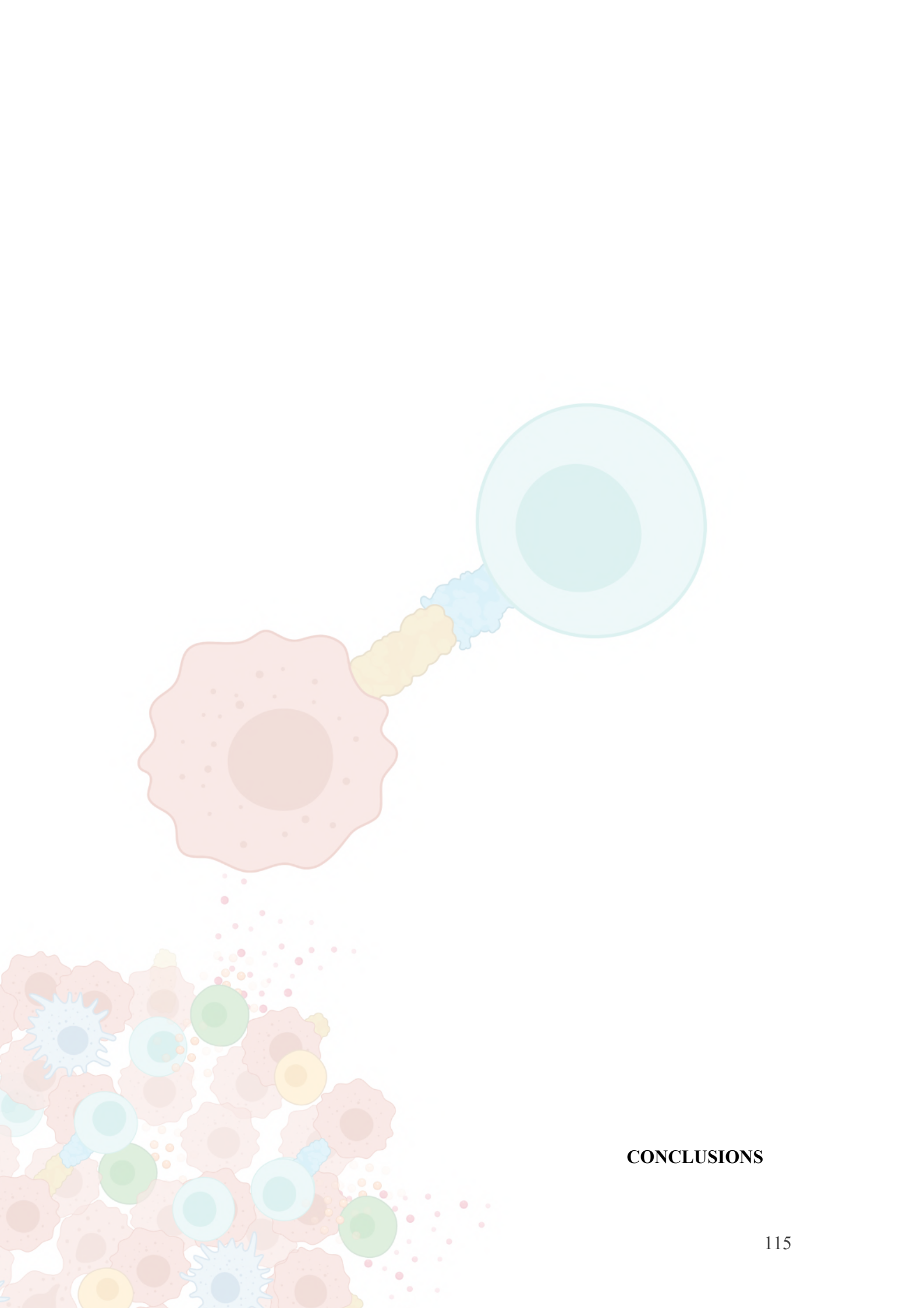
This study has a longer follow-up time than similar studies (Ahn et al., 2017; Cho et al., 2011; de Vicente et al., 2018; Kogashiwa et al., 2017; Lenouvel et al., 2021; Maruse et al., 2018; Moratin et al., 2019; Oliveira- Costa et al., 2015; Satgunaseelan et al., 2016; Straub et al., 2016; Wirsing et al., 2018), but an even longer follow-up time may allow a better assessment of the results obtained. The evaluation of the samples with different biomarkers has allowed us to more fully establish the TME. However, we consider that colocalizing biomarkers to specifically identify some expressed immunological cells and discern between their stromal or intratumoral location can yield important results that should be explored in future studies. To avoid leaving any relevant variable outside the multivariate model, the decision to include or not a variable addressed not only the statistical relationship but also the biological relevance. Although we consider the region of the floor of the mouth and tongue to be very representative of the OSCC, as they are the two main locations, these results should be studied and extrapolated with caution to other oral anatomical locations, and we also consider that the results obtained require external validation through future studies.

A prognostic biomarker provides information on the patient's overall cancer outcome, regardless of therapy, while a predictive biomarker provides information on the effect of a therapeutic intervention (Oldenhuis et al., 2008).

We can confirm that the positive expression of PD-1 suggests its role as a prognostic biomarker of OSCC, as well as the positivity of PD-L1. However, these results may question current anti-PD-1 and anti-PD-L1 therapies. With the limitations of this study, we cannot affirm its value as a predictive biomarker for this type of treatment.

Patients with PD-L1 CPS≥1 and CPS≥20 have shown better responses to anti-PD-1 therapies (Oliva et al., 2019). This indicates that the role of positively expressed PD-L1 is related to good predictive results to treatment. However, the expression of PD-L1 is dynamic, it can change depending on the moment in which it is studied since its expression is not similar at the time of diagnosis, in a recurrence or advanced disease, this heterogeneity makes it difficult to know its prognostic role, predictive and responds to the contradictory results in different studies as previously indicated (Oliva et al., 2019).

The fact that the positive expression of PD-1 acts as a biomarker of good prognosis of the OSCC, may suggest an approach regarding the use of anti-PD-1 therapies since sometimes the effect of a prognostic biomarker can be neutralized with the use. of therapies (Oldenhuis et al., 2008), however, a greater number of clinical trials are needed that take into account the expression of PD-1 to be able to confirm said data and to know its role as a predictive biomarker in anti- PD-1.



CONCLUSIONS

CONCLUSIONS

1. PD-1 is a protective survival factor that is maintained independently of PD-L1 expression. High values of PD-L1 expression also improve survival, while low values are associated with a worse prognosis. Higher expression of PD-1 is observed in smaller tumours, and higher expression of PD-L1 is more likely in women.

2. No relationship between TME and risk score was found to influence the survival patterns studied in the OSCC.

3. There is no evidence of a relationship between the histopathological features and the studied biomarkers, although the positive PD-1 and PD-L1 cases have a lower risk of a high WPOI score, and positive PD-1 expression was associated with a lower DOI.

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TABLES

Table 1. TNM classification in OSCC

T (Tumour primario)	
TX	Insufficient data to evaluate
Tis	Carcinoma in situ
T1	Tumour of ≤ 2 cm with DOI ≤ 5 mm
T2	Tumour ≤ 2 cm with DOI > 5 mm 2 to 4 cm tumour with DOI ≤ 10 mm
T3	2 to 4 cm tumour with DOI > 10 mm Tumour > 4 cm withDOI ≤ 10 mm
T4	Moderately advanced or very advanced local disease
T4a	Moderately advanced local disease
	Tumour> 4 cm DOI> 10 mm
	Invasion of adjacent structures: cortical bone, extrinsic lingual muscle in depth (genioglossus, hyoglossus, palatoglossus and styloglossus), maxillary sinus or skin
T4b	Very advanced local disease
	Invasion of the masticatory space, pterygoid process, skull base, or internal carotid
cN (Criterio clínico de los ganglios linfáticos regionales)	
NX	Insufficient data to evaluate
N0	No evidence of regional lymph node metástasis
N1	Metastasis in a single ipsilateral lymph node, ≤ 3 cm in greatest diameter and ENE (-)
N2	Metastasis in a single lymph node greater than 3 cm and less than 6 cm and ENE (-) or in multiple lymph nodes (ipsilateral or bilateral) less than 6 cm and ENE (-)
N2a	Metastasis in a single lymph node greater than 3 cm and less than 6 cm and ENE (-)
N2b	Metastasis in multiple ipsilateral nodes no larger than 6 cm in maximum dimension and ENE (-)
N2c	Bilateral lymph node metastases smaller than 6 cm and ENE(-)
N3	Metastasis in lymph nodes larger than 6 cm and ENE (-) and metastases in any lymph node with ENE (+)
N3a	Metastasis in a lymph node greater than 6 mm and ENE (-)
N3b	Lymph node metastasis and ENE (+)
M (Distant metastasis)	
Mx	Cannot be evaluated
M0	There are no distant metastases
M1	Distant metastasis

Extranodal Extension (ENE).
Depth of Invasion (DOI).
Note: Superficial erosion of the bone or tooth socket in a primary gingiva tumour is not sufficient to classify it as T4.

Table 2. Histologic Risk Score

Variable		Definition	Point Assigmentç
WPOI	Type 1	Pushing border	0
	Type 2	Finger-like growth	0
	Type 3	Large separate islands, more than 15 cells per island	0
	Type 4	Small tumour islands, 15 cells or fewer, per island	+1
	Type 5	Tumour satellites, equal to or greater than 1 mm away from main tumour (20x) or next closest satellite	+3
LHR	Type 1 (Strong)	Dense complete host response rimming tumour Lymphoid nodules at advancing edge in each 4x field	0
	Type 2 (Intermediate)	Intermediate host response Lymphoid nodules in some but not all 4x fields	+1
	Type 3 (Weak)	Little or no host response No lymphoid nodule	+3
PNI	None	None	0
	Small nerves	Tumour wrapping around nerves, <1 mm diameter	+1
	Large nerves	Tumour wrapping around nerves, equal to or greater than 1 mm diameter (20x)	+3

Abbreviations: LHR: lymphocytic host response; PNI: Perineural invasion; WPOI: WPOI: worst pattern of invasion.

Table 3. Biomarkers used in the present study.

Molecule	Antibody type	Clone name	Dilution	Source
PD-1	Mouse monoclonal	NAT105	1:4 supernatant	CNIO Monoclonal Antibody Unit, Madrid, Spain
PD-L1	Mouse monoclonal	22C3	Prediluted	Dako, Glostrup, Denmark
FOXP3	Mouse monoclonal	236A	Prediluted	CNIO Monoclonal Antibody Unit, Madrid, Spain
CD4	Mouse monoclonal	4B12	Prediluted	Dako, Glostrup, Denmark
CD8	Rat monoclonal	NOR132H	1:5 supernatant	CNIO Monoclonal Antibody Unit, Madrid, Spain
CSF1R	Mouse monoclonal	FER216	1:20 supernatant	CNIO Monoclonal Antibody Unit, Madrid, Spain
P16	Mouse monoclonal	E6H4	Prediluted	ROCHE

Table 4. Number of cases excluded according to criteria.

Exclusion criteria	**Number of cases**

Insufficient histopathological samples	3
Cases of oral carcinoma with microinvasion	6
Cases of OSCC in a location other than the floor of the mouth and tongue	6
Cases of lesion not compatible with OSCC	2
Cases of non-primary tumour	1
Date not between 2010 and 2015	1

Abbreviations: OSCC: Oral squamous cell carcinoma.

Table 5. Descriptive statistics of clinical and histopathological variables according to sex.

Variable	N	Overall, N = 65	Sex		p-value[1]
			Men, N = 40	Women, N = 25	
Age at diagnosis	65				0.178
Mean (SD)		65 (13)	64 (13)	68 (13)	
Tobacco use	63				0.001
Never smoker		30 (48%)	11 (29%)	19 (76%)	
Former smoker		16 (25%)	13 (34%)	3 (12%)	
Current smoker		17 (27%)	14 (37%)	3 (12%)	
Alcohol use	60				<0.001
Nondrinker		41 (68%)	18 (50%)	23 (96%)	
Former drinker		7 (12%)	7 (19%)	0 (0%)	
Current drinker		12 (20%)	11 (31%)	1 (4.2%)	
Mouth Primary Location	65	23 (35%)	16 (40%)	7 (28%)	0.325
Tongue Primary Location	65	48 (74%)	26 (65%)	22 (88%)	0.040
Tumour status	63				0.162
T1		13 (21%)	8 (21%)	5 (20%)	
T2		25 (40%)	11 (29%)	14 (56%)	
T3		13 (21%)	10 (26%)	3 (12%)	
T4		12 (19%)	9 (24%)	3 (12%)	
Nodal status	60				0.902
N0		36 (60%)	21 (60%)	15 (60%)	
N1		8 (13%)	4 (11%)	4 (16%)	
N2		14 (23%)	9 (26%)	5 (20%)	
N3		2 (3.3%)	1 (2.9%)	1 (4.0%)	
Metastasis status	60				0.688
M0		53 (88%)	30 (86%)	23 (92%)	
M1		7 (12%)	5 (14%)	2 (8.0%)	
Stage	63				0.656
Stage I		8 (13%)	6 (16%)	2 (8.0%)	
Stage II		13 (21%)	7 (18%)	6 (24%)	
Stage III		28 (44%)	18 (47%)	10 (40%)	
Stage IV		14 (22%)	7 (18%)	7 (28%)	
Histological Grade	65				0.513
Grade 1: WD		22 (34%)	12 (30%)	10 (40%)	
Grade 2: MD		37 (57%)	25 (62%)	12 (48%)	
Grade 3: PD		6 (9.2%)	3 (7.5%)	3 (12%)	
Oral potentially malignant disorders	65	10 (15%)	4 (10%)	6 (24%)	0.165
Lymphoplasmacytic invasion	65				0.242
Nil		2 (3.1%)	1 (2.5%)	1 (4.0%)	
Low		33 (51%)	24 (60%)	9 (36%)	
Moderate		25 (38%)	12 (30%)	13 (52%)	
Intense		5 (7.7%)	3 (7.5%)	2 (8.0%)	
Vascular invasion	65	5 (7.7%)	4 (10%)	1 (4.0%)	0.641
Perineural invasion	65	26 (40%)	19 (48%)	7 (28%)	0.118
WPOI	65				0.275
WPOI 1		4 (6.2%)	4 (10%)	0 (0%)	
WPOI 2		30 (46%)	16 (40%)	14 (56%)	
WPOI 3		20 (31%)	12 (30%)	8 (32%)	
WPOI 4		4 (6.2%)	2 (5.0%)	2 (8.0%)	
WPOI 5		7 (11%)	6 (15%)	1 (4.0%)	
Risk Score	65				0.029
0-1		24 (37%)	13 (32%)	11 (44%)	
2-3		18 (28%)	8 (20%)	10 (40%)	
4-7		23 (35%)	19 (48%)	4 (16%)	
Depth of invasion	63				0.444
Median (IQR)		9 (3, 12)	10 (4, 12)	8 (3, 10)	
Depth of invasion	63				0.278
Mild invasive		22 (35%)	13 (33%)	9 (38%)	
Moderate invasive		18 (29%)	9 (23%)	9 (38%)	
Deep invasive		23 (37%)	17 (44%)	6 (25%)	
Local recurrence	65	19 (29%)	9 (22%)	10 (40%)	0.131
Regional recurrence	65	6 (9.2%)	4 (10%)	2 (8.0%)	>0.999
Distant recurrence	65	2 (3.1%)	1 (2.5%)	1 (4.0%)	>0.999
DFS outcome	65				0.152
Alive and without recurrence		26 (40%)	14 (35%)	12 (48%)	
Death		5 (7.7%)	4 (10%)	1 (4.0%)	
Death OSCC		11 (17%)	10 (25%)	1 (4.0%)	
Local recurrence		18 (28%)	9 (22%)	9 (36%)	
Regional or Nodal recurrence		5 (7.7%)	3 (7.5%)	2 (8.0%)	
DFS event	65	34 (52%)	22 (55%)	12 (48%)	0.583
DSS outcome	65				0.081
Alive with or without recurrence		33 (51%)	16 (40%)	17 (68%)	
Death		5 (7.7%)	4 (10%)	1 (4.0%)	
Death OSCC		27 (42%)	20 (50%)	7 (28%)	
DSS event	65	27 (42%)	20 (50%)	7 (28%)	0.080
OS Outcome	65				0.028
Alive with or without recurrence		33 (51%)	16 (40%)	17 (68%)	
Death by any cause		32 (49%)	24 (60%)	8 (32%)	
Overall Survival event	65	32 (49%)	24 (60%)	8 (32%)	0.028

1 Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test.

Abbreviations: IQR: interquartile range, DFS: Disease-free survival, DSS: Disease-specific survival, MD: Moderately differentiated, OSCC: Oral squamous cell carcinoma, OS: Overall survival PD: Poorly differentiated, SD: standard deviation, WD: Well differentiated, WPOI: worst pattern of invasion.

Table 6. Clinical characteristics of patients with OPMD who developed OSCC.

OPMD	Sex	Smoke habit	Enolic habit	OSCC Localization	TNM	Exitus
Lichen planus	M	Never	Never	Tongue	T1N0M0	No
	M	Current	Current	Tongue	T2N0M0	Yes
	W	Former	Never	Mouth floor	T2N0M0	No
	W	Never	Never	Tongue	T1N2M0	No
	W	Never	Never	Tongue	T1N0	No
	W	Never	Never	Tongue	T1N0	No
Leukoplakia	W	Never	Never	Tongue	T1N0M0	No
	W	Never	Never	Mouth floor	T1N0M0	No
	W	Current	Never	Tongue	T2N1M1	Yes
	W	Never	Never	Tongue	T1N0M0	No
Chronic actinic cheilitis	M	Current	Never	Mouth floor	T1N0M0	No

Abbreviations: OSCC: Oral squamous cell carcinoma; OPMD: Oral potentially malignant disorders.

Table 7. Descriptive statistics of biomarkers according to sex.

Variable	N	Overall, N = 65	Sex		p-value[1]
			Men, N = 40	Women, N = 25	
PD-1	65				0.838
Median (IQR)		0.01 (0.00, 0.05)	0.02 (0.00, 0.05)	0.01 (0.00, 0.03)	
PD-1	65				0.202
PD-1-Negative <0%		13 (20%)	10 (25%)	3 (12%)	
PD-1-Positive >0%		52 (80%)	30 (75%)	22 (88%)	
PD-L1 CPS	65				0.138
Median (IQR)		2 (0, 15)	1 (0, 6)	3 (1, 22)	
PD-L1 CPS	65				0.279
cps[0,1)		26 (40%)	15 (38%)	11 (44%)	
cps[1,20)		16 (25%)	8 (20%)	8 (32%)	
cps[20,100]		23 (35%)	17 (42%)	6 (24%)	
PD-L1 CPS cut-off >1	65				0.194
PD-L1 Negative CPS<=1		30 (46%)	21 (52%)	9 (36%)	
PD-L1 Positive CPS>1		35 (54%)	19 (48%)	16 (64%)	
PD-L1 intensity	65				0.250
Nil		25 (38%)	19 (48%)	6 (24%)	
Low		18 (28%)	10 (25%)	8 (32%)	
Moderate		18 (28%)	9 (22%)	9 (36%)	
Intense		4 (6.2%)	2 (5.0%)	2 (8.0%)	
PD-L1 TPS	65				0.037
Median (IQR)		0 (0, 19)	0 (0, 4)	1 (0, 40)	
PD-L1 TPS cut-offs 5%/10%	65				0.131
PD-L1 <5%		43 (66%)	30 (75%)	13 (52%)	
PD-L1 [5-10%)		4 (6.2%)	2 (5.0%)	2 (8.0%)	
PD-L1[10-100%]		18 (28%)	8 (20%)	10 (40%)	
PD-L1 TPS cut-off 5%	65				0.057
PD-L1 TPS<5%		43 (66%)	30 (75%)	13 (52%)	
PD-L1 TPS>=5%		22 (34%)	10 (25%)	12 (48%)	
PD-L1 TPS cut-off 10%	65				0.153
PD-L1 TPS <=10%		48 (74%)	32 (80%)	16 (64%)	
PD-L1 TPS >10%		17 (26%)	8 (20%)	9 (36%)	
FOXP3 (%)	65				0.073
Median (IQR)		15 (10, 20)	10 (7, 16)	15 (10, 20)	
FOXP3 cut-off 10%	65				0.033
[0-10]%		29 (45%)	22 (55%)	7 (28%)	
(10-100]%		36 (55%)	18 (45%)	18 (72%)	
CD4 (%)	65				0.223
Median (IQR)		25 (15, 35)	20 (15, 31)	30 (20, 35)	
CD4	65				0.126
[0.05,0.25)		31 (48%)	23 (57%)	8 (32%)	
[0.25,0.35)		13 (20%)	7 (18%)	6 (24%)	
[0.35,0.50]		21 (32%)	10 (25%)	11 (44%)	
P16	65				>0.999
Negative		63 (97%)	39 (98%)	24 (96%)	
Positive		2 (3.1%)	1 (2.5%)	1 (4.0%)	
CSF1R (%)	65				0.619
Median (IQR)		7 (3, 10)	7 (3, 10)	7 (3, 10)	
CSF1R	65				0.217
[0-8%)		38 (58%)	21 (52%)	17 (68%)	
[8-100]		27 (42%)	19 (48%)	8 (32%)	
CD8 (%)	65				0.644
Median (IQR)		30 (20, 40)	32 (20, 41)	30 (25, 35)	
CD8	65				0.099
CD8 [0-10%) Mild		5 (7.7%)	4 (10%)	1 (4.0%)	
CD8 [10-50%] Moderate		51 (78%)	28 (70%)	23 (92%)	
CD8 >=50% Severe		9 (14%)	8 (20%)	1 (4.0%)	

1 Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test

Abbreviations: IQR: interquartile range.

Table 8. Bivariate correlation between biomarkers Spearman's rank-order correlation

Parameter 1	Parameter 2	rho (CI95%)	p-value
PD-1	PD-L1	0.251 (0.001-0.472)	0.737
PD-1	FOXP3	0.065 (−0.189-0.311)	1.000
PD-1	CSF1R	0.079 (−0.175-0.324)	1.000
PD-1	CD4	0.092 (−0.163-0.335)	1.000
PD-1	CD8	−0.080 (−0.325-0.174)	1.000
PD-L1	FOXP3	0.038 (−0.215-0.286)	1.000
PD-L1	CSF1R	0.283 (0.034-0.498)	0.452
PD-L1	CD4	−0.160 (−0.395-0.094)	1.000
PD-L1	CD8	0.018 (−0.234-0.268)	1.000
FOXP3	CSF1R	0.196 (−0.058-0.426)	1.000
FOXP3	CD4	0.341 (0.099-0.545)	0.114
FOXP3	CD8	0.049 (−0.204-0.296)	1.000
CSF1R	CD4	0.014 (−0.238-0.264)	1.000
CSF1R	CD8	0.016 (−0.236-0.266)	1.000
CD4	CD8	0.243 (−0.008-0.465)	0.818

Abbreviations: CI: Confidence Interval.

Table 9. Descriptive statistics of clinical and histopathological variables according to PD-1 expression and PD-L1 expression in TPS and CPS.

Variable	N	Overall, N = 65	PD-1 Expression		p-value[1]	PD-L1 Expression TPS		p-value[1]	PD-L1 Expression CPS		p-value[1]
			Negative, <0% = 13	Positive,> >0% N = 52		TPS <5%	TPS≥5%		CPS <= 1	CPS > 1	
Sex	65				0.202			0.057			0.194
Men		40 (62%)	10 (77%)	30 (58%)		30 (70%)	10 (45%)		21 (70%)	19 (54%)	
Women		25 (38%)	3 (23%)	22 (42%)		13 (30%)	12 (55%)		9 (30%)	16 (46%)	
Age at diagnosis	65				0.254			0.598			0.171
Mean (SD)		67 (57, 73)	65 (56, 68)	68 (59, 75)		66 (56, 73)	68 (59, 76)	0.598	66 (55, 71)	69 (60, 74)	
Tobacco use	63				0.539			0.405			0.249
Never smoker		30 (48%)	4 (33%)	26 (51%)		18 (42%)	12 (60%)		12 (40%)	18 (55%)	
Former smoker		16 (25%)	4 (33%)	12 (24%)		12 (28%)	4 (20%)		7 (23%)	9 (27%)	
Current smoker		17 (27%)	4 (33%)	13 (25%)		13 (30%)	4 (20%)		11 (37%)	6 (18%)	
Alcohol use	60				0.685			0.910			>0.999
Nondrinker		41 (68%)	7 (58%)	34 (71%)		27 (66%)	14 (74%)		20 (69%)	21 (68%)	
Former drinker		7 (12%)	2 (17%)	5 (10%)		5 (12%)	2 (11%)		3 (10%)	4 (13%)	
Current drinker		12 (20%)	3 (25%)	9 (19%)		9 (22%)	3 (16%)		6 (21%)	6 (19%)	
Mouth Primary Location	65	23 (35%)	5 (38%)	18 (35%)	>0.999	18 (42%)	5 (23%)	0.127	17 (57%)	6 (17%)	<0.001
Tongue Primary Location	65	48 (74%)	11 (85%)	37 (71%)	0.486	30 (70%)	18 (82%)	0.296	18 (60%)	30 (86%)	0.019
Tumour Size status	63				0.157			0.518			0.633
T1		13 (21%)	2 (17%)	11 (22%)		10 (24%)	3 (14%)		5 (17%)	8 (24%)	
T2		25 (40%)	2 (17%)	23 (45%)		14 (33%)	11 (52%)		10 (34%)	15 (44%)	
T3		13 (21%)	4 (33%)	9 (18%)		10 (24%)	3 (14%)		7 (24%)	6 (18%)	
T4		12 (19%)	4 (33%)	8 (16%)		8 (19%)	4 (19%)		7 (24%)	5 (15%)	
Nodal status	60				0.200			0.143			0.756
N0		36 (60%)	5 (45%)	31 (63%)		27 (68%)	9 (45%)		16 (59%)	20 (61%)	
N1		8 (13%)	3 (27%)	5 (10%)		5 (12%)	3 (15%)		4 (15%)	4 (12%)	
N2		14 (23%)	2 (18%)	12 (24%)		8 (20%)	6 (30%)		7 (26%)	7 (21%)	
N3		2 (3.3%)	1 (9.1%)	1 (2.0%)		0 (0%)	2 (10%)		0 (0%)	2 (6.1%)	
Metastasis status	60				0.330			0.404			0.222
M0		53 (88%)	8 (80%)	45 (90%)		33 (85%)	20 (95%)		21 (81%)	32 (94%)	
M1		7 (12%)	2 (20%)	5 (10%)		6 (15%)	1 (4.8%)		5 (19%)	2 (5.9%)	
Stage	63				0.574			0.612			0.680
Stage I		8 (13%)	1 (8.3%)	7 (14%)		7 (17%)	1 (4.8%)		4 (14%)	4 (12%)	
Stage II		13 (21%)	3 (25%)	10 (20%)		9 (21%)	4 (19%)		5 (17%)	8 (24%)	
Stage III		28 (44%)	7 (58%)	21 (41%)		17 (40%)	11 (52%)		15 (52%)	13 (38%)	
Stage IV		14 (22%)	1 (8.3%)	13 (25%)		9 (21%)	5 (24%)		5 (17%)	9 (26%)	
Histological Grade	65				0.478			0.239			0.456
Grade 1: WD		22 (34%)	4 (31%)	18 (35%)		17 (40%)	5 (23%)		11 (37%)	11 (31%)	
Grade 2: MD		37 (57%)	9 (69%)	28 (54%)		21 (49%)	16 (73%)		15 (50%)	22 (63%)	
Grade 3: PD		6 (9.2%)	0 (0%)	6 (12%)		5 (12%)	1 (4.5%)		4 (13%)	2 (5.7%)	
Oral potentially malignant disorders	65	10 (15%)	0 (0%)	10 (19%)	0.191	7 (16%)	3 (14%)	>0.999	6 (20%)	4 (11%)	0.493
Lymphoplasmacytic invasion	65				0.030			0.647			0.731
Nil		2 (3.1%)	0 (0%)	2 (3.8%)		2 (4.7%)	0 (0%)		1 (3.3%)	1 (2.9%)	
Low		33 (51%)	11 (85%)	22 (42%)		20 (47%)	13 (59%)		15 (50%)	18 (51%)	
Moderate		25 (38%)	1 (7.7%)	24 (46%)		18 (42%)	7 (32%)		13 (43%)	12 (34%)	
Intense		5 (7.7%)	1 (7.7%)	4 (7.7%)		3 (7.0%)	2 (9.1%)		1 (3.3%)	4 (11%)	
Vascular invasion	65	5 (7.7%)	1 (7.7%)	4 (7.7%)	>0.999	3 (7.0%)	2 (9.1%)	>0.999	3 (10%)	2 (5.7%)	0.655
Perineural invasion	65	26 (40%)	7 (54%)	19 (37%)	0.255	19 (44%)	7 (32%)	0.335	14 (47%)	12 (34%)	0.310
WPOI	65				0.313						0.016
WPOI 1		4 (6.2%)	1 (7.7%)	3 (5.8%)		4 (9.3%)	0 (0%)	0.102	4 (13%)	0 (0%)	
WPOI 2		30 (46%)	4 (31%)	26 (50%)		17 (40%)	13 (59%)		10 (33%)	20 (57%)	
WPOI 3		20 (31%)	7 (54%)	13 (25%)		13 (30%)	7 (32%)		8 (27%)	12 (34%)	
WPOI 4		4 (6.2%)	0 (0%)	4 (7.7%)		2 (4.7%)	2 (9.1%)		2 (6.7%)	2 (5.7%)	
WPOI 5		7 (11%)	1 (7.7%)	6 (12%)		7 (16%)	0 (0%)		6 (20%)	1 (2.9%)	
Risk Score	65				0.165			0.229			0.043
0-1		24 (37%)	2 (15%)	22 (42%)		17 (40%)	7 (32%)		12 (40%)	12 (34%)	
2-3		18 (28%)	4 (31%)	14 (27%)		9 (21%)	9 (41%)		4 (13%)	14 (40%)	
4-7		23 (35%)	7 (54%)	16 (31%)		17 (40%)	6 (27%)		14 (47%)	9 (26%)	
Depth of invasion	63				0.017			0.170			0.978
Median (IQR)		9 (3, 12)	12 (7, 16)	8 (3, 11)		7 (3, 11)	10 (8, 12)		7 (3, 12)	9 (4, 12)	
Local recurrence	65	65	19 (29%)	3 (23%)	0.740	10 (23%)	9 (41%)	0.139	5 (17%)	14 (40%)	0.039
Regional recurrence	65	65	6 (9.2%)	3 (23%)	0.089	5 (12%)	1 (4.5%)	0.655	3 (10%)	3 (8.6%)	>0.999
Distant recurrence	65	65	2 (3.1%)	1 (7.7%)	0.362	2 (4.7%)	0 (0%)	0.545	1 (3.3%)	1 (2.9%)	>0.999
DFS outcome	65				0.039						

Alive and without recurrence		26 (40%)	2 (15%)	24 (46%)		16 (37%)	10 (45%)		11 (37%)	15 (43%)	
Death		5 (7.7%)	1 (7.7%)	4 (7.7%)		4 (9.3%)	1 (4.5%)		4 (13%)	1 (2.9%)	
Death OSCC		11 (17%)	4 (31%)	7 (13%)		9 (21%)	2 (9.1%)		7 (23%)	4 (11%)	
Local recurrence		18 (28%)	3 (23%)	15 (29%)		10 (23%)	8 (36%)		5 (17%)	13 (37%)	
Regional or Nodal recurrence		5 (7.7%)	3 (23%)	2 (3.8%)		4 (9.3%)	1 (4.5%)		3 (10%)	2 (5.7%)	
DFS event	65	34 (52%)	10 (77%)	24 (46%)	0.047	23 (53%)	11 (50%)	0.790	15 (50%)	19 (54%)	0.730
DSS outcome	65				0.009			0.404			0.148
Alive with or without recurrence		33 (51%)	2 (15%)	31 (60%)		19 (44%)	14 (64%)		12 (40%)	21 (60%)	
Death		5 (7.7%)	1 (7.7%)	4 (7.7%)		4 (9.3%)	1 (4.5%)		4 (13%)	1 (2.9%)	
Death OSCC		27 (42%)	10 (77%)	17 (33%)		20 (47%)	7 (32%)		14 (47%)	13 (37%)	
DSS event	65	34 (52%)	10 (77%)	24 (46%)	0.004	20 (47%)	7 (32%)	0.255	14 (47%)	13 (37%)	
OS Outcome	65				0.004			0.138			0.108
Alive with or without recurrence		33 (51%)	2 (15%)	31 (60%)		19 (44%)	14 (64%)		12 (40%)	21 (60%)	
Death by any cause		32 (49%)	11 (85%)	21 (40%)		24 (56%)	8 (36%)		18 (60%)	14 (40%)	
Overall Survival event	65	32 (49%)	11 (85%)	21 (40%)	0.004	24 (56%)	8 (36%)	0.138	18 (60%)	14 (40%)	0.108

1 Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test

Abbreviations: CI: Confidence Interval. IQR: interquartile range, DFS: Disease-free survival, DSS: Disease-specific survival, HR:Hazard Ratio, MD: Moderately differentiated, OSCC: Oral squamous cell carcinoma, OS: Overall survival PD: Poorly differentiated, SD: standard deviation, WD: Well differentiated, WPOI: worst pattern of invasion.

Table 10. Demographic, clinical, **and pathological features by** FOXP3.

Variable	N	Overall, N = 65	[0.01,0.05], N = 7	(0.05,0.1], N = 22	(0.1,0.2], N = 31	(0.2,1], N = 5	p-value[J]
Age at diagnosis	65						0.189
Mean (SD)		65 (13)	68 (10)	61 (15)	68 (11)	60 (15)	
Gender	65						0.177
Men		40 (62%)	5 (71%)	17 (77%)	16 (52%)	2 (40%)	
Women		25 (38%)	2 (29%)	5 (23%)	15 (48%)	3 (60%)	
Tobacco use	63						0.126
Never smoker		30 (48%)	2 (29%)	8 (38%)	19 (63%)	1 (20%)	
Former smoker		16 (25%)	3 (43%)	8 (38%)	4 (13%)	1 (20%)	
Current smoker		17 (27%)	2 (29%)	5 (24%)	7 (23%)	3 (60%)	
Alcohol use	60						0.935
Nondrinker		41 (68%)	5 (71%)	13 (62%)	19 (70%)	4 (80%)	
Former drinker		7 (12%)	1 (14%)	4 (19%)	2 (7.4%)	0 (0%)	
Current drinker		12 (20%)	1 (14%)	4 (19%)	6 (22%)	1 (20%)	
Mouth Primary Location	65	23 (35%)	3 (43%)	8 (36%)	8 (26%)	4 (80%)	0.123
Tongue Primary Location	65	48 (74%)	6 (86%)	15 (68%)	24 (77%)	3 (60%)	0.676
Tumour status	63						0.066
T1		13 (21%)	1 (14%)	8 (38%)	3 (10%)	1 (20%)	
T2		25 (40%)	2 (29%)	4 (19%)	17 (57%)	2 (40%)	
T3		13 (21%)	3 (43%)	3 (14%)	5 (17%)	2 (40%)	
T4		12 (19%)	1 (14%)	6 (29%)	5 (17%)	0 (0%)	
Nodal status	60						0.768
N0		36 (60%)	5 (83%)	9 (47%)	19 (63%)	3 (60%)	
N1		8 (13%)	0 (0%)	3 (16%)	4 (13%)	1 (20%)	
N2		14 (23%)	1 (17%)	7 (37%)	5 (17%)	1 (20%)	
N3		2 (3.3%)	0 (0%)	0 (0%)	2 (6.7%)	0 (0%)	
Metastasis status	60						0.410
M0		53 (88%)	5 (83%)	16 (84%)	28 (93%)	4 (80%)	
M1		7 (12%)	1 (17%)	3 (16%)	2 (6.7%)	1 (20%)	
Stage	63						0.191
Stage I		8 (13%)	1 (14%)	5 (24%)	1 (3.3%)	1 (20%)	
Stage II		13 (21%)	1 (14%)	4 (19%)	7 (23%)	1 (20%)	
Stage III		28 (44%)	3 (43%)	11 (52%)	12 (40%)	2 (40%)	
Stage IV		14 (22%)	2 (29%)	1 (4.8%)	10 (33%)	1 (20%)	
Histological Grade	65						0.740
Grade 1: WD		22 (34%)	2 (29%)	7 (32%)	12 (39%)	1 (20%)	
Grade 2: MD		37 (57%)	5 (71%)	14 (64%)	15 (48%)	3 (60%)	
Grade 3: PD		6 (9.2%)	0 (0%)	1 (4.5%)	4 (13%)	1 (20%)	
Prev. Malignant disease	65	10 (15%)	0 (0%)	5 (23%)	3 (9.7%)	2 (40%)	0.126
Lymphoplasmacytic invasion	65						0.705
Nil		2 (3.1%)	0 (0%)	1 (4.5%)	1 (3.2%)	0 (0%)	
Low		33 (51%)	6 (86%)	12 (55%)	13 (42%)	2 (40%)	
Moderate		25 (38%)	1 (14%)	8 (36%)	13 (42%)	3 (60%)	
Intense		5 (7.7%)	0 (0%)	1 (4.5%)	4 (13%)	0 (0%)	
Vascular invasion	65	5 (7.7%)	0 (0%)	4 (18%)	1 (3.2%)	0 (0%)	0.261
Perineural invasion	65	26 (40%)	3 (43%)	10 (45%)	13 (42%)	0 (0%)	0.314
Invasive pattern	65						0.105
WPOI 1		4 (6.2%)	1 (14%)	1 (4.5%)	1 (3.2%)	1 (20%)	
WPOI 2		30 (46%)	4 (57%)	8 (36%)	15 (48%)	3 (60%)	
WPOI 3		20 (31%)	0 (0%)	9 (41%)	10 (32%)	1 (20%)	
WPOI 4		4 (6.2%)	0 (0%)	0 (0%)	4 (13%)	0 (0%)	
WPOI 5		7 (11%)	2 (29%)	4 (18%)	1 (3.2%)	0 (0%)	
Risk Score	65						0.157
0-1		24 (37%)	1 (14%)	8 (36%)	12 (39%)	3 (60%)	
2-3		18 (28%)	4 (57%)	3 (14%)	9 (29%)	2 (40%)	
4-7		23 (35%)	2 (29%)	11 (50%)	10 (32%)	0 (0%)	
Depth of invasion	63						0.587
Median (IQR)		9 (3, 12)	13 (10, 15)	8 (3, 11)	8 (5, 11)	5 (3, 12)	
Mild invasive		22 (35%)	1 (17%)	10 (45%)	8 (27%)	3 (60%)	
Moderate invasive		18 (29%)	1 (17%)	3 (14%)	14 (47%)	0 (0%)	
Deep invasive		23 (37%)	4 (67%)	9 (41%)	8 (27%)	2 (40%)	
Local recurrence	65	19 (29%)	3 (43%)	4 (18%)	11 (35%)	1 (20%)	0.457
Regional recurrence	65	6 (9.2%)	0 (0%)	3 (14%)	3 (9.7%)	0 (0%)	0.824
Distant recurrence	65	2 (3.1%)	0 (0%)	1 (4.5%)	1 (3.2%)	0 (0%)	>0.999
DFS outcome	65						0.688
Alive and without recurrence		26 (40%)	2 (29%)	9 (41%)	11 (35%)	4 (80%)	
Death		5 (7.7%)	0 (0%)	2 (9.1%)	3 (9.7%)	0 (0%)	
Death OSCC		11 (17%)	2 (29%)	5 (23%)	4 (13%)	0 (0%)	
Local recurrence		18 (28%)	3 (43%)	3 (14%)	11 (35%)	1 (20%)	
Regional or Nodal recurrence		5 (7.7%)	0 (0%)	3 (14%)	2 (6.5%)	0 (0%)	
DFS event	65	34 (52%)	5 (71%)	11 (50%)	17 (55%)	1 (20%)	0.381
DSS outcome	65						0.914
Alive with or without recurrence		33 (51%)	3 (43%)	11 (50%)	15 (48%)	4 (80%)	
Death		5 (7.7%)	0 (0%)	2 (9.1%)	3 (9.7%)	0 (0%)	
Death OSCC		27 (42%)	4 (57%)	9 (41%)	13 (42%)	1 (20%)	
DSS event	65	27 (42%)	4 (57%)	9 (41%)	13 (42%)	1 (20%)	0.687
OS Outcome	65						0.661
Alive with or without recurrence		33 (51%)	3 (43%)	11 (50%)	15 (48%)	4 (80%)	
Death by any cause		32 (49%)	4 (57%)	11 (50%)	16 (52%)	1 (20%)	
Overall Survival event	65	32 (49%)	4 (57%)	11 (50%)	16 (52%)	1 (20%)	0.661
CD8	65						0.867
CD8 [0-10%) Mild		5 (7.7%)	1 (14%)	2 (9.1%)	2 (6.5%)	0 (0%)	
CD8 [10-50)% Moderate		51 (78%)	6 (86%)	16 (73%)	24 (77%)	5 (100%)	
CD8 >=50% Severe		9 (14%)	0 (0%)	4 (18%)	5 (16%)	0 (0%)	
CD4	65						0.300
[0.05,0.25)		31 (48%)	6 (86%)	12 (55%)	12 (39%)	1 (20%)	
[0.25,0.35)		13 (20%)	0 (0%)	4 (18%)	8 (26%)	1 (20%)	
[0.35,0.50]		21 (32%)	1 (14%)	6 (27%)	11 (35%)	3 (60%)	
CSF1R	65						0.492
[0-8)%		38 (58%)	5 (71%)	15 (68%)	16 (52%)	2 (40%)	
[8-100)%		27 (42%)	2 (29%)	7 (32%)	15 (48%)	3 (60%)	

[J] Kruskal-Wallis rank sum test; Fisher's exact test

Table 11. Demographic, clinical, and pathological features by CD4.

Variable	N	Overall, N = 65	[0.05,0.25), N = 31	[0.25,0.35), N = 13	[0.35,0.50], N = 21	p-value[1]
Age at diagnosis	65					0.754
Mean (SD)		65 (13)	66 (14)	66 (11)	64 (13)	
Gender	65					0.126
Men		40 (62%)	23 (74%)	7 (54%)	10 (48%)	
Women		25 (38%)	8 (26%)	6 (46%)	11 (52%)	
Tobacco use	63					0.180
Never smoker		30 (48%)	13 (45%)	4 (31%)	13 (62%)	
Former smoker		16 (25%)	10 (34%)	4 (31%)	2 (9.5%)	
Current smoker		17 (27%)	6 (21%)	5 (38%)	6 (29%)	
Alcohol use	60					0.396
Nondrinker		41 (68%)	18 (62%)	8 (67%)	15 (79%)	
Former drinker		7 (12%)	5 (17%)	2 (17%)	0 (0%)	
Current drinker		12 (20%)	6 (21%)	2 (17%)	4 (21%)	
Mouth Primary Location	65	23 (35%)	9 (29%)	6 (46%)	8 (38%)	0.493
Tongue Primary Location	65	48 (74%)	23 (74%)	10 (77%)	15 (71%)	>0.999
Tumour status	63					0.509
T1		13 (21%)	8 (27%)	2 (15%)	3 (15%)	
T2		25 (40%)	8 (27%)	6 (46%)	11 (55%)	
T3		13 (21%)	6 (20%)	3 (23%)	4 (20%)	
T4		12 (19%)	8 (27%)	2 (15%)	2 (10%)	
Nodal status	60					0.419
N0		36 (60%)	17 (61%)	9 (69%)	10 (53%)	
N1		8 (13%)	3 (11%)	0 (0%)	5 (26%)	
N2		14 (23%)	7 (25%)	4 (31%)	3 (16%)	
N3		2 (3.3%)	1 (3.6%)	0 (0%)	1 (5.3%)	
Metastasis status	60					0.346
M0		53 (88%)	26 (93%)	10 (77%)	17 (89%)	
M1		7 (12%)	2 (7.1%)	3 (23%)	2 (11%)	
Stage	63					0.559
Stage I		8 (13%)	6 (20%)	1 (7.7%)	1 (5.0%)	
Stage II		13 (21%)	5 (17%)	2 (15%)	6 (30%)	
Stage III		28 (44%)	14 (47%)	7 (54%)	7 (35%)	
Stage IV		14 (22%)	5 (17%)	3 (23%)	6 (30%)	
Histological Grade	65					0.172
Grade 1: WD		22 (34%)	6 (19%)	6 (46%)	10 (48%)	
Grade 2: MD		37 (57%)	22 (71%)	6 (46%)	9 (43%)	
Grade 3: PD		6 (9.2%)	3 (9.7%)	1 (7.7%)	2 (9.5%)	
Prev. Malignant disease	65	10 (15%)	3 (9.7%)	2 (15%)	5 (24%)	0.354
Lymphoplasmacytic invasion	65					0.143
Nil		2 (3.1%)	2 (6.5%)	0 (0%)	0 (0%)	
Low		33 (51%)	20 (65%)	5 (38%)	8 (38%)	
Moderate		25 (38%)	8 (26%)	6 (46%)	11 (52%)	
Intense		5 (7.7%)	1 (3.2%)	2 (15%)	2 (9.5%)	
Vascular invasion	65	5 (7.7%)	3 (9.7%)	1 (7.7%)	1 (4.8%)	0.846
Perineural invasion	65	26 (40%)	13 (42%)	4 (31%)	9 (43%)	0.748
Invasive pattern	65					0.037
WPOI 1		4 (6.2%)	2 (6.5%)	2 (15%)	0 (0%)	
WPOI 2		30 (46%)	16 (52%)	3 (23%)	11 (52%)	
WPOI 3		20 (31%)	10 (32%)	7 (54%)	3 (14%)	
WPOI 4		4 (6.2%)	2 (6.5%)	0 (0%)	2 (9.5%)	
WPOI 5		7 (11%)	1 (3.2%)	1 (7.7%)	5 (24%)	
Risk Score	65					0.215
0-1		24 (37%)	8 (26%)	8 (62%)	8 (38%)	
2-3		18 (28%)	11 (35%)	1 (7.7%)	6 (29%)	
4-7		23 (35%)	12 (39%)	4 (31%)	7 (33%)	
Depth of invasion	63					0.930
Median (IQR)		9 (3, 12)	9 (3, 12)	8 (6, 12)	7 (3, 12)	
Mild invasive		22 (35%)	12 (40%)	3 (25%)	7 (33%)	
Moderate invasive		18 (29%)	6 (20%)	5 (42%)	7 (33%)	
Deep invasive		23 (37%)	12 (40%)	4 (33%)	7 (33%)	
Local recurrence	65	19 (29%)	10 (32%)	3 (23%)	6 (29%)	0.937
Regional recurrence	65	6 (9.2%)	2 (6.5%)	2 (15%)	2 (9.5%)	0.650
Distant recurrence	65	2 (3.1%)	0 (0%)	2 (15%)	0 (0%)	0.037
DFS outcome	65					0.580
Alive and without recurrence		26 (40%)	10 (32%)	5 (38%)	11 (52%)	
Death		5 (7.7%)	4 (13%)	1 (7.7%)	0 (0%)	
Death OSCC		11 (17%)	6 (19%)	2 (15%)	3 (14%)	
Local recurrence		18 (28%)	10 (32%)	3 (23%)	5 (24%)	
Regional or Nodal recurrence		5 (7.7%)	1 (3.2%)	2 (15%)	2 (9.5%)	
DFS event	65	34 (52%)	17 (55%)	7 (54%)	10 (48%)	0.871
DSS outcome	65					0.262
Alive with or without recurrence		33 (51%)	12 (39%)	8 (62%)	13 (62%)	
Death		5 (7.7%)	4 (13%)	1 (7.7%)	0 (0%)	
Death OSCC		27 (42%)	15 (48%)	4 (31%)	8 (38%)	
DSS event	65	27 (42%)	15 (48%)	4 (31%)	8 (38%)	0.516
OS Outcome	65					0.178
Alive with or without recurrence		33 (51%)	12 (39%)	8 (62%)	13 (62%)	
Death by any cause		32 (49%)	19 (61%)	5 (38%)	8 (38%)	
Overall Survival event	65	32 (49%)	19 (61%)	5 (38%)	8 (38%)	0.178
CD8	65					0.270
[0-10%) Mild		5 (7.7%)	3 (9.7%)	2 (15%)	0 (0%)	
[10-50)% Moderate		51 (78%)	25 (81%)	8 (62%)	18 (86%)	
>=50% Severe		9 (14%)	3 (9.7%)	3 (23%)	3 (14%)	
FOXP3	65					0.300
[0.01,0.05]		7 (11%)	6 (19%)	0 (0%)	1 (4.8%)	
(0.05,0.1]		22 (34%)	12 (39%)	4 (31%)	6 (29%)	
(0.1,0.2]		31 (48%)	12 (39%)	8 (62%)	11 (52%)	
(0.2,1]		5 (7.7%)	1 (3.2%)	1 (7.7%)	3 (14%)	
CSF1R	65					0.540
[0-8)%		38 (58%)	16 (52%)	8 (62%)	14 (67%)	
[8-100)%		27 (42%)	15 (48%)	5 (38%)	7 (33%)	

[1] Kruskal-Wallis rank sum test; Pearson's Chi-squared test; Fisher's exact test.

Table 12. Demographic, clinical, and pathological features by CD8.

Variable	N	Overall, N = 65	CD8 [0-10%) Mild, N = 5	CD8 [10-50)% Moderate, N = 51	CD8 >=50% Severe, N = 9	p-value[1]
Age at diagnosis	65					0.729
Mean (SD)		65 (13)	65 (7)	66 (14)	63 (11)	
Gender	65					0.099
Men		40 (62%)	4 (80%)	28 (55%)	8 (89%)	
Women		25 (38%)	1 (20%)	23 (45%)	1 (11%)	
Tobacco use	63					0.856
Never smoker		30 (48%)	2 (40%)	23 (46%)	5 (62%)	
Former smoker		16 (25%)	1 (20%)	14 (28%)	1 (12%)	
Current smoker		17 (27%)	2 (40%)	13 (26%)	2 (25%)	
Alcohol use	60					0.830
Nondrinker		41 (68%)	3 (60%)	33 (69%)	5 (71%)	
Former drinker		7 (12%)	0 (0%)	6 (12%)	1 (14%)	
Current drinker		12 (20%)	2 (40%)	9 (19%)	1 (14%)	
Mouth Primary Location	65	23 (35%)	1 (20%)	19 (37%)	3 (33%)	0.897
Tongue Primary Location	65	48 (74%)	4 (80%)	38 (75%)	6 (67%)	0.875
Tumour size status	63					0.443
T1		13 (21%)	2 (40%)	9 (18%)	2 (29%)	
T2		25 (40%)	1 (20%)	21 (41%)	3 (43%)	
T3		13 (21%)	2 (40%)	11 (22%)	0 (0%)	
T4		12 (19%)	0 (0%)	10 (20%)	2 (29%)	
Nodal status	60					0.089
N0		36 (60%)	4 (80%)	31 (63%)	1 (17%)	
N1		8 (13%)	1 (20%)	6 (12%)	1 (17%)	
N2		14 (23%)	0 (0%)	11 (22%)	3 (50%)	
N3		2 (3.3%)	0 (0%)	1 (2.0%)	1 (17%)	
Metastasis status	60					0.379
M0		53 (88%)	4 (80%)	44 (90%)	5 (83%)	
M1		7 (12%)	1 (20%)	5 (10%)	1 (17%)	
Stage	63					0.503
Stage I		8 (13%)	2 (40%)	6 (12%)	0 (0%)	
Stage II		13 (21%)	1 (20%)	11 (22%)	1 (14%)	
Stage III		28 (44%)	1 (20%)	22 (43%)	5 (71%)	
Stage IV		14 (22%)	1 (20%)	12 (24%)	1 (14%)	
Histological Grade	65					0.830
Grade 1: WD		22 (34%)	1 (20%)	17 (33%)	4 (44%)	
Grade 2: MD		37 (57%)	4 (80%)	28 (55%)	5 (56%)	
Grade 3: PD		6 (9.2%)	0 (0%)	6 (12%)	0 (0%)	
Prev. Malignant disease	65	10 (15%)	2 (40%)	7 (14%)	1 (11%)	0.286
Lymphoplasmacytic invasion	65					0.297
Nil		2 (3.1%)	1 (20%)	1 (2.0%)	0 (0%)	
Low		33 (51%)	2 (40%)	26 (51%)	5 (56%)	
Moderate		25 (38%)	1 (20%)	21 (41%)	3 (33%)	
Intense		5 (7.7%)	1 (20%)	3 (5.9%)	1 (11%)	
Vascular invasion	65	5 (7.7%)	0 (0%)	3 (5.9%)	2 (22%)	0.179
Perineural invasion	65	26 (40%)	2 (40%)	19 (37%)	5 (56%)	0.604
Invasive pattern	65					0.390
WPOI 1		4 (6.2%)	1 (20%)	3 (5.9%)	0 (0%)	
WPOI 2		30 (46%)	3 (60%)	25 (49%)	2 (22%)	
WPOI 3		20 (31%)	1 (20%)	14 (27%)	5 (56%)	
WPOI 4		4 (6.2%)	0 (0%)	4 (7.8%)	0 (0%)	
WPOI 5		7 (11%)	0 (0%)	5 (9.8%)	2 (22%)	
Risk Score	65					0.244
0-1		24 (37%)	2 (40%)	18 (35%)	4 (44%)	
2-3		18 (28%)	2 (40%)	16 (31%)	0 (0%)	
4-7		23 (35%)	1 (20%)	17 (33%)	5 (56%)	
Depth of invasion	63					0.586
Median (IQR)		9 (3, 12)	9 (2, 11)	8 (3, 12)	10 (8, 12)	
Mild invasive		22 (35%)	2 (40%)	18 (37%)	2 (22%)	
Moderate invasive		18 (29%)	1 (20%)	14 (29%)	3 (33%)	
Deep invasive		23 (37%)	2 (40%)	17 (35%)	4 (44%)	
Local recurrence	65	19 (29%)	2 (40%)	14 (27%)	3 (33%)	0.693
Regional recurrence	65	6 (9.2%)	0 (0%)	5 (9.8%)	1 (11%)	>0.999
Distant recurrence	65	2 (3.1%)	0 (0%)	2 (3.9%)	0 (0%)	>0.999
DFS outcome	65					0.916
Alive and without recurrence		26 (40%)	2 (40%)	20 (39%)	4 (44%)	
Death		5 (7.7%)	1 (20%)	4 (7.8%)	0 (0%)	
Death OSCC		11 (17%)	0 (0%)	9 (18%)	2 (22%)	
Local recurrence		18 (28%)	2 (40%)	13 (25%)	3 (33%)	
Regional or Nodal recurrence		5 (7.7%)	0 (0%)	5 (9.8%)	0 (0%)	
DFS event	65	34 (52%)	2 (40%)	27 (53%)	5 (56%)	0.908
DSS outcome	65					0.666
Alive with or without recurrence		33 (51%)	3 (60%)	25 (49%)	5 (56%)	
Death		5 (7.7%)	1 (20%)	4 (7.8%)	0 (0%)	
Death OSCC		27 (42%)	1 (20%)	22 (43%)	4 (44%)	
DSS event	65	27 (42%)	1 (20%)	22 (43%)	4 (44%)	0.810
OS Outcome	65					>0.999
Alive with or without recurrence		33 (51%)	3 (60%)	25 (49%)	5 (56%)	
Death by any cause		32 (49%)	2 (40%)	26 (51%)	4 (44%)	
Overall Survival event	65	32 (49%)	2 (40%)	26 (51%)	4 (44%)	>0.999
CD4	65					0.270
[0.05,0.25)		31 (48%)	3 (60%)	25 (49%)	3 (33%)	
[0.25,0.35)		13 (20%)	2 (40%)	8 (16%)	3 (33%)	
[0.35,0.50]		21 (32%)	0 (0%)	18 (35%)	3 (33%)	
FOXP3	65					0.867
[0.01,0.05]		7 (11%)	1 (20%)	6 (12%)	0 (0%)	
(0.05,0.1]		22 (34%)	2 (40%)	16 (31%)	4 (44%)	
(0.1,0.2]		31 (48%)	2 (40%)	24 (47%)	5 (56%)	
(0.2,1]		5 (7.7%)	0 (0%)	5 (9.8%)	0 (0%)	
CSF1R Median	65					>0.999
[0-8)%		38 (58%)	3 (60%)	30 (59%)	5 (56%)	
[8-100)%		27 (42%)	2 (40%)	21 (41%)	4 (44%)	

[1] Kruskal-Wallis rank sum test; Fisher's exact test

Table 13. Demographic, clinical and pathological features by CSF1R.

Variable	N	Overall, N = 65	[0-8)%, N = 38	[8-100)%, N = 27	p-value[1]
Age at diagnosis	65				0.785
Mean (SD)		65 (13)	65 (13)	66 (13)	
Gender	65				0.217
Men		40 (62%)	21 (55%)	19 (70%)	
Women		25 (38%)	17 (45%)	8 (30%)	
Tobacco use	63				0.766
Never smoker		30 (48%)	19 (50%)	11 (44%)	
Former smoker		16 (25%)	10 (26%)	6 (24%)	
Current smoker		17 (27%)	9 (24%)	8 (32%)	
Alcohol use	60				0.708
Nondrinker		41 (68%)	26 (70%)	15 (65%)	
Former drinker		7 (12%)	5 (14%)	2 (8.7%)	
Current drinker		12 (20%)	6 (16%)	6 (26%)	
Mouth Primary Location	65	23 (35%)	15 (39%)	8 (30%)	0.413
Tongue Primary Location	65	48 (74%)	26 (68%)	22 (81%)	0.238
Tumour size status	63				0.720
T1		13 (21%)	8 (21%)	5 (20%)	
T2		25 (40%)	17 (45%)	8 (32%)	
T3		13 (21%)	7 (18%)	6 (24%)	
T4		12 (19%)	6 (16%)	6 (24%)	
Nodal status	60				0.707
N0		36 (60%)	21 (58%)	15 (62%)	
N1		8 (13%)	4 (11%)	4 (17%)	
N2		14 (23%)	10 (28%)	4 (17%)	
N3		2 (3.3%)	1 (2.8%)	1 (4.2%)	
Metastasis status	60				>0.999
M0		53 (88%)	33 (89%)	20 (87%)	
M1		7 (12%)	4 (11%)	3 (13%)	
Stage	63				>0.999
Stage I		8 (13%)	5 (13%)	3 (12%)	
Stage II		13 (21%)	8 (21%)	5 (20%)	
Stage III		28 (44%)	17 (45%)	11 (44%)	
Stage IV		14 (22%)	8 (21%)	6 (24%)	
Histological Grade	65				0.064
Grade 1: WD		22 (34%)	12 (32%)	10 (37%)	
Grade 2: MD		37 (57%)	25 (66%)	12 (44%)	
Grade 3: PD		6 (9.2%)	1 (2.6%)	5 (19%)	
Prev. Malignant disease	65	10 (15%)	7 (18%)	3 (11%)	0.503
Lymphoplasmacytic invasion	65				0.610
Nil		2 (3.1%)	2 (5.3%)	0 (0%)	
Low		33 (51%)	17 (45%)	16 (59%)	
Moderate		25 (38%)	16 (42%)	9 (33%)	
Intense		5 (7.7%)	3 (7.9%)	2 (7.4%)	
Vascular invasion	65	5 (7.7%)	2 (5.3%)	3 (11%)	0.642
Perineural invasion	65	26 (40%)	14 (37%)	12 (44%)	0.538
Invasive pattern	65				0.910
WPOI 1		4 (6.2%)	3 (7.9%)	1 (3.7%)	
WPOI 2		30 (46%)	17 (45%)	13 (48%)	
WPOI 3		20 (31%)	11 (29%)	9 (33%)	
WPOI 4		4 (6.2%)	2 (5.3%)	2 (7.4%)	
WPOI 5		7 (11%)	5 (13%)	2 (7.4%)	
Risk Score	65				0.878
0-1		24 (37%)	15 (39%)	9 (33%)	
2-3		18 (28%)	10 (26%)	8 (30%)	
4-7		23 (35%)	13 (34%)	10 (37%)	
Depth of invasion	63				0.732
Median (IQR)		9 (3, 12)	9 (3, 11)	8 (3, 14)	
Mild invasive		22 (35%)	12 (32%)	10 (38%)	
Moderate invasive		18 (29%)	13 (35%)	5 (19%)	
Deep invasive		23 (37%)	12 (32%)	11 (42%)	
Local recurrence	65	19 (29%)	11 (29%)	8 (30%)	0.952
Regional recurrence	65	6 (9.2%)	2 (5.3%)	4 (15%)	0.224
Distant recurrence	65	2 (3.1%)	1 (2.6%)	1 (3.7%)	>0.999
DFS outcome	65				0.804
Alive and without recurrence		26 (40%)	16 (42%)	10 (37%)	
Death		5 (7.7%)	2 (5.3%)	3 (11%)	
Death OSCC		11 (17%)	7 (18%)	4 (15%)	
Local recurrence		18 (28%)	11 (29%)	7 (26%)	
Regional or Nodal recurrence		5 (7.7%)	2 (5.3%)	3 (11%)	
DFS event	65	34 (52%)	20 (53%)	14 (52%)	0.951
DSS outcome	65				0.553
Alive with or without recurrence		33 (51%)	21 (55%)	12 (44%)	
Death		5 (7.7%)	2 (5.3%)	3 (11%)	
Death OSCC		27 (42%)	15 (39%)	12 (44%)	
DSS event	65	27 (42%)	15 (39%)	12 (44%)	0.689
OS Outcome	65				0.390
Alive with or without recurrence		33 (51%)	21 (55%)	12 (44%)	
Death by any cause		32 (49%)	17 (45%)	15 (56%)	
Overall Survival event	65	32 (49%)	17 (45%)	15 (56%)	0.390
CD8	65				>0.999
CD8 [0-10%) Mild		5 (7.7%)	3 (7.9%)	2 (7.4%)	
CD8 [10-50)% Moderate		51 (78%)	30 (79%)	21 (78%)	
CD8 >=50% Severe		9 (14%)	5 (13%)	4 (15%)	
CD4	65				0.540
[0.05,0.25)		31 (48%)	16 (42%)	15 (56%)	
[0.25,0.35)		13 (20%)	8 (21%)	5 (19%)	
[0.35,0.50]		21 (32%)	14 (37%)	7 (26%)	
FOXP3	65				0.492
[0.01,0.05]		7 (11%)	5 (13%)	2 (7.4%)	
(0.05,0.1]		22 (34%)	15 (39%)	7 (26%)	
(0.1,0.2]		31 (48%)	16 (42%)	15 (56%)	
(0.2,1]		5 (7.7%)	2 (5.3%)	3 (11%)	

[1] Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test

Table 14. Univariate analysis of clinical and histopathological variables for the different survival rates (OS, DSS, DFS)

	Cox proportional Hazard Model					
	OS: HR(CI95%)	p-value	DSS: HR(CI95%)	p-value	DFS: HR(CI95%)	p-value
Age at diagnosis						
	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
NA	1.02 (0.99-1.05)	0.107	1.01 (0.98-1.04)	0.373	1.02 (0.99-1.04)	0.177
Gender						
Men	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Women	0.35 (0.15-0.82)	0.015	0.36 (0.14-0.89)	0.028	0.80 (0.40-1.63)	0.549
Tobacco use						
Never smoker	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Former smoker	1.50 (0.59-3.84)	0.395	1.96 (0.73-5.31)	0.184	1.40 (0.62-3.19)	0.418
Current smoker	2.11 (0.90-4.90)	0.084	2.47 (0.97-6.27)	0.056	1.14 (0.50-2.59)	0.757
Alcohol use						
Nondrinker	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Former drinker	1.73 (0.64-4.68)	0.280	2.07 (0.75-5.73)	0.162	1.78 (0.72-4.44)	0.211
Current drinker	1.28 (0.51-3.25)	0.596	1.25 (0.45-3.45)	0.667	1.13 (0.46-2.83)	0.787
Tumour location						
Tongue	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Base of the Mouth	0.87 (0.36-2.05)	0.741	0.92 (0.36-2.35)	0.857	0.67 (0.29-1.57)	0.358
Tongue and Base of the Mouth	1.16 (0.34-3.93)	0.810	1.39 (0.40-4.78)	0.603	1.61 (0.55-4.68)	0.384
Tumour status						
T1	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
T2	1.46 (0.47-4.54)	0.516	1.43 (0.38-5.31)	0.596	1.06 (0.40-2.83)	0.912
T3	2.16 (0.63-7.41)	0.222	2.43 (0.60-9.76)	0.211	1.04 (0.34-3.23)	0.944
T4	2.29 (0.69-7.65)	0.177	3.00 (0.79-11.38)	0.107	2.21 (0.80-6.10)	0.124
Nodal Status						
N0	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
N1	1.21 (0.40-3.67)	0.741	1.07 (0.30-3.84)	0.912	1.57 (0.57-4.31)	0.384
N2/N3	1.04 (0.45-2.40)	0.920	1.12 (0.46-2.76)	0.795	0.88 (0.40-1.98)	0.764
Metastasis status						
M0	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
M1	5.84 (2.18-15.64)	0.000	6.00 (2.02-17.81)	0.001	3.11 (1.15-8.44)	0.026
Histological Grade						
Grade 1: WD	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Grade 2: MD	1.26 (0.54-2.95)	0.596	1.77 (0.64-4.92)	0.276	1.63 (0.72-3.71)	0.242
Grade 3: PD	5.07 (1.73-14.84)	0.003	8.09 (2.43-26.97)	0.001	5.15 (1.75-15.15)	0.003
Stage						
Stage I	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Stage II	1.43 (0.28-7.39)	0.674	2.75 (0.32-23.63)	0.358	1.41 (0.36-5.48)	0.617
Stage III	2.24 (0.52-9.74)	0.280	3.88 (0.51-29.46)	0.190	1.56 (0.46-5.36)	0.478
Stage IV	1.71 (0.35-8.29)	0.503	2.39 (0.28-20.48)	0.430	1.32 (0.34-5.12)	0.689
Oral potentially malignant disorders						
No	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Yes	0.81 (0.28-2.32)	0.697	0.46 (0.11-1.94)	0.289	0.74 (0.26-2.10)	0.569
Perineural invasion						
No	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Yes	1.21 (0.58-2.51)	0.610	1.25 (0.56-2.79)	0.575	1.04 (0.52-2.08)	0.912
Vascular invasion						
No	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Yes	0.68 (0.16-2.95)	0.610	0.81 (0.18-3.59)	0.787	0.59 (0.14-2.53)	0.478
Lymphoplasmacytic invasion						
Nil/Low	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Moderate	0.86 (0.40-1.89)	0.711	0.67 (0.28-1.64)	0.384	0.90 (0.43-1.88)	0.779
Intense	1.04 (0.24-4.61)	0.952	1.16 (0.26-5.19)	0.841	0.73 (0.17-3.15)	0.674
Margins affected						
No	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Yes	0.50 (0.07-3.71)	0.503	0.61 (0.08-4.53)	0.631	0.44 (0.06-3.22)	0.418
Risk Score						
0-1	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
2-3	0.92 (0.36-2.37)	0.865	1.02 (0.37-2.86)	0.968	1.02 (0.43-2.41)	0.968
4-7	1.26 (0.54-2.94)	0.582	1.35 (0.53-3.41)	0.529	1.17 (0.52-2.63)	0.704
WPOI 1+2+3 VS 4+5						
WPOI 1+2+3	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
WPOI 4+5	2.34 (1.00-5.47)	0.050	1.92 (0.72-5.12)	0.194	1.76 (0.72-4.28)	0.211
Depth of Invasion						
Mild invasive	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Moderate invasive	0.93 (0.39-2.23)	0.873	0.89 (0.34-2.36)	0.818	1.07 (0.48-2.42)	0.865
Deep invasive	0.64 (0.26-1.57)	0.332	0.69 (0.26-1.81)	0.453	0.51 (0.21-1.20)	0.124

Abbreviations: CI: Confidence Interval. IQR: interquartile range, DFS: Disease-free survival, DSS: Disease-specific survival, HR:Hazard Ratio, MD: Moderately differentiated, OSCC: Oral squamous cell carcinoma, OS: Overall survival PD: Poorly differentiated, SD: standard deviation, WD: Well differentiated, WPOI: worst pattern of invasion.

Table 15. Univariate analysis of biomarkers for the different survival rates (OS, DSS, DFS)

	Cox proportional Hazard Model					
	OS: HR(CI95%)	p-value	DSS: HR(CI95%)	p-value	DFS: HR(CI95%)	p-value
PD-1 expression						
PD-1-Negative 0%	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
PD-1-Positive>0%	0.47 (0.22-1.00)	0.050	0.43 (0.19-0.98)	0.044	0.47 (0.22-0.99)	0.047
PD-L1 CPS						
[0, 1)	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
[1, 20)	0.78 (0.36-1.67)	0.522	0.94 (0.43-2.01)	0.873	0.59 (0.23-1.51)	0.276
[20, 100)	0.25 (0.08-0.803)	0.019	0.59 (0.23-1.51)	0.276	0.23 (0.06-0.82)	0.024
PD-L1 CPS cut-off >1%						
PD-L1 Negative CPS<=1%	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
PD-L1 Positive CPS >1	0.44 (0.21-0.92)	0.031	0.53 (0.24-1.17)	0.119	0.89 (0.45-1.76)	0.749
PD-L1 TPS cut-off 10%						
PD-L1 <=10%	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
PD-L1 >10%	0.36 (0.14-0.94)	0.037	0.33 (0.11-0.98)	0.046	0.71 (0.32-1.57)	0.401
PD_L1 TPS cut-off 5%						
PD-L1 <5%	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
PD-L1 >=5%	0.41 (0.17-0.95)	0.038	0.42 (0.16-1.05)	0.063	0.80 (0.39-1.65)	0.549
PD-L1 TPS cut-offs 5%/10%						
PD-L1 <5%	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
PD-L1 [5-10%)	1.01 (0.24-4.30)	0.992	1.26 (0.29-5.46)	0.757	0.88 (0.21-3.74)	0.857
PD-L1[10-100%]	0.32 (0.12-0.86)	0.024	0.31 (0.10-0.91)	0.033	0.79 (0.36-1.71)	0.549
PD-L1 intensity						
Nil	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Low	0.50 (0.20-1.23)	0.134	0.53 (0.20-1.41)	0.204	0.80 (0.34-1.86)	0.603
Moderate	0.43 (0.17-1.05)	0.063	0.45 (0.17-1.20)	0.112	0.62 (0.26-1.48)	0.280
Intense	0.21 (0.03-1.66)	0.139	0.26 (0.03-2.08)	0.204	1.02 (0.29-3.57)	0.968
FOXP3						
[0.02,0.1]	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
(0.1,0.15]	0.75 (0.32-1.77)	0.516	0.65 (0.25-1.71)	0.384	0.73 (0.31-1.71)	0.472
(0.15,0.3]	0.73 (0.31-1.73)	0.478	0.73 (0.29-1.84)	0.509	1.03 (0.47-2.29)	0.936
CD4						
[0.05,0.25)	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
[0.25,0.35)	0.80 (0.29-2.20)	0.674	0.84 (0.27-2.58)	0.757	1.37 (0.56-3.34)	0.484
[0.35,0.50]	0.78 (0.34-1.81)	0.569	1.01 (0.42-2.45)	0.976	1.01 (0.46-2.21)	0.984
P16						
Negative	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Positive	4.45 (1.04-19.05)	0.044	2.69 (0.36-20.13)	0.337	1.29 (0.17-9.48)	0.803
CSF1R						
[0-8)%	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
[8-100)%	1.17 (0.57-2.38)	0.667	1.03 (0.47-2.26)	0.944	1.00 (0.50-1.98)	0.992
CD8						
CD8 [0-10%) Mild	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
CD8 [10-50)% Moderate	1.51 (0.35-6.45)	0.582	2.59 (0.34-19.53)	0.358	1.60 (0.38-6.74)	0.522
CD8 >=50% Severe	1.19 (0.21-6.62)	0.849	2.44 (0.27-22.50)	0.430	1.37 (0.26-7.09)	0.711

Abbreviations: CI: Confidence Interval, DFS: Disease-free survival, DSS: Disease-specific survival, HR: Hazard Ratio, OS: Overall survival.

Table 16. Multivariate analysis of clinical and histopathological variables for the different survival rates (OS, DSS, DFS)

	Cox proportional Hazard Model					
	OS: HR(CI95%)	p-value	DSS: HR(CI95%)	p-value	DFS: HR(CI95%)	p-value
Gender						
Men	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Women	0.49 (0.18-1.31)	0.157	0.52 (0.18-1.47)	0.215	1.01 (0.44-2.30)	0.988
Tobacco use						
Never smoker	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Former smoker	1.77 (0.51-6.06)	0.367	2.71 (0.75-9.81)	0.128	1.61 (0.59-4.40)	0.350
Current smoker	1.12 (0.35-3.51)	0.850	1.24 (0.36-4.27)	0.738	0.64 (0.23-1.76)	0.385
Tumour size status						
T1+T2	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
T3+T4	1.10 (0.37-3.33)	0.861	0.99 (0.32-3.11)	0.990	1.30 (0.50-3.40)	0.586
Metastasis status						
M0	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
M1	8.49 (1.90-37.97)	0.005	9.39 (1.85-47.60)	0.007	6.53 (1.59-26.83)	0.009
Histological Grade						
Grade 1: WD	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Grade2+3:MD+PD	2.96 (1.06-8.29)	0.039	5.29 (1.48-18.96)	0.010	3.46 (1.32-9.03)	0.011
Stage						
Stage I	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
Stage II	1.14 (0.18-7.05)	0.887	1.98 (0.20-19.70)	0.558	0.72 (0.16-3.22)	0.667
Stage III	1.16 (0.20-6.88)	0.870	2.07 (0.21-20.26)	0.533	0.65 (0.14-2.97)	0.582
Stage IV	1.25 (0.23-6.77)	0.793	1.98 (0.22-18.06)	0.543	0.99 (0.25-4.02)	0.994
WPOI						
WPOI 1+2+3+4	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
WPOI 5	2.47 (0.56-10.90)	0.232	3.41 (0.74-15.79)	0.117	2.14 (0.51-9.03)	0.301
PD-1						
PD-1-Negative <0%	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
PD-1-Positive >0%	0.54 (0.20-1.48)	0.232	0.52 (0.18-1.51)	0.232	0.36 (0.14-0.93)	0.034
PD-L1 TPS cut-off 10%						
PD-L1 <=10%	Ref.Cat.	-	Ref.Cat.	-	Ref.Cat.	-
PD-L1 >10%	0.35 (0.11-1.14)	0.081	0.40 (0.12-1.35)	0.139	0.87 (0.35-2.13)	0.757

Abbreviations: CI: Confidence Interval, DFS: Disease-free survival, DSS: Disease-specific survival, HR: Hazard Ratio, MD: Moderately differentiated, OS: Overall survival PD: Poorly differentiated, SD: standard deviation, WD: Well differentiated, WPOI: worst pattern of invasion.