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Implications of PD-L1 Expression in Oral Squamous Cell Carcinomas

Implicaciones de la expresión de PD-L1 en Carcinomas Orales de Células Escamosas

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To Irene,

Without whom this thesis could never have happened

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And finally, to Ana Alonso del Pozo,

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Acronyms

AJCC – American Joint Committee on Cancer

AEs – Adverse Effects

irAEs- immune related Adverse Effects

CI – Confidence Interval

CPS – Combined Positive Score

DFS – Disease Free Survival

DSS – Disease Specific Survival

FDA – United States Food and Drug Administration

HNSCC – Head and neck squamous cell carcinoma

HNC – Head and neck cancer

HPV – Human Papilloma virus

HR – Hazard ratio

IARC – International Agency for Research on Cancer

ICD – International classification of diseases

LRFS – Local recurrence free survival

LUSCC – Lung Squamous Cell Carcinoma

mRNA – micro RNA

NCCN – National Comprehensive Cancer Network

NSCLC- Non-Small Cell Lung Cancer

OSCC – Oral squamous cell carcinoma

OPSCC – Oropharyngeal squamous cell carcinoma

OPMD – Oral Potentially Malignant Disorders

OR – Odds Ratio

OS – Overall Survival

PFS – Progression free survival

PD-1 – Programmed cell Death protein 1

PD-L1 – Programmed Death Ligand 1

TIL – Tumor infiltrating lymphocytes

TMA – Tissue Microarrays

TNM – Tumor, Node, Metastasis Classification of Malignant Tumors

TPS – Tumour Positive Score

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INTRODUCTION

1. ORAL CANCER

I. DEFINITIONS

Oral cancer can refer to any malignant neoplasm of the oral cavity. However, the great majority (approximately 90%) of oral cancers are malignancies arising from the lining of the oral cavity known as squamous cell carcinomas (OSCC)[1]. The remaining 10% include adenocarcinomas of the salivary glands, lymphomas, sarcomas, melanomas and malignancies of the dental tissues[2]. The oral cavity includes the labial mucosa, buccal mucosa, the hard palate, the alveolar ridges including the gingiva, the floor of the mouth extending to the retromolar trigone and the anterior two-thirds of the tongue (from the circumvallate papillae)[3]. This is codified in the 10th revision of the International Classification of Diseases (ICD-10) as sites C00, C02, C03, C04, C05, and C06[4]. The oral cavity borders the oropharynx at the junction of the hard and soft palate superiorly and the circumvallate papillae inferiorly. The oropharynx includes the posterior one-third (base) of the tongue, palatine tonsils, palatoglossal folds, valleculae, and the posterior pharyngeal wall and is codified as C01, C09 and C10. Although the term oral cancer may be used for both cancers of the oral cavity and the oropharynx, it is important to differentiate they appear to have different incidences and aetiologies[5].

II. INCIDENCE OF ORAL SQUAMOUS CELL CARCINOMA

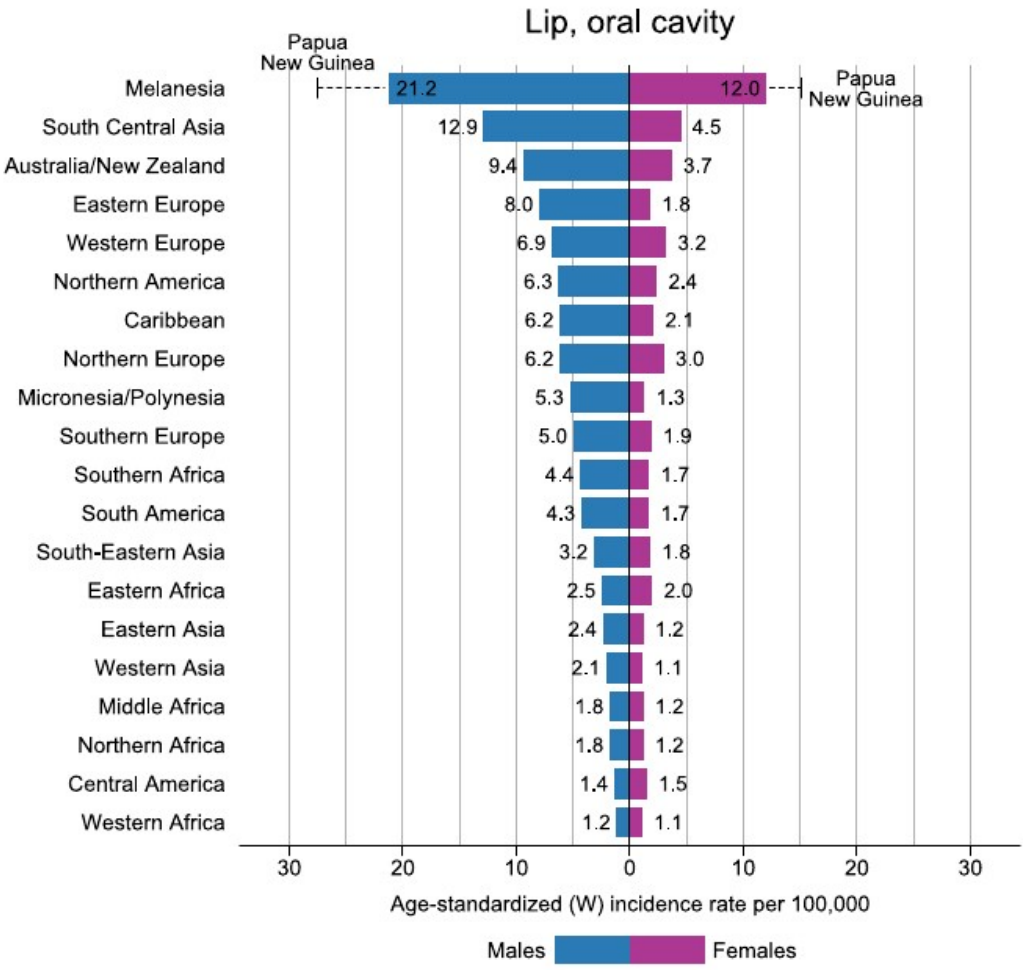
A. The global burden

According to GLOBOCAN estimates, there was approximately 355,000 new diagnoses and 177,000 deaths from cancers of the lip, tongue and mouth in 2018[6]. About 90% of these are OSCC[7], and although GLOBOCAN includes cancers of the tongue base and soft palate in this measurement it can give us an approximate idea of the global incidence and death rate[3]. When oral and oropharyngeal cancers are considered together, GLOBOCAN estimated nearly 448,000 new cancers each year, accounting for 2.5% of all cancer[6]. There is considerable variation in both incidence and survival around the world. The most effected regions are the Pacific Islands and South Asia with the lowest incidence seen in Africa and eastern Asia[6] (**Figure 1**). The trends in oral cancer incidence tend to follow the trend in risk factors, namely alcohol and tobacco use. Globally the incidence of lip and oral cancer is more common in men (2.9% of all cancers) than in women (1.0% of all cancers)[7]. The difference in incidence between genders is reduced in countries where women are more likely to use tobacco or excessive alcohol[7]. The average age of presentation is between 60-65 years, but an increasing number of tongue cancers are occurring in younger patients with less association to the risk factors of alcohol and tobacco[3]. In Europe and North America, the incidence of HPV-positive oropharyngeal cancer has been rising as alcohol and tobacco use reduces. HPV infection has been identified as a risk factor for oropharyngeal (oropharynx, base of tongue, tonsils) but not oral cavity carcinoma[6,7]. The mortality of oral cancer remains unacceptably high at around 50%[8].

B. The Spanish context

In Spain, there were approximately 7200 new cases and 2200 deaths from both oral and oropharyngeal cancer in 2010[9]. The rate of oral cancer has been decreasing and oropharyngeal cancers have increased[9]. In 2018 there were 4526 new cases of oral/lip cancer and 1165 new cases of oropharyngeal cancer with 1211 and 601 corresponding deaths[10]. The province of Granada had 458 new oral cancers and 166 new oropharyngeal cancers from 1988-2002.

Figure 1. The age-standardized incidence of lip/oral cavity cancer by gender and region. Taken from Bray et al. 2018[6]



III. RISK FACTORS

The traditional risk factors for oral cancer are tobacco, alcohol and betel quid. In recent year human papilloma virus (HPV) has been shown to be an important aetiology factor in a majority of oropharyngeal cancers[3].

A. Tobacco

Tobacco use continues to be the greatest known risk factor for developing OSCC. The International Agency for Research on Cancer (IARC) classifies tobacco (both smoked and smokeless) as carcinogenic to humans for the oral cavity[11]. Case-control studies show that compared to non-users tobacco, in all consumed forms, results in an increased risk of oral cancer (Cigarettes OR=2.87, 95% CI=2.60-3.18[12], Cigar OR=2.83, 95% CI=1.91-4.17[12], Pipe OR=2.51, 95% CI=1.68-3.75[12], Bidi smoking OR=4.0, 95% CI=2.7-4.4[13], Chewing tobacco 3.01, 95% CI=1.63-5.55[14]). The risk is dose dependant as measured by frequency and the total amount consumed. For those that quit their tobacco habit the risk of developing an oral cancer reduces after a year and returns to the risk of non-smokers after 20 years[15]. Involuntary smoking has been found to be associated with other cancers of the upper aerodigestive tract, but remains to be established for oral cavity cancer[16]. It is estimated that tobacco accounts for 71% of all deaths caused by oral cancer in high-income countries, and 37% in low- and medium-income countries[17]. There is a lack of studies of oral cavity cancer and tobacco smoked through water pipes, as well as a need to investigate the new phenomenon of e-cigarettes and oral cancer.

Smokeless tobacco can be consumed as wet/moist snuff, dry snuff and chewing tobacco. The risk of malignant transformation appears higher with dry snuff compared to other modalities of smokeless tobacco[18]. Some of the carcinogenic properties are attributed to nitrosamines in the tobacco, and nitrosamine levels are lower in dry snuff (snus) and modern American moist snuff which some studies have failed to show an increased risk of oral cancer. While these modes have been advertised as less dangerous then alternatives, caution and further research is advised[3].

B. Betel Quid

Betel quid is a recognized risk factor for oral cancer that is consumed globally by approximately 600-1200 million people, predominately in South Asia and Taiwan[19]. It is typically mixed with areca nut, catechu and slaked lime wrapped in a *Piper betle* leaf and may or may not be combined with tobacco. A meta-analysis of studies in India found that when combined with tobacco the relative risk (RR) increased to 8.47 and without tobacco it increased by a RR of 2.41. In Taiwan where it is consumed without tobacco the RR was 10.98[20].

C. Alcohol

Alcohol is a separate independent risk factor for developing oral cancer. The risk is dose dependant and is proportional to the frequency and length of time alcohol is consumed. Alcohol can be measured in grams of pure alcohol per drink. A meta-analysis has found an increased risk when compared to non-drinkers, and this increased with alcohol consumption: light-drinkers ($\leqslant$12.5g alcohol/day, OR=1.13, 95% CI=1.00-1.26), moderate drinkers ($\leqslant$50g/day, OR=1.82, 95% CI=1.62-2.07), and heavy drinkers (>50g/day, OR=5.12, 95% CI, 4.31-6.10). Reducing alcohol abuse can return the risk back to that of non-drinkers after 10 years[21].

D. Combinations

The combination of alcohol, tobacco and betel quid are known to have a synergistic effect for developing oral cancer. An OR of 15.49 was found in those that both drink 20/day and 3 or more alcoholic beverages per day. When betel quid habits are added to alcohol and tobacco then the risk is 40x greater (OR=40.09, 95% CI=35.06-45.83).

E. Human Papilloma Virus (HPV)

HPV is a double stranded DNA virus, which strictly targets epithelial cells. It is known to cause cancers of the cervix, vagina, vulva, penis and rectum as well as the oropharynx[22,23]. There are over 100 subtypes of HPV capable of infecting humans. Those associated with cancers are termed high-risk HPV and include HPV-16/18/31/33/45/52/58[24]. HPV infection appears to be an early event in oncogenesis. The virus' oncogenic potential seems linked to two of its genes, E6 and E7. When the virus becomes integrated in the host cell's genome the transcription of E6 and E7 becomes unregulated. E6 and E7 respectively inactivate p53 and pRb (retinoblastoma protein), which are two tumour suppressor proteins involved in normal cell cycle control. This therefore alters cell cycle regulation of infected cells and may initiate carcinogenesis[22,25]. HPV-positive tumours rarely have mutations in p53 as E6 inactivates p53[23]. In contrast p53 mutations are present in 60-80% of HPV-negative HNSCC, a finding closely linked to tobacco use[25]. HPV detection can be done through PCR, which amplifies HPV DNA if present. This technique does not allow for quantification of transcriptional activity and can confuse integrated HPV DNA (with oncogenic potential) with non-integrated HPV DNA undergoing replication (assumed to not have an oncogenic potential). Another option is detecting E6 and E7, which reflects viral transcriptional activity[25–27]. The most widely accepted technique is based on p16INK4A detection through immunohistochemistry (IHC) and PCR detection of HPV DNA[25,28].

The evidence from epidemiological, clinical, histopathological and molecular studies have demonstrated that HPV is an important aetiological factor in a subset of HNSCC. The majority of HPV-positive HNSCC are found in the oropharynx including the tonsils and base of the tongue[29]. Approximately 70% of oropharyngeal cancers are thought to be caused by HPV, accounting for an estimated 13,500 cases in the United States alone[30]. There are 200 types of HPV that can infect humans, but only a select few are considered high risk for oncogenesis. For the HPV+ SCC of the oropharynx (OPSCC), 90% are caused by HPV-16, with the next most common caused by HPV-18 and 6% having HPV 31/33/45/52/58[29]. Epidemiologically, HPV-positive OPSCC are more likely to occur in younger patients (<40 years old), who have an absence of traditional risk factors (alcohol, tobacco, betel quid), and are healthier and wealthier. HPV-positive tumours do not appear to cause field cancerization and respond better to treatment with radio-chemotherapy[31]. As such HPV infection in OPSCC can serve as a prognostic and predictive marker. HPV transmission in the head and neck is thought to be spread through sexual practices, as the incidence of HPV infection correlates well with the number of sexual partners, a history of oro-genital sex, and the age of sexual onset[32].

An oncogenic role of HPV in SCC of the oral cavity has not been demonstrated. A metanalysis of HPV in normal mucosa found 4.5% of subjects reviewed were positive, with 3.5% positive for high risk HPV[33,34]. Systematic reviews of HPV expression in OSCC have shown increased HPV expression compared to normal tissue. HPV expression was found to increase the risk of OSCC with an OR of 3.89, 95% CI= 2.62-6.02[35]. Kansy et al. found 25% of OSCC had HPV 16 positive samples and 18% were HPV-18 positive[36]. However, criticism of the included studies includes that anatomic sites may be including oropharyngeal tissue as part of the lingual tonsils or base of tongue. HPV also fails to show a prognostic benefit as it does in OPSCC. At the current time HPV cannot be deemed a major risk factor for oral cavity SCC[37].

F. Additional risk factors

Socio-economic status has been assessed as a risk factor for oral cancer. When measured by education, income or occupational class, higher socio-economic level had a lower risk of developing oral cancers. Low socio-economic status is linked with higher alcohol and tobacco use, however the associated risk persisted even when controlling for behavioral confounders. It is not clear if these confounders were not fully

captured or if other unknown risk factors are what cause socio-economic status to act as an independent predictor of oral cancer[38].

Numerous other agents have been suggested in the aetiology of OSCC. Mate is a hot tea common in south America and 2 meta-analyses have found an association between mate drinking and an increased risk of oral (OR=2.11, 95% CI=1.39-3.19) and upper aerodigestive tract (including oral, OR=2.24 95% CI=1.74-2.87) cancers. Chronic mechanical trauma has been postulated as a risk factor. Experimental models have been used to show trauma may promote tumour formation[39] but at present the evidence is insufficient to fulfil the Bradford Hill causality criteria[40]. Microbes within the oral cavity have been researched as a risk factor for oral oncogenesis. Some species contain enzymes, such as alcohol dehydrogenase, which may convert alcohol into carcinogenic acetaldehyde in the oral cavity. Furthermore, OSCC often contain higher levels of Porphyromonas and Fusobacterium the adjacent normal mucosa. However, there is still insufficient evidence to conclude on the role of microbes in oral oncogenesis[41]. Immunodeficiencies (HIV positive patients), the role of dietary protection (carotenes, vitamin C and E, folate, flavonoids, fiber and lycopene), vitamin and mineral deficiencies (vitamin D and iron), environmental contamination (arsenic), occupational exposure (metal, woods and cement), and hereditary conditions (Falconi's anemia) are all subjects of interest but further studies are needed to establish any association with oral oncogenesis[3].

IV. Diagnosis

A. Clinical Appearance

In western countries over 50% of OSCC affect the tongue or floor of mouth[3]. In India, and Indian populations, the buccal-gingival mucosa is the most common site of oral malignancy[42]. OSCC appearance can be divided in early and late presentations. Early OSCC are typically under 2cm in size and can present as a delimitated red and/or white area with a loss of elasticity. Ulcers are rare in early lesions but may appear as lesion continues to grow. An indurated ulcer with a rolled edge is a classic description of OSCC. Clinically, lesions may grow into the oral cavity (exophytic) or into the structures underlying the mucosa (endophytic) and very often have a mix of the two presentations. Early lesions are typically free of pain or other symptoms. As lesions grow, they may affect local nerve supply (resulting in pain or numbness), cause excess salivation, and invade into adjacent structures[43]. If left untreated the disease will result in death. Cause of death may be due to metastatic disease, treatment complications, or from uncontrolled local tumour invading vital structure such ass the great arteries of the neck[44].

B. Biopsy

The gold standard for the diagnosis of OSCC is a tissue biopsy. The sample is taken from the clinically suspicious area including adjacent mucosa of normal appearance. The sample is typically fixed in formalin and embedded in paraffin to later be stained with haematoxylin and eosin. The slide can then be visualized and the diagnosis of OSCC can be made[45].

V. Staging

Staging is an import step in the treatment of all cancers. Staging allows clinicians and researchers to describe the extent of the disease and to plan treatments. The TNM staging system, as described by the American Joint Committee on Cancer (AJCC), is a validated staging system used internationally in cancer research and treatment. The 6th edition was published in 2001 is made up of three elements: The first, *T*, describes the extent of the primary tumour. The second, *N*, describes the extent of regional metastases to the lymph nodes. The third, *M*, describes the extent of distant metastases[46]. When these three variables are taken together a stage can be determined. The exact definitions for the TNM system and staging can be found in **Table 1** and **Table 2**. The 7th edition was published in 2009 and for the purpose of oral cavity cancers is essentially unchanged from the 6th edition. The 8th edition came into effect in 2018 and contains considerable changes. The first major change is that the *T* category now includes a measure of tumour depth, in 5mm increments. Secondly, the *N* category includes a measure of Extra-nodal extension. This update has proved to better prognosticate patient survival[47].

Table 1. TNM Definitions

Primary Tumour (T)	
TX	The tumour cannot be examined
T0	No evidence of the primary tumour
Tis	Carcinoma in situ
T1	Tumour of 2 cm or less in greatest dimension
T2	Tumour more than 2 cm but not more than 4 cm in greatest dimension
T3	Tumour more than 4 cm in greatest dimension
T4a (lip)	Tumour invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin (chin or nose)
T4a (oral cavity)	Tumour invades through cortical bone, into deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), maxillary sinus, or skin of face
T4b (lip and oral cavity)	Tumour invades masticator space, pterygoid plates, or skull base; or encases internal carotid artery
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 2. Stage Grouping

0		Tis N0 M0
I		T1 N0 M0
II		T2 N0 M0
III		T3 N0 M0
		T1/2/3 N1 M0
IV		T4a N0 M0
		T4a N1 M0
	IVa	T1/2/3/4a N2 M0
		T4b Any N M0
	IVb	Any T N3 M0
	IVc	Any T Any N M1

VI. Treatment of OSCC

Treatment of OSCC is multidisciplinary and should include specialist in surgery, oncology, radiotherapy, nursing, and speech therapists[3]. The guidelines of the National Comprehensive Cancer network (NCCN) is based on the TNM staging[48]. The majority of early stage OSCC are treated surgically with a clear margin of 1-2 cm[3]. Removal of the cervical lymphatic nodes, known as a neck dissection, is carried out to remove affected lymph nodes and may be done when the risk of nodal spread is considered to be high. Stage III and IV cancers typically require surgery, as a primary treatment, combined with adjuvant radiotherapy or chemotherapy, especially in the event of negative findings (e.g. positive surgical margins, lymphatic or perineural invasion, N2 or N3 nodes, or extracapsular spread)[3,48]. Optimal treatment of a patient without signs of nodal metastasis (cN0) remains controversial. The decision to operate on the lymph nodes of a cN0 patient is based on the perceived risk of undetected lymphatic metastasis. Risk factors for nodal metastasis include: an advanced T stage, a high histological grade, tumour width and the presence of lymphovascular or perineural invasion[49]. In a published case series of cN0 patients treated with an elective neck dissection, the incidence of occult lymphatic metastasis varied between 6-25% in T1 tumours and 20-32% in T2 tumours. When observational studies are included the rate of occult nodal metastasis rises to 40-50%, although there may be an element of patient selection bias, where patients more likely to have nodal metastasis are offered elective surgery[49]. In relation to anatomic subsite, it appears that lingual OSCC high a higher rate of regional metastasis when compared to those in the floor of the mouth[50]. Treatment options include observation, elective neck dissection, radiotherapy, and sentinel node biopsy. This last option involves the biopsy of the first draining nodes from the cancer origin and can detect metastases in 86-94% of cases of cN0 and can help patients avoid the morbidity associated with neck dissection[51].

In relation to surgical technique various modalities are possible including open resection, transoral robotic surgery and laser microsurgery. The core operating principal is nevertheless the same: removal of the tumour with a clear margin of at least 1cm[3]. Non-surgical treatment has been advised in cases where the surgical removal would result in high morbidity and loss of quality of life, for example in bilateral tumours of the base of the tongue[3].

VII. Pathophysiology

A. OSCC

The pathophysiology of OSCC is complex. The current dogma is that cancer is a genetic disease caused by genetic changes can be both inherited and acquired[52]. Numerous molecular alterations have been associated with the disease, and it is hoped they could be used to personalise treatment. Targeting aberrant molecular signaling pathways seems to be a promising therapeutic approach[53]. A new paradigm is that of the cancer stem cell. This holds that stem cells in the basal layer of the oral epithelium are responsible for the clonal repopulation of the epithelium. This would imply that only changes in the stem cells are responsible for the development of cancer[54].

An important aspect of OSCC is the development of multiple tumours. Between 17-30% of patients with OSCC develop a second oral tumour and this has been attributed to genetically altered premalignant patches throughout the oral cavity, a concept known as field cancerization[53]. Diagnostic techniques used to try to determine premalignant areas include analysing the loss of heterozygosity of 3p, 9p and 17p, or the presence of TP53 mutations. These techniques are expensive, complex and not universally available which limits their routine application[53].

B. Oral Potentially Malignant Disorders (OPMD)
An import concept in the aetiology of OSCC are OPMD as these may be precursor lesions to the cancer. These may be asymptomatic and picked up by screening or on routine dental examination.

Nomenclature and concept
In 2005, the WHO Collaborating Centre for Oral Cancer and Precancer decided on the term *Oral Potentially Malignant Disorders* (OPMD) to describe a lesion or disorder of the oral mucosa that has an increased risk of developing into an oral cancer. These conditions include leukoplakias, erythroplakias, palatal lesions in reverse smokers, submucous fibrosis, actinic keratosis, lichen planus, discoid lupus erythematosus as well as hereditary conditions such as dyskeratosis congenita and epidermolysis bullosa[55].

Prognostic factors for malignant transformation
An OPMD may develop into an OSCC, but not all cases do. An OPMD can persist without changing clinically throughout the patient's life or it may change in size or even disappear completely. Approximately 40% of OPMD will decrease in size or disappear, which is often associated with tobacco cessation[56]. Malignant transformation rates can vary by tobacco consumption and geographic location[17], and paradoxically, transformation is increased in non-smokers[57]. In 2003, Petti calculated a malignant transformation for leukoplakias of 1.36%/year[19] and a recent systematic review found a mean transformation rate of 3.5% (range 0.13%-34%)[58]. Important factors for transformation include advanced age, female gender, leukoplakia exceeding 200mm^2, higher grades of dysplasia and a non-homogenous appearance[58].

Treatment of OPMD
In the absence of evidence, surgical elimination is often recommended for high-risk oral leukoplakias. Removal of the lesion does not guarantee that it will not recur or that it will not transform into a cancer in the future[59]. As such these patients may need lifelong follow-up to diagnosis any transformations in the earliest stages. The frequency of reviews depends of patient and lesion risk factors and should be tailored to each patient. All patients should be advised to stop any tobacco habits. At the moment there is no medical treatments to reduce the malignant transformation of oral leukoplakias[59].

Molecular alterations in OSCC
In 1996 the multistep model of progressive genetic damage was proposed for HNSCC[60]. This model used genetic changes to explain the morphological changes in the epithelium that occurred during carcinogenesis. The loss of heterozygosity in chromosomes 3p, 9p and 17p appeared to occur in dysplastic tissue, which reflected an early step in carcinogenesis. Loss of chromosomes 11q, 4q and 8 were more likely to be present in established carcinomas[60].

In general, carcinogenesis occurs as a result of multiple genetic and epigenetic changes to the genes controlling vital signalling pathways. This results in changes to the cell's structure and function which have been resumed into different *hallmarks of cancer*. The first hallmarks described were: self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, limitless replicative potential and tissue invasion/metastasis[61]. After a decade a further two were added: reprogramming energy metabolism and evading immune destruction[62]. Underlying the hallmarks is genome instability which generates the genetic changes that result in the hallmarks of cancer. This process is aided by inflammation which fosters the hallmark's functions[62].

The immortalization of a cancer cell is regulated by pathways involving p53 and pRb, which control the cell cycle, and they are in turn influenced by the expression of telomerase reverse transcriptase (TERT)[63].

Mutations to EGFR are a frequent finding in OSCC, resulting in self-sustaining growth pathways being activated[25]. EGFR is a receptor tyrosine kinase found in the cell membrane. It binds with ligands, which act as cell messengers, forming homodimers or heterodimers and activating growth pathways, including MAPK, PI3K and phospholipidase C[25]. EGFR bound to the ligand EGF has also been found to migrate to the nucleus and act as a transcription factor or co-activator of other transcription factors, like STAT[64,65].

Most OSCC escape the inhibitory pathway mediated by TGFβ, either by mutation or chromosomic loss of key genes[25]. TGFβ1 signals through TGFβ receptors which results in phosphorylation of SMAD2 and SMAD3 which, acting with SMAD4, regulates the transcription of genes controlling proliferation, survival and apoptosis[25]. The TGFβ appears to be interconnected with the NF-κB pathway, of great importance to cancer as it signals for cell survival[66,67]. The PI3K-PTEN-AKT-mTOR pathway is also implicated in many cancers including OSCC. PI3K are enzymes implicated in cell growth and survival and is comprised of four classes, with each class having subunits. In OSCC the class IA PI3K molecules are often mutated[25]. For example, the class IA subunit p110α, which codes for PIK3CA, is mutated in 7% of OSCC[68].

All solid tumours have mechanisms to induce neo-angiogenesis to supply themselves with oxygen and nutrients. Normally a tumour will produce angiogenic factors which induce growth factors in endothelial cells allowing for vascular growth to the tumour. There are numerous angiogenic growth factors but a prominent example is VEGF (Vascular Endothelial Growth Factor)[69]. The presence of VEGF in HNSCC is associated with a worse survival[70].

OSCC will most commonly metastasize to loco-regional lymph nodes before developing distant metastases. The number of affected lymph nodes, the size and the presence of extracapsular spread are all important factors for prognosis. In order to invade and metastasize a series of steps must occur including the degradation of the extracellular matrix[25,71]. Matrix metalloproteinases (MMP) are enzymes involve in the dissolution of the extracellular matrix. However treatment targeting MMP has not been very successful[25,72]. Genetic analysis of metastatic cancers show alterations to genes involved in epithelial-mesenchyme transition (EMT)[73]. EMT is an essential process in embryonic cells, allowing epithelial cells transform into mesenchymal cells. When present in cancerous cell it is associated with cellular migration and, consequently, metastasis[73].

VIII Prognostic Markers

A. Clinical Markers

Clinical staging has historically been the most important determinant of prognosis. The TNM system is the most well known and widely used system to establish a stage, with advanced stages having worse prognosis. The newest edition of staging OSCC now includes extra-nodal extension and depth of invasion as important prognostic features. To obtain these findings tumours must be assessed with microscopy and histopathological stains and so will not be available from the initial clinical exam[47,74].

B. Histopathological Markers

Histological grading is a system used to classify tumours adopted by the World Health Organisation (WHO)[75,76]. This system grades tumours by the amount of keratinization, cellular and nuclear pleomorphism and mitotic activity. This results in 3 categories: well-differentiated, moderately differentiated and poorly differentiated (which has the worst prognosis)[75,76]. Some tumours have areas of varying grades of differentiation, and the worst area is always taken. This system has been implemented since the 1970s but now is seen to poorly predict survival and response to therapy in individual patients[75]. It has many limitations including the subjective nature of measurement, the

heterogeneity of differentiation in tumours and the dependence on structural appearance and not functional. For this reason, approximately 90% of OSCC are graded as moderately differentiated[75].

Other histological prognostic factors are: the size of the tumour, the invasive front of the tumour, the presence of lymphovascular or perineuronal invasion, involvement of adjacent bone or cutaneous tissue, and the presence of histologically clear surgical margins[75]. Likewise, histological examination of affected lymph nodes may reveal more information including: the size of the metastasis, the extent of extra-nodal spread, the presence of embolization within the lymphatic vessels[75].

C. Molecular Markers

In the last decade there has been a considerable amount of research into molecular prognostic markers in OSCC. Some of the most researched have been summarized in a recent systematic review. While there are many limitations to using these molecular markers (subjective interpretation, economic cost, time and training needed to perform the tests) there is reason to believe some may help prognostic and guide treatment. **Table 3** resumes some of these markers and their functions in health and in cancer[77]

Table 3. Potential Prognostic Molecular Markers in OSCC. Taken from Rivera et al. 2017[77]

Name of the gene	Biological function	Function in cancer
MKI67	Cell cycle. Cellular proliferation.	Marker of growth fraction for a certain cell population. The labelling index is considered one of the best prognostic factors of the survival rate and recurrence.
CDKN2A	Cell cycle. Cell cycle arrest.	This gene is frequently mutated or deleted in a wide variety of tumours, and is known to be an important tumour suppressor gene.
HPV16	High risk HPV type.	Is emerging as an important factor in the rise of oropharyngeal tumours affecting non-smokers in developed countries.
DLC1	Negative regulation of cell proliferation and migration	Acts as a tumour suppressor in a number of common cancers, including liver cancer.
CYR61	Regulate cell growth and adhesion.	Acts as a tumour suppressor, depending on the origin of the cancer.
TP53	Cell cycle Cell cycle arrest	Tumour suppressor protein. Mutations in this gene are associated with a variety of human cancers.
CA9	Response to hypoxia	Is the most widely expressed gene in response to hypoxia. Its role in intracellular pH maintenance represents the means by which cancer cells adapt to the toxic conditions of the extracellular environment.
CCND1	Cell cycle. Cell division.	Is frequently deregulated in cancer and is a biomarker of cancer phenotype and disease progression
EGFR	Positive regulation of cell proliferation	EGFR overexpression is a significant finding in cancer, particularly in head and neck cancer, where it is also associated with a poor prognosis.
RB1	Cell cycle. Cell cycle arrest	Tumour suppressor protein. Defects in the gene are a cause of childhood retinoblastoma, bladder cancer and osteogenic sarcoma
MYC	Positive regulation of cell proliferation	Its oncogenic reputation stems from its frequent deregulation in a host of human cancers and from a suite of activities that place this protein at the nexus of cell growth, proliferation, metabolism and genome stability.
ALDH1A1	Ethanol oxidation	Plays a key role in the regulation and differentiation of both normal tissue stem cells and cancer stem cells
PROM1	Retina layer formation	Maintaining stem cell properties by supressing differentiation
S100-A2	Endothelial cell migration	In epithelial tissue, S100-A2 expression is decreased remarkably in tumours compared with normal specimens. S100-A2 promotes p53 transcriptional activity, and its loss of expression has been associated with a poorer prognosis.
CDC20	Cell cycle. Positive regulation of cell proliferation.	The role of CDC20 expression in tumours is not known, but many studies have reported that CDC20 regulates apoptosis, leading to genetic instability
MAP1LC3A	Autophagy	Strong positive expression in the peripheral area of pancreatic cancer tissue had a shorter overall survival
FAS/ FASLG	Apoptotic process	Cancer cells can never lose FAS or FASLG. FAS and/or FASLG expression promotes tumour growth and favors the establishment of tumour metastases.
HMOX1	Angiogenesis	Many human tumours produce HMOX1, and its expression is usually higher in cancer cells compared to the surrounding healthy tissues.
PDPN	Lymphangiogenesis	Marker of lymphatic endothelial differentiation in vascular endothelial neoplasms and lymphatic invasion by tumours. It is also a marker of cancer-associated fibroblasts.

CTTN	Cell motility. Focal adhesion assembly.	Is overexpressed in HNSCC and breast cancer.
PTK2	Angiogenesis	Promotes tumour progression and metastasis through effects on cancer cells, as well ass stromal cells of the tumour microenvironment.
MUC4	Cell adhesion	An aberrant expression of MUC4 has been reported in various carcinomas.
CTNND1	Cell adhesion	The complete loss, downregulation of CTNND1 correlates with the progression of different types of human tumours.
ACTA2	Mesenchyme migration	Patients with lung adenocarcinoma and high ACTA2 expression showed significantly enhanced distant metastasis and unfavorable prognosis.
MMP1/2	Proteolysis. Angiogenesis. Response to hypoxia.	The imbalance of matrix metalloproteinases and their inhibitors play an important role in the progression of HNC.
VIM	Movement of cell or subcellular component	A marker of epithelial-mesenchyme transition. Overexpression in cancer correlates well with accelerated tumour growth, invasion and poor prognosis.
CDH1	Cell adhesion	Loss of function of this gene is thought to contribute to increasing proliferation, invasion and metastasis.
VCAN	Cell adhesion	Is strongly associated with a poor outcome for many different cancers. Depending on the cancer nature it is expressed by cancer cells or by the stromal cells surrounding the tumour.
AMFR	Movement of the cell or subcellular components	Is a tumour motility-stimulating protein secreted by tumour cells
MUC1	DNA damage response. Cell cycle arrest.	Is aberrantly glycosylated and overexpressed in various epithelial cancers and plays a crucial role in the progression of the disease. MUC1 is often used as a diagnostic marker for metastatic progression.
HIF1A	Angiogenesis. Response to hypoxia.	Up-regulates the expression of proteins that promote angiogenesis, anaerobic metabolism and many other survival pathways.
SLC2A1	Glucose transport	Correlates with depth of invasion and clinical stage in patients with gastric cancer.
IL4R	Immune system process and regulation of cell proliferation	The IL4/IL4R signaling axis is a strong promoter of pro-metastatic phenotypes in epithelial cancer cells including enhanced migration, invasion, survival and proliferation.
IL13RA1	Cell surface receptor signalling pathway	Glioblastomas present higher IL13RA1 and IL13RA2.
CXCL8	Angiogenesis. Movement of cell or subcellular component. Chemotaxis.	Affects neovascularization of the tumour microenvironment.
CD163	Inflammatory response	Could be used as a general anti-inflammatory myeloid marker with prognostic impact for breast cancer patients.
MPO	Defense response	Myeloperoxidase-positive cell infiltration in colorectal carcinogenesis is an indicator of colorectal cancer risk
SERPINB3	Positive regulation of cell proliferation	Promotes oncogenesis and epithelial-mesenchymal transition.
CRP	Inflammatory response	Patients with high baselines CRP had a greater risk of early death compared to those with low CRP levels.

D. Immune regulatory markers

Of the 41 prognostic markers identified by Rivera et al. only three are directly involved in the immune response (IL4R, CD163 and CRP)[77]. However, the ability to evade immune destruction is a hallmark of cancer[62]. In recent years several markers have been found to be exploited in cancers including a class known as immune checkpoint inhibitors. One such protein is Programmed Death Ligand 1 (PD-L1), which has been found in many human cancers, including OSCC. Targeted treatment of it's receptor, Programmed Cell Death Protein 1 (PD-1), has lead to improved survival times in recurrent or metastatic HNSCC[78].

2. Programmed Death Ligand 1 (PD-L1)

I. Overview of PD-L1

A. Structure, regulation and expression of PD-L1

PD-L1 (also known as B7-H1) is a transmembrane protein. It is a member of the B7 protein family, which is a family of structurally related cell surface ligands that bind to receptors on lymphocytes in order to regulate immune responses[79]. PD-L1 is encoded by the *CD274* gene, located on chromosome 9p24.1 and is made up of 290 amino acids[80]. *CD274* contains seven exons that encode the different parts of the PD-L1 protein. The first six exons encode the 5'UTR, the signal sequence, an IgV-like domain, an IgC-like domain, the hydrophobic transmembrane domain, and the intracellular domain. The seventh exon encodes a portion of the intracellular domain and the 3'UTR (**Figure 2**)[81,82].

In mice, PD-L1 is constitutively present in naïve and activated T and B lymphocytes, dendritic cells, macrophages, mast cells, and mesenchymal stem cells as well as in non-hematopoietic cells, including cells in the cornea, lungs, placenta, vascular endothelium, pancreatic islet cells and keratinocytes. In humans, PD-L1 is absent in naïve T-cells but can be induced in activated T-cells and has been found in dendritic cells, monocytes, lung, placenta, vascular endothelium and keratinocytes [81]. The presence of PD-L1 in healthy tissues suggests a role in the prevention of auto-immunity and regulation of inflammatory responses in target organs [84,85]. PD-L1 expression is primarily regulated by inflammatory signaling through various cytokines or microbial molecules acting on cell surface receptors [86] (**Figure 3**).

Figure 2. Representation of PD-L1 from gene to mRNA (figure based on an illustration in Chen et al. 2016)[83]

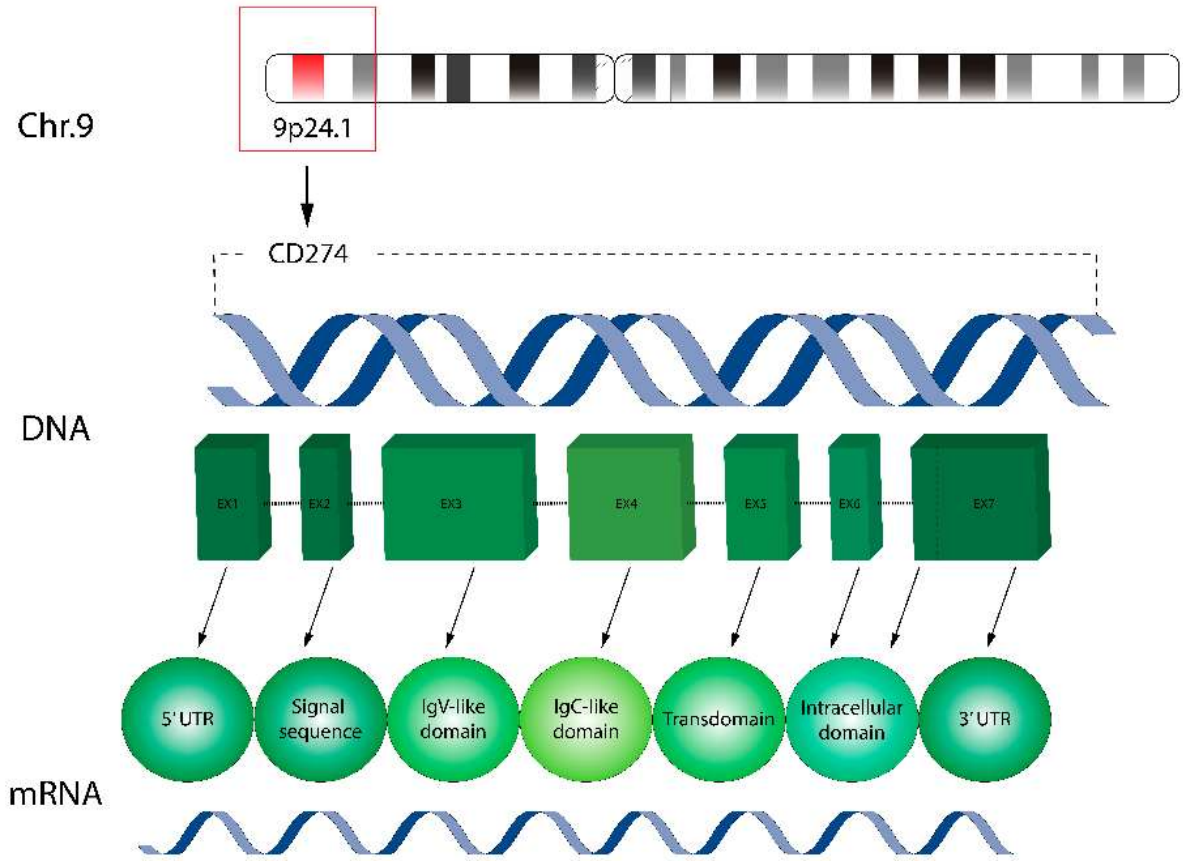
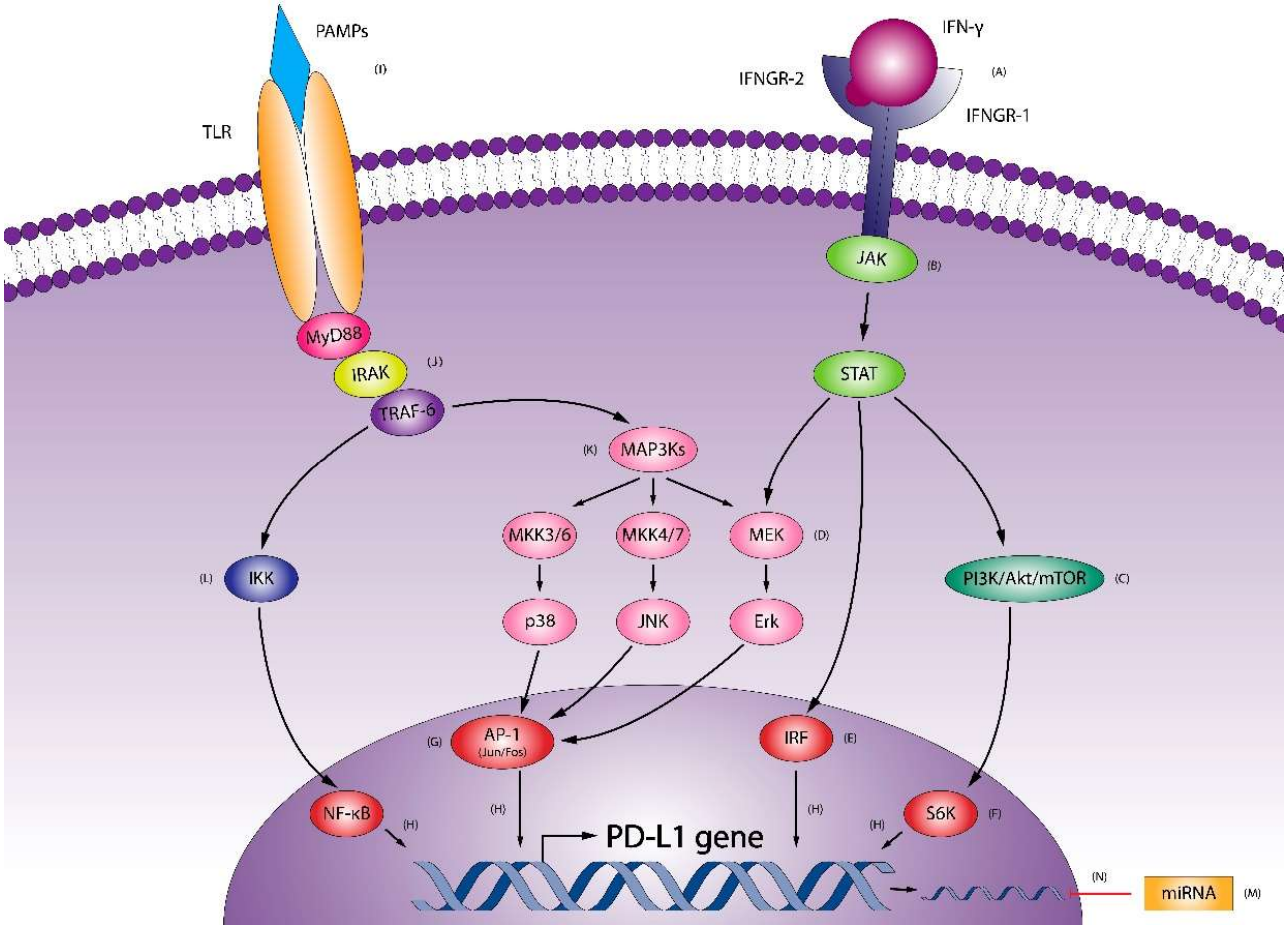


Figure 3. Two different pathways associated with the physiological expression of PD-L1.



INFγ is considered the most potent inducer of PD-L1 expression [87,88]. The process begins when INFγ binds to the INFγ receptor complex, a heterodimer of two subunits (INFGR-1, INFGR-2) (A), producing autophosphorylation and activation of the JAK/STAT pathway (B). In either a direct manner or *via* the PI3K/Akt/mTOR (C) and MEK/Erk (D) pathways, this causes the nuclear translocation of their respective transcription factors IRF (E), S6K (F) and AP-1 (Jun/Fos)(G) and the subsequent transcription of mRNA from the *CD274* gene encoding PD-L1 (H)[87,89–91]. PD-L1 has also been shown to be expressed *via* activation of Toll-like Receptors (TLRs), which are activated on binding to Pathogen Associated Molecular Patterns (PAMPs) such as lipopolysaccharide (I) [92,93]. PAMP binding can cascade through MyD88/IRAK/TRAF-6 (J). TRAF-6 can activate MAP3K (K), allowing translocation of the transcription factor AP-1 (Jun/Fos) (G) via different pathways (MEK-Erk, MKK4/7-JNK, MKK3/6-p38) and activation of PD-L1 transcription (H) [94]. Alternatively, TRAF-6 can activate IKK (L), which facilitates the translocation of NF-κB, a known transcription factor of PD-L1, to the nucleus. PD-L1 can be negatively regulated by the binding of microRNAs (M) to the 3'UTR of PD-L1 mRNA, inhibiting translation (N) [95].

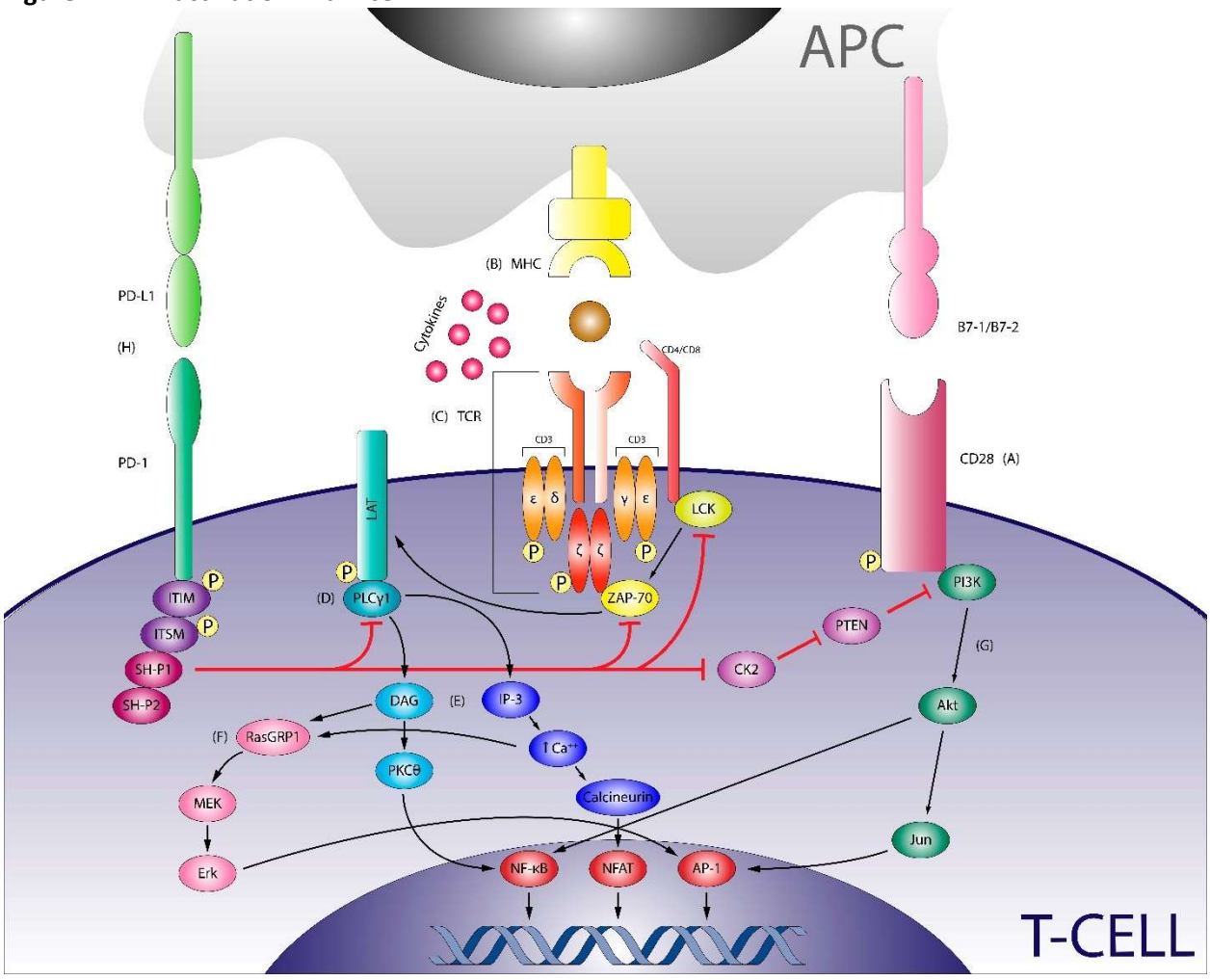
B. PD-1: PD-L1 Axis Functions

PD-L1 is the natural ligand of PD-1, a 288aa transmembrane receptor protein encoded by *PDCD1* at 2q37[96,97]. PD-1 can be found on Natural Killer T cells, B cells, activated monocytes and dendritic cells and can be induced in CD4+ and CD8+ T-cells [81,98]. Its structure comprises an IgV-like domain with a 20aa stalk forming the extracellular domain, followed by a transmembrane region and a cytoplasmic tail containing an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) and Immunoreceptor Tyrosine-based Switch Inhibitory motif (ITSM)[99]. **Figure 4** depicts the inhibitory action resulting from the binding of a PD-L1 positive Antigen Presenting Cell (APC) to a PD-1 positive T-cell.

PD-L1 is involved in the regulation of antigen tolerance through T-cell inhibition, both centrally (in primary lymphatic organs) and peripherally (in secondary lymphatic organs). This process appears crucial for protecting peripheral tissues against an autoimmune attack. The expression of PD-L1 in pancreatic β cells has been shown to protect against diabetes in the, and treatment of these mice with an anti-PD-L1 antibody accelerated diabetes onset [100]. Similar results have been obtained in Experimental Autoimmune Encephalomyelitis mouse models, with PD-L1 -/- mice showing greater susceptibility to the disease [101]. In other mouse studies, treatment with anti-PD-1: PD-L1 antibodies promoted the rejection of corneal allografts, while treatment with PD-L1 protein improved graft survival *in vivo* [102]. PD-1: PD-L1 has also been implicated in maternal-fetal tolerance, preventing a T-cell attack against placental cells [103]. The PD-1: PD-L1 pathway can also provide protection from autoimmunity in peripheral tissues through the induction or enhancement of T regulatory cells (Tregs). Tregs are largely identified by CD4+, CD25+, and FOXP3+ (forkhead box P3) expression and result in immune suppression by raising the threshold for T-cell activation. Tregs have been shown to express both PD-1 and PD-L1, and ligation of PD-L1 can increase the conversion of naïve T-cells into Tregs as well as aiding in their maintenance and suppressive capabilities. [104105].

Another important role of the PD-1: PD-L1 axis is in the regulation of the immune response to chronic infections leading to T-cell exhaustion. Immune exhaustion is characterized by a reduction in cytokine production, proliferation, and cytotoxic activity. This can prevent tissue damage from a cytotoxic attack during chronic infections and is pathogenically linked to PD-1: PD-L1 interactions [106]. This interaction is active in HIV and chronic hepatitis C infections and in chronic bacterial infections such as tuberculosis[107–109]. These findings raise the possibility of targeting the PD-1: PD-L1 axis to restore T-cell function in chronic infection.

Figure 4. PD-1 activation in a T-cell.



CD28 (A) is a costimulatory receptor present in the membrane of T-cells. It is necessary, along with the signal emitted by the binding of the T-cell Receptor (TCR) (C) to the antigen-MHC (Major Histocompatibility Complex) (B) and the presence of cytokines, for the activation of T-cells [110,111]. When the TCR is activated, the kinase Lck, linked to CD4 or CD8, can phosphorylate the CD3 chains, permitting the binding of another kinase, ZAP-70[112,113]. This kinase phosphorylates LAT (Linker of Activated T-cells) (D), thereby anchoring PLCγ1, which hydrolyzes phosphatidylinositol into DAG (Diacylglycerol) and IP-3 (Inositol trisphosphate) (E)[114–116]. IP-3 releases calcium, allowing calcineurin to translocate NFAT into the nucleus and stimulate IL-2 transcription [117,118]. DAG activates PKCθ, which releases NF-κB, allowing transcription of multiple genes encoding cytokines [119]. PLCγ1, mediated by DAG and Ca^{++}, also activates RasGRP1, which activates the MEK/Erk pathway for transcription factor AP-1 [114]. CD28, on binding to CD80 (B7-1) or CD86 (B7-2), causes autophosphorylation and PI3K recruitment, activating Akt and Jun and the eventual activation of AP-1 and NF-κB [120,121]. The inhibitory action of PD-1: PD-L1 counters the stimulatory signaling caused by TCR/MHC binding. PD-1, on binding to PD-L1, phosphorylates ITSM and ITIM, which recruits the phosphatases SHP-1 and SHP-2 to ITSM [122,123]. As a consequence of this Lck, ZAP-70, and PLCγ1 dephosphorylate and their downstream signals are halted [123]. PD-L1 binding to PD-1 also eliminates CK2, thereby increasing PTEN activity, which inhibits the PI3K pathway [124]. The end-effect on T-cells is a reduction in proliferation, inhibition of cytokine secretion and the induction of apoptosis [88,125].

C. The B7 protein family

PD-L1 belongs to the B7 protein family which includes six other members (CD80, CD86, ICOS-L, PD-L2, B7-H3, and B7-H4). These have been studied in humans and mice but have also been observed in birds and lower vertebrates [126]. The key function of this family is to regulate the immune response, as demonstrated by the immune deficiency disorders and autoimmune pathologies produced in mice with a knockout of B7 family genes [79]. B7 family members share a common structure, as they are transmembrane proteins (except for B7-H4: a glycosylphosphatidylinositol [GPI]-linked protein) with extracellular IgV and IgC domains, a transmembrane domain, and a cytoplasmic tail. The exact function of the cytoplasmic tail is unclear but as it contains serine and threonine it may be involved in phosphorylation and signaling. At the cell surface the B7 family probably forms homodimers [127].

All B7 family ligands are expressed on professional antigen-presenting cells (macrophages, dendritic cells, and B cells). The B7 family contributes to the immune response either through co-stimulatory or co-inhibitory signaling. Co-stimulatory signals act in conjunction with antigen-receptor signals to induce cellular activation, growth factor production and cellular expansion, increasing survival. Co-inhibitory signals attenuate the antigen-receptor signal, reducing cellular activation and inhibiting growth factor production and cell cycle progression, potentially leading to cell death. As an example, the binding of either CD80 or CD86 to CD28 produces co-stimulatory signals, whereas binding of the same CD80 or CD86 to CTLA-4 produces co-inhibitory signals [111,128].

PD-L1 has been shown to interact directly with the B7 family member CD80 (B7-1) which can be found on antigen presenting cells and activated T and B cells as well as keratinocytes. Mouse and in vitro experiments have shown that PD-L1 and CD80 binding can lead to the inhibition of T-cells[129,130].

II. PD-L1 in Cancer

PD-L1 is overexpressed in a wide range of human cancers and can attenuate the T-cell-specific response to tumour cells, leading to tumour escape from the immune system [131]. Studies of various mouse cancer cell lines showed that treatment with anti-PD-L1 and anti-PD-1 antibodies recovered immune function against tumour cells[132]. PD-1/PD-L1 induced escape mechanisms include: functional anergy and apoptosis of effector T-cells [131], the promotion of Tregs [133], and resistance to T-cell-mediated killing when tumour cells expressing PD-L1 bind with either PD-1 or CD80 on effector T-cells [134,135].

III. PD-L1 overexpression

PD-L1 expression in cancer cells is regulated in a complex manner and may result from: the aberrant activation of signaling pathways, genetic alterations or the loss of regulation during transcription, translation or post-translation phases. (**Figure 5**)

A. Aberrant activation of signaling pathways

Various oncogenic pathways lead to PD-L1 overexpression. The MAPK/Erk and PI3K/Akt pathways are known to have a key role in promoting cell survival and proliferation and have also been implicated in immune evasion through PD-L1 upregulation [83]. The specific pathways appear to vary among tumour types.

The MAPK pathway may be over-activated by a mutation in its upstream proteins; for example, mutations in the gene *BRAF* can result in increased MAPK signaling. *BRAF* mutations are rare in OSCC (2%)[136] but are found in approximately 50% of melanomas [137]. Increased PD-L1 expression was observed in melanoma cell lines with the V600E BRAF mutation, and the addition of a BRAF-inhibitor reduced PD-L1 expression, which then rose again after resistance to the BRAF-inhibitor was attained. Further addition of a MEK-inhibitor, which blocks downstream from BRAF, produced a new decrease in PD-L1 expression [138]. PD-L1 can also be overexpressed through over-activation of MAPK pathway receptors. Thus, in Non-Small Cell Lung Cancer (NSCLC), oncogenic activation of the EGFR receptor by its mutation or an increase in EGF ligand resulted in PD-L1 expression and T-cell inhibition [139,140]. Other tumour types with EGFR mutations (e.g., breast cancer and bronchial carcinoma) also show an increase in PD-L1 [141]. The role of EGFR in PD-L1 overexpression is of particular interest in OSCC because EGFR overexpression is reported in 80% of HNSCC, although this overexpression rarely shows mutations [142]. In HNSCC cell lines, overexpression of wild-type EGFR significantly correlated with an increase in PD-L1, although the JAK2/STAT1 rather than the MAPK pathway was found to be involved [141]. On the other hand, no correlation was observed between EGFR overexpression and PD-L1 levels in an immunohistochemistry study of oropharyngeal carcinomas [143]. Interestingly, PD-L1 expression *via* the MAPK pathway was reported in cell lines after low-dose chemotherapy (paclitaxel and cisplatin) [144,145]. However, this effect might not translate to the clinical setting, where PD-L1 levels have been found to decrease, increase, or remain unchanged after platinum-based chemotherapy [146]. This effect remains of interest, given that drugs such as cisplatin are recommended by the National Comprehensive Cancer Network (NCCN) for cases of unresectable or metastatic HNSCC, and immune checkpoint therapy targeting the PD-1: PD-L1 axis are often used after progression following chemotherapy with platinum-based drugs [147].

Activation of the PI3K/Akt pathway through increased extrinsic signaling or loss of its negative regulator (PTEN) can result in increased PD-L1 expression [83]. Experimental blockade of PTEN in colon cancer cell lines results in the activation of Akt and an increase in PD-L1 protein expression without increasing PD-L1 mRNA. Subsequent blockade of Akt produces a new fall in PD-L1 protein levels, suggesting that Akt may be involved in the post-translational control of PD-L1 expression [148].

The JAK/STAT pathway plays an important role in PD-L1 overexpression because it is the main signaling pathway for IFNγ, the most potent cytokine inducer of PD-L1. IFNγ signaling via its receptor phosphorylates JAK kinases [86]. It has been shown that increased JAK2 upregulates STAT1 but not STAT3 in HNSCC cell lines, resulting in increased PD-L1 [141]. Another study in a different HNSCC cell line (CAL27) found that blockade of STAT3 produced the loss of PD-L1 overexpression [149]. These contrasting results illustrate the importance of different JAK/STAT pathways in distinct HNSCC cell lines.

Transcription factors involved in the overexpression of PD-L1

Various transcription factors have been implicated in PD-L1 overexpression. An increase in HIF-1α (Hypoxia-Inducible Factor-1-alpha) is associated with PD-L1 overexpression suggesting that hypoxic environments, typical of tumour microenvironments, may encourage immune evasion as a survival mechanism through the PD-1: PD-L1 axis [150]. STAT1 and STAT3, as mentioned above, have been identified as transcriptional factors causing PD-L1 overexpression through IFNγ signaling. STAT3 can also be increased by mutations of tyrosine kinase receptor NPM-ALK (nucleophosmin-anaplastic lymphoma kinase) [151]. Silencing STAT3 with siRNA (small interfering RNA) has been used to inhibit STAT3 protein expression, resulting in reduced PD-L1 expression [152]. NF-κB is an important transcription factor of PD-L1 and can be induced by INFγ. Inhibition of NF-κB can abolish the expression of PD-L1 induced by INFγ [153]. Finally, MYC is an import transcription factor that is dysregulated in up to 70% of human cancers [154]. MYC can bind to the PD-L1 promotor and regulate PD-L1 transcription, whereas blockade of MYC produces a decrease of PD-L1 expression [155].

Epigenetic Regulation of PD-L1

Many microRNAs (miRNAs) act as epigenetic control points involved in PD-L1 production. These miRNAs are small non-coding single RNA strands that bind to a target RNA to block translation [156]. The miRNAs-513, -570, -34a, and -200 can all bind to the 3'UTR of PD-L1 mRNA, effectively inhibiting translation of the protein. In this way, mutations of the PD-L1 3'UTR can escape these controls and result in excessive PD-L1 expression [157]. Other miRNAs regulate upstream targets in the PD-L1 pathway, including IFNγ, IFNGR-1, PTEN, IRF-1, c-Fos, and STAT1, and their dysregulation could also produce an increase in PD-L1[158].

Alterations in the PD-L1 gene (CD274)

Both amplifications and translocations of CD274 (9p24.1) have been demonstrated in Hodgkin's lymphoma and primary mediastinal B-cell lymphoma [159] [160]. Amplification of *CD274* has also been found in NSCLC, triple negative breast cancer, colon cancer, gastric cancer, glioblastomas, and cases of OSCC [86,161,162]. A study of 80 OSCC using both immunohistochemistry (IHC) and fluorescent in-situ hybridization (FISH) found that 36/80 were positive for PD-L1 expression using IHC but amplification was only seen in 15/80 cases. Only 73% of the cases with amplification showed positive IHC. This discordance shows the importance of mechanisms other than amplification in the over-expression of PD-L1 [163]. Interestingly, chromosome 9p also includes *JAK2,* the encoding gene of the aforementioned JAK2 kinase, which is upstream of PD-L1 production[86]. Co-amplification of *JAK2* and *CD274* has been observed in lung cancers and could potentiate the expression of PD-L1[164].

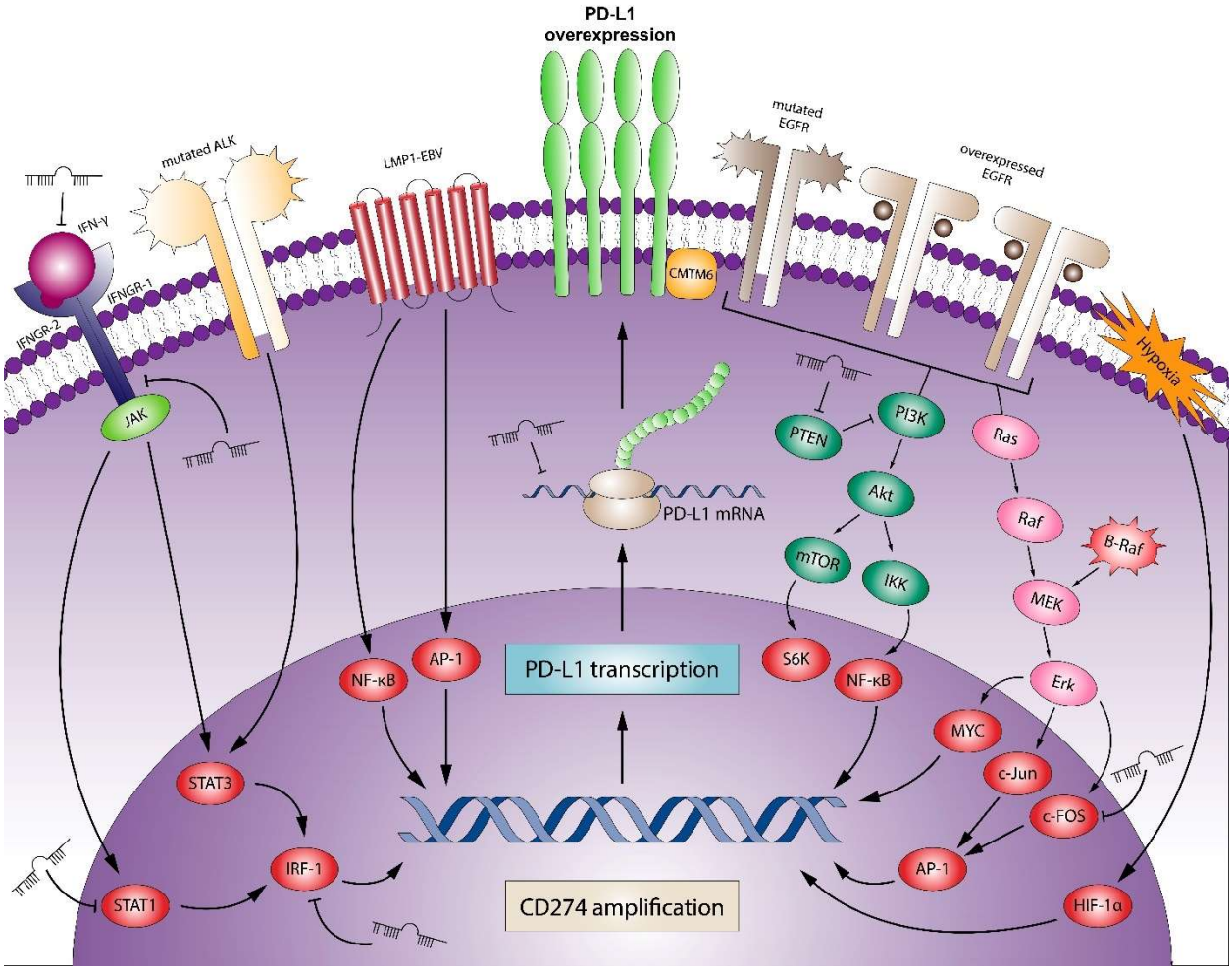
Viral Regulation

A known function of the Epstein-Barr Virus (EBV) is the production of its oncoprotein LMP1, which activates AP-1 and JAK/STAT [165]. This could, in turn, increase the expression of PD-L1. In classic Hodgkin's lymphoma, an increase in PD-L1 has been recorded in EBV-positive cases [166167]. However, it may be that the increase in PD-L1 may be due to viral-activated pathways or to an inflammatory response to the virally infected cells mediated by IFNγ [86].

Post-Translational Regulation

Proteins inducing post-translational control of PD-L1 expression have been reported. CMTM6 is a membranous protein that can bind to PD-L1, and it is believed that this stable binding prevents the ubiquitination and recycling of PD-L1, thus enhancing its effects. Increased PD-L1 expression due to the presence of CMTM6 has been described in pancreatic cancer, breast cancer, thyroid cancer, colon cancer, NSCLC, and melanoma. Besides CMTM6, the proteins CDK4, GSK3b, and CSN5 can also alter the post-translation expression of PD-L1 [86].

Figure 5. Mechanisms of overexpression of PD-L1



*miRNA and their respective targets (IFNγ, IFNGR-1, PD-L1, PTEN, c-Fos, STAT1, IRF-1) regulate PD-L1 production. Mutations in these miRNAs may cause a loss of function resulting in increased PD-L1 expression. **1** Mutations in PD-L1 upstream receptors may result in PD-L1 overexpression; **2** Epstein-Barr viral oncoproteins signaling may increase PD-L1 production; **3** Overexpression of PD-L1 pathway receptors; **4** Amplification of the PD-L1 gene: *CD274*; **5** Proteins stabilizing PD-L1; **6** Overexpressed PD-L1

B. Clinical Implications of PD-L1 overexpression in Cancer

PD-L1 expression is often studied as a clinical prognostic marker and predictor of PD-1: PD-L1-targeted therapy. IHC is a relatively simple and economical method to study PD-L1 expression, and varying expression levels have been reported in different tumour types. Positive PD-L1 expression is observed in 24% - 49% of melanoma cases and is associated with increased vertical growth and a lower overall survival (OS) [168,169]. PD-L1 has been detected in >50% of tumour cells in 20-30% of NSCLC cases [170] and this overexpression is associated with poor recurrence-free survival and OS [171]. In a study of PD-L1 in ovarian cancer, cases with high PD-L1 had a lower 5-year survival rate and OS [172].

PD-L1 overexpression has been described in ductal and lobular breast cancers [173] along with an increase in PD-L1 mRNA, which has been associated with the absence of hormone receptors, HER-2 positivity, tumour grade and positive Ki-67 expression [174]. In triple-negative breast cancer, PD-L1 is overexpressed in 20% of cases and can be due to transcriptional activation following a loss of PTEN [175]. In HNSCC, PD-L1 expression has been associated with distant metastases and a worse prognosis, independent of tumour size [176]. PD-L1 is overexpressed in varying degrees in gastro-oesophageal, colorectal, hepatic, pancreatic and bile duct carcinomas as well as in Hodgkin's lymphoma and acute myeloid leukaemia, among others, and is usually associated with a poor prognosis [177–183].

C. Clinical implications of PD-L1 expression in OSCC

PD-L1 overexpression has been reported in OSCC varying between 18% and 96% of cases **(Table 4).** This variance in expression may be due to different sample populations, different preparation methods, and the lack of a standardized scoring method for determining positivity. These studies have related PD-L1 expression levels to clinical-pathological features of the patient and disease. [163,184–195]

Table 4. Immunohistochemistry studies of PD-L1 positivity in OSCC

REFERENCES	Sample Size	Fixation	Clone	Ab	Staining	PD-L1 Cut-off for positivity/Scoring system	%PD-L1 Positive
Mattox et al. 2017	53	FFPE	Clone 5H1	Mo	M	Cut-off > 1 %	79%
Cho YA et al. 2011	45	FFPE	ab82059 (abcam)	P	C+M	Cut-off: ≥1%	87%
Satgunaseelan L et al. 2016	217	FFPE	E1L3N-XP-Rb(CST)	Mo	M	Cut-off: ≥ 5%	18%
Schneider et al. 2018	36	FFPE	Clone 5H1	Mo	M	Cut-off: ≥5%	25%
Troeltzsch M et al. 2017	88	FFPE	E1L3N (CST, USA)	Mo	n.s	Cut-off: ≥ 5%	29%
Straub M et al. 2016	80	FFPE	E1L3N (CST, USA)	Mo	M	Cut-off: ≥ 5%	45%
Kogashiwa Y et al. 2017	84	PE	SP142 (Spring Bioscience)	Mo	C+M	Cut-off: ≥5%	52.4%
Oliveira-Costa et al. 2015	96	FFPE	ab28753 (abcam)	P	C+M	Cut-off: ≥ 5%	56%
Maruse Y et al. 2018	97	FFPE	E1L3N (CST, USA)	Mo	n.s	Cut-off: ≥ 5%	65%
Chen T-C et al. 2015	218	PE	n.s; (Proteintech)	n.s	N+C	Cut-off: ≥ 5%	64%
Hanna GJ et al. 2017	23*	FFPE	9 A11	Mo	C+M	Cut-off: ≥10%	87%
Ahn H et al. 2017	68	FFPE	ab153991 (abcam)	P	C+M	0: no staining or staining in <10% of the tumour cells 1+: staining in ≥10% of the tumour cells with weak positivity 2+: moderate to strong positivity in tumour cells	0: 33.8% 1+: 33.8% 2+: 32.4%.
Hirai M et al. 2017	24	FFPE	n.s (abcam)	n.s	M	Low: labelling in <10% of tumour cells, or weak labelling in >10% of tumour cells High: Moderate/intense labelling in>10% of tumour cells	Low: 46% High: 54%
Lin Y-M et al. 2015	305	FFPE	GTX104763 (GeneTex)	P	C+M	Cut-off: n.s; Divided between low and high expression	Low: 56% High: 43%
Stasikowska-Kanicka O et al. 2017	78	FFPE	n.s (abcam)	P	C	Cut-off: n.s; Staining: 0:none; 1: weak; 2: moderate; 3: strong	79%
Stasikowska-Kanicka O et al. 2018	70	FFPE	n.s (abcam)	P	C	Cut-off: ≥5% with moderate or strong staining intensity of	96%
Kouketsu et al. 2017	106	FFPE	SP142	Mo	C+M	Weakly positive at any percent	68%

FFPE: formalin-fixed paraffin-embedded; PE: paraffin-embedded; FT: Frozen tissue; n.s: not specified; Mo: Monoclonal, P: Polyclonal; C: Cytoplasmic; M: Membranous; N: Nuclear; *female patients only

The significance of gender on PD-L1 expression varies. Lin et al. [184] reported significantly increased cytoplasmic staining intensity for PD-L1 in men compared to women (p=0.0062), and their multivariate analysis correlated high expression with a worse prognosis in male smokers. In contrast, Satgunaseelan et al. [188] found a significantly higher level of PD-L1 in women, while two other studies observed no significant relationship between sex and PD-L1 expression [163,189].

PD-L1 expression has been reported to vary among tumour sites. Significantly higher PD-L1 expression was observed in OSCC arising in mandibular structures or the tongue compared to the maxilla or soft palate (p=0.039) [189] and in those localized in lingual and buccal mucosa *versus* gingiva and floor of mouth (p=0.05) [188]. There have also been discordant results on the relationship between tumour size and PD-L1 expression, with some studies showing no correlation [163,184] and others finding a significant relationship between PD-L1 overexpression and tumour size [195,196].

One of the most important prognostic factors in OSCC is the spread of the disease to cervical lymph nodes [197]. Two studies[163,189] associated tumour PD-L1 overexpression with an increased risk of lymph node metastases. Maruse et al. reported a significantly higher rate of distant metastasis in tumours with PD-L1 and PD-1 overexpression, suggesting that PD-L1 expression may be an independent marker of lymph node metastasis[191]. In a similar finding, OSCC patients with increased circulating blood PD-L1 levels have shown a higher frequency of lymph node metastasis [198].

Studies relating PD-L1 levels to survival and clinical progression have published diverging results. Worse OS and disease control were found in PD-L1 positive *versus* negative OSCC [163,190,191,195]. Similarly, Chen et al. found that patients with both tumour necrosis and PD-L1 overexpression had a worse OS [199]. However, six studies failed to find a relationship between OS or tumour associated death and PD-L1 positivity [184,188,189,192,196,200]. Furthermore, Ahn et al. found a better prognosis in OSCC with both PD-L1 overexpression and high miR-197 levels[185]. Kogashiwa et al. also found an improved OS in OSCC cases that were both PD-L1 positive and locally advanced [194]. Two meta-analyses have failed to find an association between PD-L1 and OS [201,202]. However, the issue of data heterogeneity and identifying appropriate PD-L1 positivity cut-off values may affect the findings of these meta-analyses [203].

Tumor-infiltrating lymphocytes (TILs) are an important component of the host immune response but can be evaded by cancer cells through the induction of functional defects in these lymphocytes [204]. Conflicting findings have been reported by studies on the relationship between TILs and PD-L1 levels in OSCC. Lower TIL rates were found in PD-L1 positive OSCC [190,192], suggesting that the PD-1: PD-L1 interaction may inhibit T-cell activation in the tumour microenvironment. However, some studies observed an increased infiltration of T-cells in OSCC with PD-L1 overexpression, often associated with lower recurrence rates and longer survival [189,190,194]. Tregs, a subset of T-cells involved in immunosuppression, have been positively correlated with PD-L1 expression [185], which has been associated with a worse prognosis [187], suggesting that Tregs may play a role in OSCC progression via the PD-1: PD-L1 axis.

Human Papillomavirus (HPV) is an important etiological factor in oropharyngeal cancers but its relation to PD-L1 expression is unclear. It has been noted that high levels of PD-L1 expression are found in noncancerous tonsillar crypts where HPV positive tumours often originate. It has been hypothesized that HPV infection of tonsillar crypts may exploit the PD-L1 expression leading to a carcinoma with immune privilege. In a small sample size of 27 patients, Lyford-Pike et al. showed an increase in PD-L1 in HPV positive cases of oropharyngeal carcinoma. In larger sample sizes both Ukpo et al. and Badoual et al. have shown a strong trend that HPV positive oropharyngeal carcinomas overexpress PD-L1

(p=0.08)[205,206]. In contrast, Kim et al. as well as Scognamiglio and Chen have shown no correlation between PD-L1 and HPV expression in oropharyngeal tumours [207,208]. In studies of oral cavity OSCC, two studies both failed to find a significant correlation between PD-L1 and HPV expression [163,188].

Clinical Implications of PD-L1 expression in Oral Potentially Malignant Disorders (OPMD)

Given the prevalence of PD-L1 in OSCC there has been growing interest in the role PD-L1 may play in oral carcinogenesis. Several studies have measured PD-L1 in OPMD with divergent findings. Yagyuu et al. assessed 120 biopsies of oral epithelial dysplasia for PD-L1 expression using IHC. They found a significant association between both the epithelial and subepithelial PD-L1 levels and malignant transformation as well as malignant-free survival [209]. Koukatsu et al. assessed 106 OSCC and 79 oral leukoplakias with varying levels of dysplasia. They found a significantly higher expression of PD-L1 in OSCC than in the leukoplakias and theorized that PD-L1 may play a role in the progression of leukoplakia to OSCC. They failed to find a significant difference in PD-L1 expression between different grades of dysplasia [196]. Glass et al. studied OSCC and oral epithelial dysplasias and found 2 of 12 dysplastic lesions showed PD-L1 staining in the epithelium [210]. Gonçalves et al. studied multiple immune regulatory molecules including PD-L1 in OSCC, oral leukoplakias, and healthy oral mucosa. They claimed all cases of oral leukoplakia showed overexpression of PD-L1 which was greater than the healthy controls [211]. Stasikowska-Kanicka et al. also studied PD-L1 expression in OSCC, oral leukoplakias, and healthy controls. They found a significant increase in PD-L1 positive cell numbers in OSCC compared to oral leukoplakia and in oral leukoplakia compared to controls [212]. Seivvilanen et al studied dysplastic oral lesions and healthy controls for PD-L1 levels and followed a portion of these patients for up to 36 months. They found the levels of PD-L1 in the lamina propria in inflammatory cells to be greater than in healthy controls. However, they did not find any PD-L1 expression within the epithelium. The PD-L1 expression in the lamina propria fluctuated over time and was not reliable to monitor dysplasia progression [213]. PD-L1 has also been investigated in oral lichen planus. One study found increased levels of PD-L1 in lymphocytes and keratinocytes as compared to normal mucosa. [214]. Du et al. however did not find a significant increase in PD-L1 levels in lichen planus mucosa compared to healthy controls[215]. PD-L1 expression appears to be raised in some OPMD and any potential role in oral carcinogenesis merits further research.

IV. PD-1: PD-L1 as a therapeutic target in OSCC

Targeting of the PD-1: PD-L1 axis in HNSCC is based on two clinical trials (KEYNOTE-012 and CheckMate 141) that led the US Food and Drug Administration (FDA) to approve pembrolizumab and nivolumab. These are humanized monoclonal IgG antibodies targeting the PD-1 receptor and are part of a new class of drugs known as immune checkpoint inhibitors (ICI) [216,217]**.** Both drugs were initially included in National Comprehensive Cancer Network (NCCN) clinical guidelines as a fourth treatment line (after surgery, radiotherapy, and chemotherapy), and are indicated for patients with recurrent or metastatic HNSCC (R/M HNSCC) with disease progression after treatment with a platinum-based chemotherapy agent [147]. Following the KEYNOTE-048 trial, the FDA approved pembrolizumab as a first line agent in R/M HNSCC with sufficient PD-L1 expression[218].

Pembrolizumab

In a phase Ib clinical trial (KEYNOTE-012), pembrolizumab was administered to 60 patients (11 with OSCC) with metastatic or recurrent HNSCC whose disease had progressed after platinum-based chemotherapy; at least 1% of tumour cells were positive for PD-L1 in all patients [219]. The overall response rate (ORR) was 18%, mean progression-free survival (PFS) was 2 months and mean OS was 13 months, with grade 3+ adverse events (AEs) recorded in 17% of patients. A reduced pembrolizumab dosing schedule was administered to 132 patients (17 with OSCC) in an expansion cohort of the

KEYNOTE-012 trial, obtaining an ORR of 18%, which was significantly higher in the patients with PD-L1-positive tumours. The PFS was 2 months and OS was 8 months, with grade 3+ AEs in 9% of the expansion cohort [220]. In a phase II single-arm trial (KEYNOTE-055) of 171 patients with metastatic or recurrent HNSCC (28 with OSCC), an ORR of 18 % was observed in with 12% having grade 3+ AEs [221]. Follow-up results of the KEYNOTE-12 trial showed an OS at 12 months for 38% of patients. The authors highlight the durability of the response showing 2 patients who achieved a complete response, 6 patients who completed 2 years of pembrolizumab treatment and 18 patients that continued with pembrolizumab treatment. A higher response rate, PFS and OS was not seen in patients overexpressing PD-L1 in tumour cells but was seen in patients overexpressing PD-L1 in all cells (including lymphocyte, macrophages, and tumour cells). A higher response was seen in patients overexpressing PD-L2 as well as those with both PD-L1 and PD-L2 positive tumours. Responses were observed regardless of HPV status[222]. The KEYNOTE-048 randomized 882 R/M HNSCC to pembrolizumab, pembrolizumab + chemotherapy or cetuximab + chemotherapy. PD-L1 expression was scored as a percentage of all cells to express PD-L1 (not just tumour cells) which was called combined positive score (CPS). OS was significantly improved in the pembrolizumab + chemotherapy versus cetuximab + chemotherapy (HR 0.77; 95% CI: 0.63-0.93; p=0.0067). Pembrolizumab on its own significantly improved OS compared to cetuximab + chemotherapy in patients with CPS≥1 (HR=0·78 [0·64–0·96], p=0·0086) and CPS≥20 (HR=0·61 [95% CI 0·45–0·83], p=0·0007) and was non-inferior in the total population (HR=0·85 [0·71−1·03]). PFS was not significantly different between groups[223].

Nivolumab

In a phase III randomized control trial (CheckMate-141), nivolumab was administered to 240 patients with metastatic or recurrent HNSCC (108 with OSCC) with disease progression at ≤6 months after platinum-based chemotherapy, irrespective of PD-L1 levels, comparing results with those obtained in 121 control patients (67 with OSCC) receiving chemotherapy of the attending oncologist's choice [224]. An overall survival of ≥24 months was observed in 17% of the nivolumab group *versus* 6% of controls, and grade 3+ AEs occurred in 15% of the nivolumab group *versus* 37% of the control group [225]. Benefit in OS was independent of the level of PD-L1 expression (as measured in tumour cells only). OS benefit was observed in HPV positive and negative cases with the greatest benefit in PD-L1 and HPV positive cases (although the authors cautioned this comes from a small sample size with limited power).

Durvalumab

Durvalumab is an IgG1 monoclonal antibody that targets PD-L1. A phase I clinical trial of durvalumab in 50 patients with recurrent or metastatic HNSCC reported an ORR of 24% and recorded grade 3+ AEs in 5% of patients [226]. A phase II/III clinical trial of durvalumab in 62 patients with recurrent or metastatic HNSCC reported an ORR of 12% globally and of 25% in the PD-L1 positive subgroup, recording grade 3+ AEs in 7% of patients, with no drug-related deaths [227]. The ORR appeared to be superior with PD-1 inhibitors (pembrolizumab and nivolumab) than with the PD-L1 inhibitor (durvalumab) in patients with recurrent or metastatic HNSCC, although there was a higher frequency of AEs. One explanation for this improved clinical effect is the capacity of anti-PD-1 drugs to inhibit both PD-L1 and PD-L2 interactions.

Adverse events

All immune checkpoint inhibitors target the mechanisms responsible for regulating T-cell quiescence and activation. By removing these check points T-cell are activated which not only can attack tumour cells but can cause inflammatory adverse events which are distinctive to this new pharmacological class. They are known as immune-related adverse events (irAEs). Any organ can be affected but the most common sites of irAEs are the gastrointestinal tract, the endocrine system, the skin, the liver and the lungs. Inflammation of these tissues can range from mild to fatally severe [217,228]. PD-1 inhibitors appear

to most commonly cause pneumonitis and thyroiditis. PD-1 inhibitors also appear to have less frequent and less severe irAEs than another class of ICI: CTLA-4 inhibitors [217]. Combination therapy of more than one ICI leads to more frequent and more severe irAEs. Most irAEs occur in the first weeks of treatment but may appear after a year of treatment or even after treatment has been discontinued. The pathophysiology of irAE is not fully understood but may arise from a loss of self-tolerance in the immune system, cross-reactivity between tumour neoantigens and self-antigen or an increase in cytokines such as IL-17 [217,228]. Treatment of the irAEs is based on immunosuppression with glucocorticoids being used as a first-line treatment. Rarely this needs to be escalated to more a potent immunosuppressant such as tumour necrosis factor alpha inhibitors or mycophenolate mofetil. Irreversible irAEs have been described most commonly affecting the endocrine system where patients may develop insulin-dependent diabetes mellitus or primary adrenal failure and require lifelong treatment. Fatalities are rare but can occur through myocarditis, pneumonitis, colitis or neurological events. It is worth noting that in the CHECKMATE-141 trial there was a lower rate of AEs in the nivolumab group versus the control group receiving the prescribing oncologist's choice of chemotherapy. This improved AE profile compared to standard chemotherapy has also been shown in other types of cancers [228].

Neoadjuvant use of PD-1: PD-L1 inhibitors

An important question arising from the initial PD-1: PD-L1 trials is determining exactly how these drugs work in vivo and why only some patients respond. One way of obtaining excellent information is through neoadjuvant window trials. Window trials (short for a *window of opportunity*) are an alternative study design aimed at introducing new drugs or drug combinations in a patient before commencing the standard treatment regime. They take advantage of the *window of opportunity* provided by a patient that has not had previous treatments as this could confound how the tumour reacts. Most OSCC patients will have an initial biopsy followed by surgical resection. Interventions are carried out between the biopsy and definitive surgical resection giving two tissues samples to study the in vivo actions of the intervention on the tumour tissue. The patients can also be compared for clinical outcomes following these treatments. As there is a risk of AE and potential treatment delay, window trials must be carefully executed. When properly planned and monitored window trials can be a safe option to uncover the potential of new drugs, new treatment combinations or personalized markers for more precise treatment [229,230]. One such clinical trial (NCT02488759) is investigating newly diagnosed resectable HNSCC and giving nivolumab on day 1 and day 15 before preceding to definitive surgery on day 29±7. They have so far published 23 evaluable patients with no delays or grade3/4 AEs. 48% showed shrinkage of the tumour with one shrinking 75% of its original size [231]. Uppaluri et al. have published results from a trial (NCT02296684) with results from 21 patients with stage III/IV HNSCC receiving 1 dose of pembrolizumab before surgery and standard of care treatment. They report no delays or AEs and no locoregional recurrences in the first 10 patients at 1 year. 48% of the patients had clinical-to-pathological downstaging. 11 of 19 evaluable samples were PD-L1 positive as were 7 of the 8 pathological responders [232]. These and other trials continue and will likely help us to understand the exciting potential of these new agents.

3. Summary

OSCC is a disease affecting hundreds of thousands around the world while continuing to have an unacceptable mortality rate. Many prognostic markers are being researched but it is not yet possible to predict with accuracy the evolution of all OSCC. PD-L1 is a recently discovered, transmembrane protein which can be found in human cancers. PD-L1 interacts with PD-1 to act as an immune checkpoint, especially in immune privileged tissues. It is theorized that cancers can use this mechanism to evade immune destruction. The existing literature is so far unclear whether PD-L1 has a prognostic value in OSCC. However, it is clear that blocking the PD-1/PD-L1 pathway in OSCC can improve survival. There is, therefore, a need for further research into the value of PD-L1 in OSCC.

Objectives

Following this narrative review of the literature we derived three research questions to answer:

1) What proportion of OSCC express PD-L1?
2) What is the significance of PD-L1 expression on the prognosis of OSCC?
3) What are the clinico-pathological associations of PD-L1 expression in OSCC?

Research Plan

We planned to answer these questions in two way:

1)Conducting a systematic review and meta-analysis to synthesize the existing research.

2)Perform an observational experiment using archived OSCC stained for PD-L1

Methods

I. Systematic Review and Meta-Analysis

Protocol:

We followed PRISMA guidelines in conducting and reporting this review[233,234]. A research protocol has been registered with the international prospective register of systematic reviews (PROSPERO, CRD42019133935).

Eligibility:

We defined our research question as: "What is the prognostic and clinicopathological significance of PD-L1 overexpression in patients with oral squamous cell carcinoma?" To remove the confounding etiological factor of HPV infection the review selected only patients with OSCC of the oral cavity (ICD-10: C02-C06). The index prognostic factor of interest was PD-L1 overexpression (however defined in each original article) in OSCC tumours measured by immunohistochemistry. The comparison groups were patients without PD-L1 overexpression. The primary outcomes of concern were overall survival (OS), disease-free survival (DFS), disease specific survival (DSS), progression free survival (PFS) or local-regional progression free survival (LRFS).

Search strategy:

A search was performed in PubMed, Embase, Web of Science and Scopus for all studies, posters and communications published at any point until March 02 2019. Searches were carried out using controlled vocabulary terms (MeSH and EMTREE) combined with free terms. Full search strategies can be found in **Table 5.** Hand searching was done of the bibliographies of any relevant studies.

Study selection criteria

Inclusion criteria were: 1) English language publication. 2) Evaluating PD-L1 in OSCC of the oral cavity (ICD 02-06) by IHC. 3) Analysis of PD-L1 and at least one of the following variables: OS, DFS, DSS, PFS, LRFS, TMN status, histological grade, clinical stage, gender, alcohol use, smoking status.

Exclusion criteria were: 1) Studies where data on OSCC cases was not obtainable 2) Clinical trials 3) In vitro or animal trials 4) Techniques other than IHC 5) Studies that do not analyse the effect of PD-L1 on survival or clinicopathological parameters 6) Studies with insufficient data to estimate Odds Ratios or Hazard Ratios relevant to PD-L1.

Data collection

Two authors (DL and PRG) independently screened titles and abstracts from the search results and then retrieved articles from this initial selection for full read. Any disagreement was resolved by a third investigator (MAGM). Risk of bias was assessed using the Quality in Prognostic studies (QUIPS) tool. A standardized QUIPS form was used to equally assess sources of bias in each study and can be found in **Table 6.** In cases where survival data had been collected but a hazard ratio was not reported nor possible to estimate, then an email was sent to the corresponding author or first author if no corresponding author exists[188,200,235–245]. When authors responded the data was included[188,200,235–237,245].

Statistical Analysis

Hazard Ratios (HRs) and 95% confidence intervals (CIs) were taken from the included studies to estimate the effect of PD-L1 overexpression on time-to-event variables (OS, DFS, DSS, PFS and LRFS). Multivariate analysis HRs were preferentially taken over univariate HRs. If data was not explicitly stated but an estimate of the HR was possible using the techniques described in Tierney et al. then a HR was

estimated using the available information[246]. When extracting a hazard ratio from a Kaplan-Meier Curve then the tracings were done digitally using Engauge Digitizer 12.0 (open-source digitizing software developed by M. Mitchell). If p-values were used in the calculation of an estimated HR but an exact p value was not given then a conservative estimate was used (e.g. if the article states p<0.05, then HR was calculated using p=0.049). Odds ratios (ORs) with 95% CIs were calculated for clinical and pathological variables of interest.

Table 5. – Search Strategies and number of articles returned

Database	Query	March 2 2019
Pubmed	(("mouth"[MeSH Terms] OR "mouth"[All Fields] OR "oral"[All Fields] OR "buccal"[All Fields] OR "tongue"[All Fields] OR "alveolar"[All Fields] OR "gingiva*"[All Fields] OR "retromolar"[All Fields] OR "palat*"[All Fields] OR "head and neck"[All Fields] OR "Head and Neck Neoplasms"[Mesh] OR "mouth neoplasms"[MeSH Terms]) AND ("carcinoma, squamous cell"[MeSH Terms] OR ("carcinoma"[All Fields] AND "squamous"[All Fields] AND "cell"[All Fields]) OR "squamous cell carcinoma"[All Fields])) AND ("PD-L1" OR "PDL1" OR "PDL-1" OR "B7-H1" OR "B7H" OR "B7H1" OR "CD274" OR "PDCD1L1" OR "PDCD1LG1" OR ("programmed death" AND "-ligand 1" OR "receptor ligand-1" OR "ligand 1" OR "receptor ligand 1") OR ("B7-H1 Antigen"[Mesh]) OR ("CD274 protein, human" [Supplementary Concept]))	**306**
Embase	('mouth tumor'/exp OR 'mouth tumor' OR 'head and neck tumor'/exp OR 'head and neck tumor' OR 'mouth'/exp OR 'mouth' OR 'cheek' OR 'tongue' OR 'gingiva*' OR 'alveolar ridge' OR 'retromolar' OR 'oral' OR 'head and neck') AND ('squamous cell carcinoma'/exp OR 'squamous cell carcinoma' OR (squamous AND ('carcinoma'/exp OR carcinoma) AND ('cell'/exp OR cell))) AND ('pd l1' OR pdl1 OR 'b7 h1' OR cd274 OR pdcd1l1 OR pdcd1lg1 OR ('programmed death' AND ('ligand one' OR 'ligand 1')) OR 'pd l1 gene'/exp OR 'pd l1 gene' OR 'pd l1 protein'/exp OR 'pd l1 protein' OR 'pd l1 antibody'/exp OR 'pd l1 antibody' OR 'programmed death 1 ligand 1'/exp OR 'programmed death 1 ligand 1' OR 'cd274 protein human'/exp OR 'cd274 protein human')	**841**
WOS	TS=(mouth OR oral OR buccal OR tongue OR gingiv* OR alveolar OR retromolar OR palat* OR "head and neck") AND TS=("squamous cell carcinoma*" OR (squamous AND cell AND carcinoma)) AND TS=(PD-L1 OR PDL1 OR B7-H1 OR B7H1 OR B7H OR CD274 OR PDCD1L1 OR PDCD1LG1 OR (("programmed cell" OR programmed death OR programmed cell death) AND "ligand 1" OR "-ligand 1" OR "ligand one" OR "-ligand one" OR receptor ligand 1 OR receptor ligand one))	**530**
Scopus	TITLE-ABS-KEY ((mouth OR oral OR buccal OR tongue OR gingiv* OR alveolar OR retromolar OR palat* OR "head and neck") AND ("squamous cell carcinoma" OR (squamous AND cell AND carcinoma)) AND (PD-L1 OR PDL1 OR B7-H1 OR B7H1 OR B7H OR CD274 OR PDCD1L1 OR PDCD1LG1 OR (("programmed cell" OR "programmed death" OR "programmed cell death") AND "ligand 1" OR "-ligand 1" OR "ligand one" OR "-ligand one" OR "receptor ligand 1" OR "receptor ligand one")))	**435**
Total		**2,212**

Table 6 - Blank QUIPS form

Each line is answered as Yes/No/Not available and then scored. Scoring: the lack of "No" was low bias, 1 "No" was moderate and more than 1 "No" was a high risk of bias.

1: participation: The study sample adequately represents the population of interest

Adequate participation in the study by eligible persons	
Description of the source of the population of interest	
Description of the baseline study sample	
Adequate description of the sampling frame and recruitment	
Adequate description of the period and place of recruitment	
Adequate description of inclusion and exclusion criteria	

2: Attrition: The study data is available (not lost to follow up) for the study sample

Adequate response rate for study participants	
Description of attempts to collect information on participants that drop out	
Reasons for loss to follow up are given	
Adequate description of participants lost to follow up	
No important differences between those that finished study and those that did not	

3: PF measurement: Measured in the same way for all participants

Clear definition of the PF is given	
Method of PF measurement is adequately valid and reliable	
Continuous variables are reported or appropriate cut off points are used	
Methods of measuring the PF is the same for all participants	
Adequate proportion of the sample has data for the PF	
Appropriate methods of imputation are used for missing PF data	

4: outcome measurement: The outcome of interest is measured the same for all patients

A clear definition is given of the outcome	
Method of outcome measurement is adequately valid and reliable	
Method and setting of outcome measurement is the same for all patients	

5: confounding: Important potentially confounding factors are accounted for

All the important confounders are measured	
Clear definitions of confounders are given	
Measurement of confounders is valid and reliable	
Measurement of confounding is the same for all patients	
Appropriate methods are used for missing confounding factor data	
Important potential confounders are accounted for in the analysis	
Important potential confounders are accounted for in the study design	

6: Statistical analysis: Stats are appropriate and all primary outcomes are reported

Sufficient presentation of data to assess the adequacy of the analytic strategy	
Strategy for model building is appropriate and based on a conceptual framework or model	
The statistical model is appropriate for the study	
There is no selective reporting of results	

For meta-analysis, outcomes were grouped together and analysed using Review Manager 5 version 5.2.8 (Cochrane Collaboration, Copenhagen, Denmark; 2014). The different IHC scoring methods used in each study were accounted for using a random effects model. Where a study presented two different HRs based on using different IHC measuring criteria then both HRs were entered as if separate studies and denominated as 1 and 2. This was done to not favour any one methodology over another and to reduce bias in the inclusion of data. Heterogeneity was calculated using Cochran's Q test ($p<0.1$) and Higgins I^2, using 25%, 50% and 75% as respectively indicative of low, moderate and high heterogeneity. Sub-group analyses were performed to identify sources of heterogeneity including grouping by cut-off and IHC staining pattern. A meta-analysis of proportions was conducted using MetaXL (version 5.3, EpiGear Int.) to assess what proportion of patients was positive for PD-L1. This analysis was carried out using a double arcsine transformation and 95% confidence intervals.

Sensitivity analysis was carried out to detect the influence of individual studies[247]. This was done by repeating the meta-analysis and systematically removing one study at a time (*leave-one-out analysis*). Meta-analysis results that lost their significance after removing one study were considered non-robust. For studies that gave two different HRs these were removed first individually and then on second pass as a block together. Publication bias was assessed with funnel plots and Eggers regression test ($p_{Egger} < 0.1$)[248].

II. Immunohistochemistry study

Description of patients

We conducted a retrospective study of 55 patients using archived OSCCs exclusively of the oral cavity. Ethical Approval was granted by the CEIM/CEI Provincial of Granada. Oropharyngeal SCCs were excluded to remove HPV infection as a confounding factor (PD-L1 expression has been shown to be increased in HPV positive oropharyngeal SCCs[249,250]). PD-L1 expression can be heterogenous, so both micro-invasive and carcinoma-in-situ tumours were excluded as the small size of these tumours may distort the scoring of PD-L1. Patients were aged between 42-87 years old at the time of diagnosis (mean: 66.8y, median: 68y., SD: 12y.) and came from the University Hospital of Jaen, Spain (26 patients) and the Virgen de las Nieves Hospital in Granada, Spain (29 patients). Patient demographic characteristics were gathered from the hospital medical history and included age, sex, smoking habit and alcohol consumption (**Table 5**). The tumour characteristics recorded were: location, TNM status, clinical stage, and grade of differentiation. Multiple tumours were present in 9 patients but only the first tumour was used. When two tumours were present from the same year (1 patient in this study) then only one tumour was randomly chosen and assessed in each patient.

Inclusion criteria were: 1) Having an OSCC of the oral cavity (International Classification of Diseases, ICD-10: C02-C06[4]), 2) Available formalin-fixed paraffin-embedded tumour, 3) Existence of essential clinical records corresponding to the tumour. Exclusion criteria were: 1) Lack of essential survival data, 2) Lack of sufficient quantity of tumour tissue in the paraffin block, 3) Microinvasive carcinomas or *carcinoma in situ*, 4) Previous carcinoma of the head and neck.

Patient survival data was collected from clinical records and the length of time from the date of diagnosis until the event of interest was measured to the nearest month. Events were: death from oral cancer, death from other cause, recurrence (no distinction between local or distant) or alive without recurrence. An event for Disease Specific Survival (DSS) was when the patient died from the oral cancer (recurrences and death from another cause were not considered events). Events for Disease Free Survival (DFS) were either recurrence or death from cancer (dead from another cause was not an event).

Overall Survival information was only available for the 29 patients from the Granada Virgen de las Nieves hospital and an event was defined as death by any cause.

Immunohistochemistry (IHC)

Immunohistochemistry was carried out in the Pathology Department of the San Cecilio University Hospital, Granada, Spain. Peroxidase-antiperoxidase and avidin-biotin techniques were applied to a 4-µm section from each paraffin block including tumour. No serial sections were taken. PD-L1 was stained using an anti-PD-L1 mouse monoclonal antibody (22C3 clone, Dako, Carpinteria, CA, USA) and an Autostainer Link system with EnVision FLEX reagents (K8002) (Dako, Carpinteria, CA, USA) following the manufacturer's instructions. This system allows dewaxing and rehydration followed by heat-induced epitope recovery. The reproducibility of the process was ensured by loading the whole coverslip, guaranteeing identical heating of all sections in each cycle. The manufacturer does not recommend a dilution of the anti-PD-L1 antibody and therefore the antibody was used at the supplied concentration (approximately 3µL/mL protein concentration). A negative control was performed by replacing the primary antibody with phosphate buffered saline. A positive control from the manufacturer was included in each staining run (Supplementary Figure 3). The slides were digitized using the Philips IntelliSite Ultra-Fast Scanner (Philips Digital Pathology Solutions, Best, The Netherlands). A separate haematoxylin and eosin (H&E) stain of each tumour was obtained.

Scoring Methods

All scoring variables were measured by three researchers independently and a consensus was reached in cases of disagreement (PRG, IRA, DL). Full tumour slides were assessed and scored for PD-L1 expression (brown labelling). H&E slides were viewed to ensure at least 100 tumour cells were present. Dysplasia and differentiation were graded using the WHO guidelines[251,252]. Tumour infiltrating lymphocytes (TILs) were measured in the stroma and categorized as mild, moderate or severe (<10%, 10-50%, >50% of cells present, respectively). PD-L1 staining of TILs was recorded as low or high (<10% or ≥10% of TILs present, respectively). The tumour was then split into 4 equal quadrants and PD-L1 staining in tumour cells was considered exclusively in the membrane. A percentage of all tumour cells staining positive for PD-L1 was given to each quadrant and the average of the 4 quadrants was calculated to give the tumour proportion score (TPS). The PD-L1 staining pattern was determined as induced (if only affecting the cells adjacent to inflammatory cells) or constitutive (if affecting most cells not adjacent to inflammatory cells). The tumours where grouped as PD-L1 positive or negative using a cut-off point of TPS ≥5%[253]. Finally, the epithelium, both adjacent (<10mm) and distant (>10mm) from the point of invasion[254,255], was assessed for PD-L1 staining and reported as present (at any level) or absent. Treatment variables included three binary categories, indicating if the treatment was given or not: surgery, chemotherapy and radiotherapy.

Statistics

SPSS windows 24.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. The chi-squared test was used for clinicopathological variables with categorical data unless any category had a value less than 5, in which case Fisher's exact test was used. Continuous data was analysed using either Mann-Whitney's U or Kruskal-Wallis's H tests. Survival outcomes were calculated using Kaplan-Meier curves and the log-rank test. Cox proportional hazards models were used to calculate hazard ratios for PD-L1 (as it was the focus of the study) and any other factor that had a Log-rank p-value of less than 0.1. A p-value less than 0.05 was considered statistically significant for all tests.

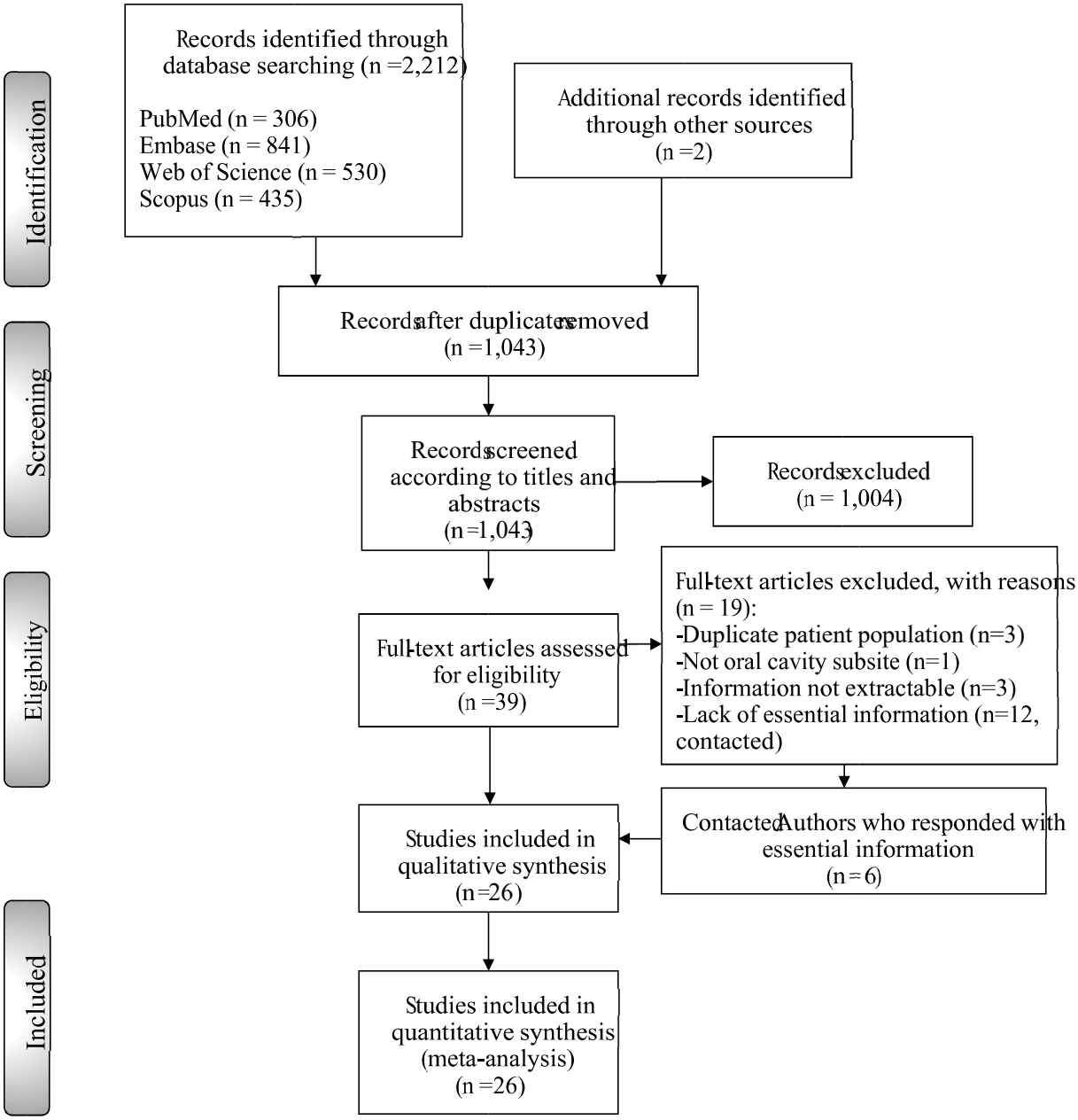
Results

I. Systematic Review and Meta-Analysis

Literature search

Our search returned 2,212 records from PubMed (306), Embase (841), Web of Science (530) Scopus (435) and 2 from screening reference lists. After excluding duplicates 1,043 articles remained. Titles and abstracts were reviewed and 39 studies were included for full reading. Sources lacking essential information were contacted by email and 6 additional studies were entered when authors replied. After applying inclusion and exclusion criteria 26 studies remained[163,184,186,188–190,192,194,195,199,200,235–237,243–245,256–264] as seen in the flow diagram, **Figure 6.**

Figure 6. Flow diagram of the literature search for systematic review



Study Characteristics

The pertinent information of the 26 included studies is summarized in **Table 7**. Two studies reported findings using different antibodies and were therefore considered as two separate studies[256,263]. One study reported findings using two different IHC scoring methods and this was also treated as two separate studies[195]. One study reported HRs using two different IHC cut off points and these were also treated as separate studies[245]. Two studies[194,259] were carried out at the same unit and may have overlapping patients, but as the methodology was different these were both included and considered as separate studies. The total number of patients from the included 26 studies was 2,532. Twenty-one studies assessed PD-L1 overexpression on survival parameters involving 2,221 patients. Nineteen studies assessed clinicopathological associations with PD-L1 overexpression and involved 2,027 patients. Studies included all continents with 11 in Asia, 10 in Europe, 4 in the Americas and 1 in both Oceania and Africa. Study sizes ranged from 20 to 305 patients.

Survival data extraction

PD-L1 time-to-event HRs for common survival parameters are summarized in **Table 8**. A total of 20 studies had survival data either reported or that could be calculated, of which 6 were available thanks to contact from the study authors. Only 9 of the studies clearly stated that survival analysis was multivariate.

Table 7. Basic information of the 26 studies included in the systemic review

Study	Year	Country	Sample Size (additional information)	Follow-up Range (months)	Anti-PD-L1 Antibody	IHC Staining (M, C, N, NS)	IHC Cutoff for High PD-L1 (%, NS, H)	IHC Intensity Assessed (Y, N,)	Cells considered for positive IHC (TC, IC, E, NS)	Survival Parameter Assessed (Y/N)	Clinical Parameter assessed (Y/N)
Cho YA et al.	2011	Korea	45	25-125	Abcam (clone ab82059)	C&M	NS	Y	TC	Y	Y
Oliveira-Costa et al.1	2015	Brazil	96 (TMA)	4-108, mean: 20	Abcam (clone ab28753)	C	5%	N	TC	Y	Y
Oliveira-Costa et al.2	2015	Brazil	96 (TMA)	4-108, mean: 20	Abcam (clone ab28753)	M	5%	N	TC	Y	Y
Lin Y-M et al.	2015	Taiwan	305 (TMA)	1-133, mean: 45.6	GTX104763, GeneTex	C&M	NS	Y	NS	Y	Y
Chen T-C et al.	2015	Taiwan	218 (all Stage III/IV, N+)	1-128, mean: 31	Proteintech Group Inc., Chicago, IL, USA	M	≥5%	N	TC	Y	Y
Straub M et al.	2016	Germany	80 (TMA)	2-63, mean: 31	E1L3N, Cell Signaling	M	≥5%	Y	TC	Y	Y
Satgunaseelan et al.	2016	Australia	217 (TMA)	1-144, mean: 22	E1L3N, Cell Signaling	M	Positive >5%	Y	TC	Y	Y
Mattox et al.	2017	USA	53 (all tongue subsite)	NS	5H1 clone	M	>1%	N	TC or IC	Y	Y
Troeltzsch M et al.	2017	Germany	88 (TMA)	NS	E1L3N, Cell Signaling	NS	≥5%	Y	TC	N	Y
Kogashiwa Y et al.	2017	Japan	84	4-90, mean: 40.6	SP142 Spring Bioscience	M&C	≥5%	N	TC or IC	Y	N
Foy JP et al. 1	2017	France	44	NS	clone SP142, Roche	NS	1%, 5%, 10%	N	TC	N	Y
Foy JP et al. 2	2017	France	44	NS	clone 28.8, Dako	NS	1%, 5%, 10%	N	TC	N	Y
Balermpas et al.	2017	Germany	41	NS	E1L3N, Cell Signaling	NS	≥5%	N	TC or IC	Y	N
Ahn H et al.	2017	Korea	68 (TMA)	2-122, mean:	Abcam (clone	M&C	≥10%	Y	TC	Y	Y
Hirai M et al.	2017	Japan	24	NS	Abcam	M	>10%	Y	TC	N	Y
Udeabor et al.	2018	Nigeria	20	NS	28-8 Abcam	NS	H	Y	TC	N	Y
Kouketsu et al.	2017	Japan	106	36-75	SP142, Spring	M&C	1%,	Y	TC and E	Y	Y
Yoshida et al.	2018	Japan	135 (TMA, all tongue subsite)	NS	28-8 Abcam	M	50%	N	TC	N	Y
Hanna et al. 1	2018	USA	23 (all	1-227, median:	Clone 9A11	M&C	H	Y	TC	Y	N
Hanna et al. 2	2018	USA	31 (all post	0.1-	Clone 9A11	M&C	H	Y	TC	Y	N
Wirsing et al.	2018	Norway	45	0-60	Clone SP263,	M&C	>10%	N	TC	Y	Y
Maruse et al.	2018	Japan	97	0-60	E1L3N, Cell	NS	>5%	N	TC or IC	Y	N
Schneider et	2018	Austria	36 (TMA)	0-60	Clone 5H1	M	>5%	Y	TC	Y	N
De Vicente et	2018	Spain	125 (TMA)	1-230, median:	22C3, Dako	NS	>10	N	TC	Y	Y
De Vicente et	2018	Spain	125 (TMA)	1-230, median:	E1L3N, Cell	NS	>10%	N	TC	Y	Y
Manikhas et	2018	Russia	82	NS	clone	NS	>1%	N	TC and IC	Y	N
Manikhas et	2018	Russia	82	NS	clone	NS	>5%	N	TC and IC	Y	N
Naruse et al.	2019	Japan	121 (all	0-120	Abcam (clone	C or N	>5%	Y	TC	Y	Y
Tsai et al.	2019	Taiwan	173 (TMA, Stage III / IV, M0)	0-120	NS	NS	H	Y	NS	Y	N
Moratin et al.	2019	Germany	175 (TMA)	0-60	Cell Signaling	NS	H	Y	NS	Y	Y

Abbreviations: IHC: Immunohistochemistry; M: Membranous; C: Cytoplasmic; N: Nuclei; NS : Not stated; TC: Tumor Cell; IC: Immune Cell; E: Epithelial Cell; N+: N status positive; M0: M status negative; Y: Yes, N: No; HSCT: Hematopoietic Stem Cell Transplantation; TMA: Tissue Microarray, H: H-Score (A score made by adding or multiplying the intensity score and proportion score of cells stained)

Table 8. Data extraction of the effect of PD-L1 on common survival parameters

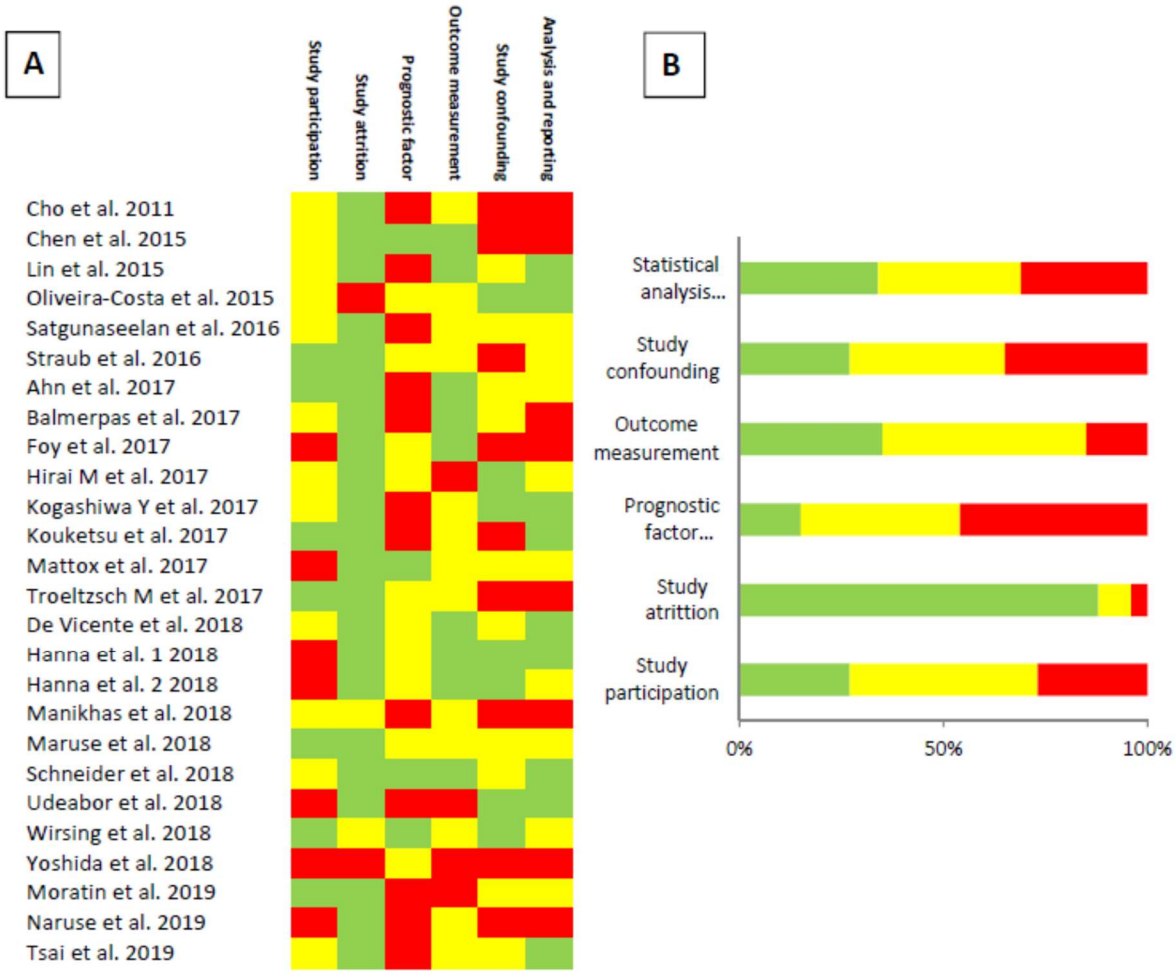
Study	OS (HR, 95%CI)		DFS/PFS*/LRFS** (HR, 95%CI)		DSS (HR, 95%CI)		Reported or Estimated	Multivariate or Univariate
Cho YA et al.	1.35	0.57-3.20	-	-	-	-	Estimated	Univariate
Oliveira-Costa et al.1	-	-	-	-	0.43	0.19-0.98	Reported	Multivariate
Oliveira-Costa et al.2	-	-	-	-	2.63	0.47-14.61	Reported	Multivariate
Lin Y-M et al.	1.35	0.99-1.83	-	-	-	-	Reported	Multivariate
Chen T-C et al.	1.32	0.94-1.85	1.49	1.07-2.10	1.21	0.85-1.71	Estimated	Multivariate
Straub M et al.	-	-	2.11	1.00-4.43	3.1	1.31-7.31	Estimated	Univariate
Satgunaseelan et al.	1.24	0.58-2.63	1.17	0.63-2.14	1.51	0.57-3.98	Estimated***	Univariate
Mattox et al.	1.62	0.50-4.46	-	-	-	-	Reported***	Univariate
Kogashiwa Y et al.	0.26	0.10-0.65	0.54*	0.28-0.89*	-	-	Reported	Multivariate
Balmerpas et al.	0.59	0.20-1.74	-	-	-	-	Estimated***	Univariate
Ahn H et al.	0.32	0.11-0.94	0.25	0.06-1.12	-	-	Reported	Univariate
Hanna et al. 1	0.58	0.45-0.74	-	-	-	-	Reported	Multivariate
Hanna et al. 2	0.93	0.74-1.16	-	-	-	-	Reported	Multivariate
Wirsing et al.	-	-	-	-	0.50	0.17-1.46	Reported***	Univariate
Maruse et al.	-	-	-	-	2.79	1.09-7.16	Estimated	Univariate
Schneider et al.	-	-	3.10	1.10-9.10	-	-	Reported	Univariate
De Vicente et al. 1	-	-	-	-	2.05	1.02-4.11	Reported	Multivariate
De Vicente et al. 2	-	-	-	-	1.91	0.86-4.24	Reported	Univariate
Manikhas et al. 1	0.76	0.35-1.65	-	-	-	-	Reported***	Univariate
Manikhas et al. 2	1.14	0.53-2.48	-	-	-	-	Reported***	Univariate
Naruse et al.	-	-	-	-	2.79	1.24-6.28	Estimated	Univariate
Tsai et al.	-	-	0.71**	0.30-1.68**	-	-	Reported	Multivariate
Moratin et al.	3.02	1.06-8.64	1.61*	0.68-3.82*	-	-	Reported***	Univariate

Abbreviations: OS: Overall Survival; DFS: Disease-free Survival; DSS: Disease Specific Survival; HR: Hazard Ratio; CI: Confidence Interval, Univariate analysis is assumed unless clearly stated by authors, *Progression-free Survival, ** Local Recurrence-free Survival; ***from email correspondence with one of the authors

Quality Analysis

Risk of bias and quality assessment was done using the QUIPS tool and is summarized in **Figure 7**.

Figure 7 – Quality analysis using QUIPS tool



A) QUIPS tool showing each included study B) Bar chart summarizing each element of study bias assessed. Green, yellow and red represent low, moderate and high risk of bias, respectively.

Quantitative Analysis - Survival Parameters

Overall Survival (OS)

OS was assessed with Hazard Ratios in 13 studies from a combined 1,380 patients. All 13 studies were included and one[245] was treated as two separate studies as two HRs were available (one for a 1% PD-L1 cut-off and one for a 5% PD-L1 cut-off). No significant effect was seen in Overall Survival (HR=1.00, 95% CI=0.75-1.35, p=0.98) as shown in **Figure 8**. There was significant heterogeneity in the results with an I^2 value of 74% (p<0.00001).

Disease-free Survival(DFS)/Progression-free Survival(PFS)/Local-Regional Progression-free Survival (LRFS)

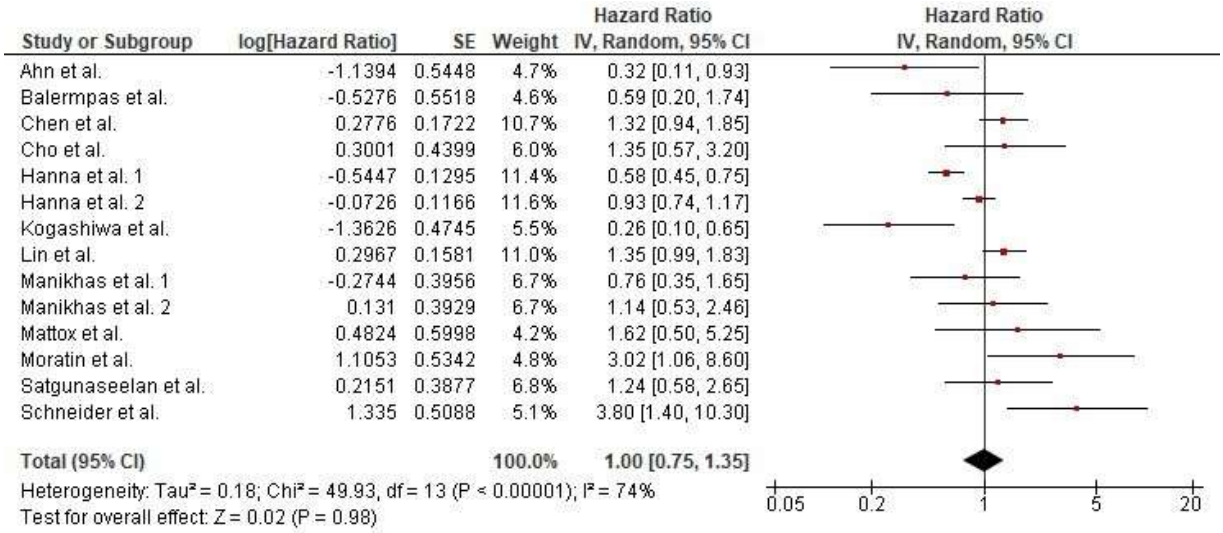
DFS was assessed in 5 studies with 619 patients and was not statistically significant (HR= 1.42, 95% CI=0.88-2.28, p=0.15) **Figure 8**. There was substantial heterogeneity (I^2 =56%, p=0.05) mostly due to one study[257]. Three studies with 432 patients reported PFS or LRFS, two of which only included locally advanced stage III/IV patients[194,264]. While clear definitions of PFS and LRFS were not given these were considered as sufficiently similar to DFS for the purpose of meta-analysis. The combined DFS/PFS/LRFS meta-analysis again showed no significant finding (HR=1.16, 95% CI=0.76-1.75, p=0.49) and had significant heterogeneity (I^2 =64%, p=0.008) which did not change with leave-one-out sensitivity analysis **Figure 9**.

Disease Specific Survival (DSS)

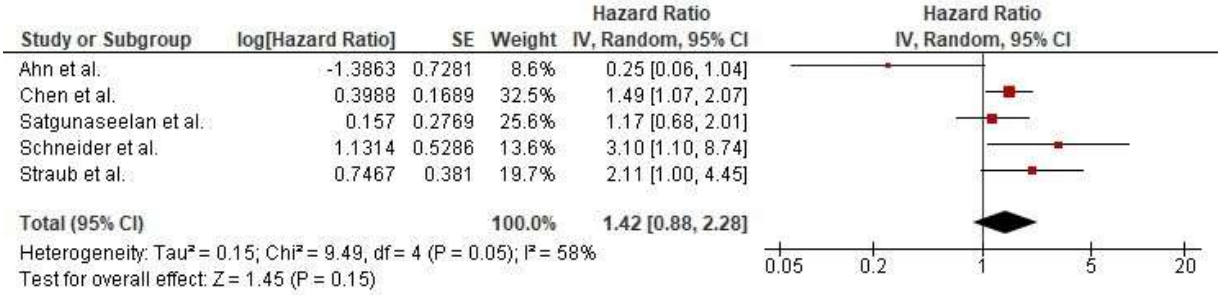
DSS was reported in 8 studies including 999 patients. Two studies reported two different HR each and these were entered as separate studies (de Vicente et al. used two different antibodies[263] and Oliveira-Costa et al. used two different IHC scoring methods[195]). A statistically significant result was found (HR= 1.54, 95% CI=1.03-2.28, p=0.03) with significant heterogeneity (I^2=58%, p=0.01). This result was not robust to sensitivity analysis **Figure 8**.

Figure 8. Forest plot of survival parameters

Overall Survival



Disease Free Survival



Disease Specific Survival

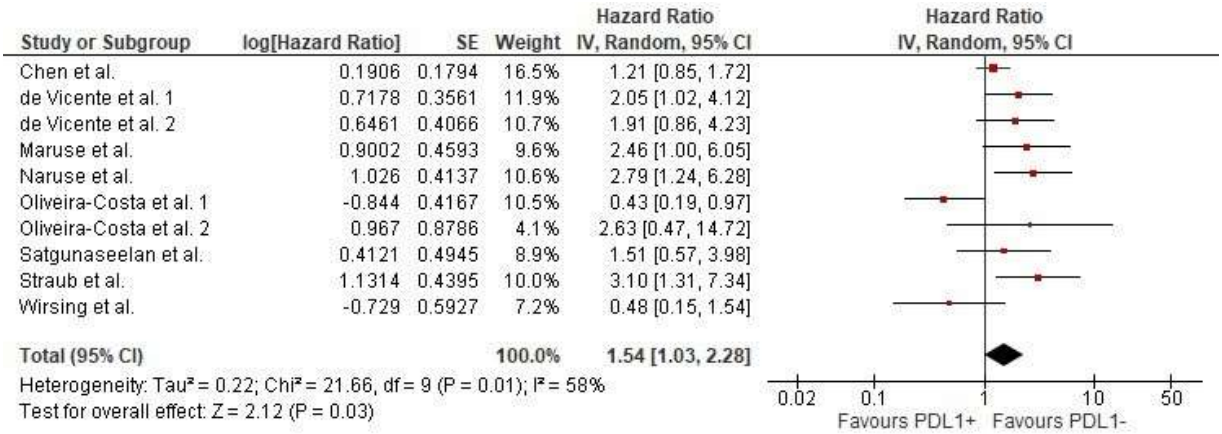
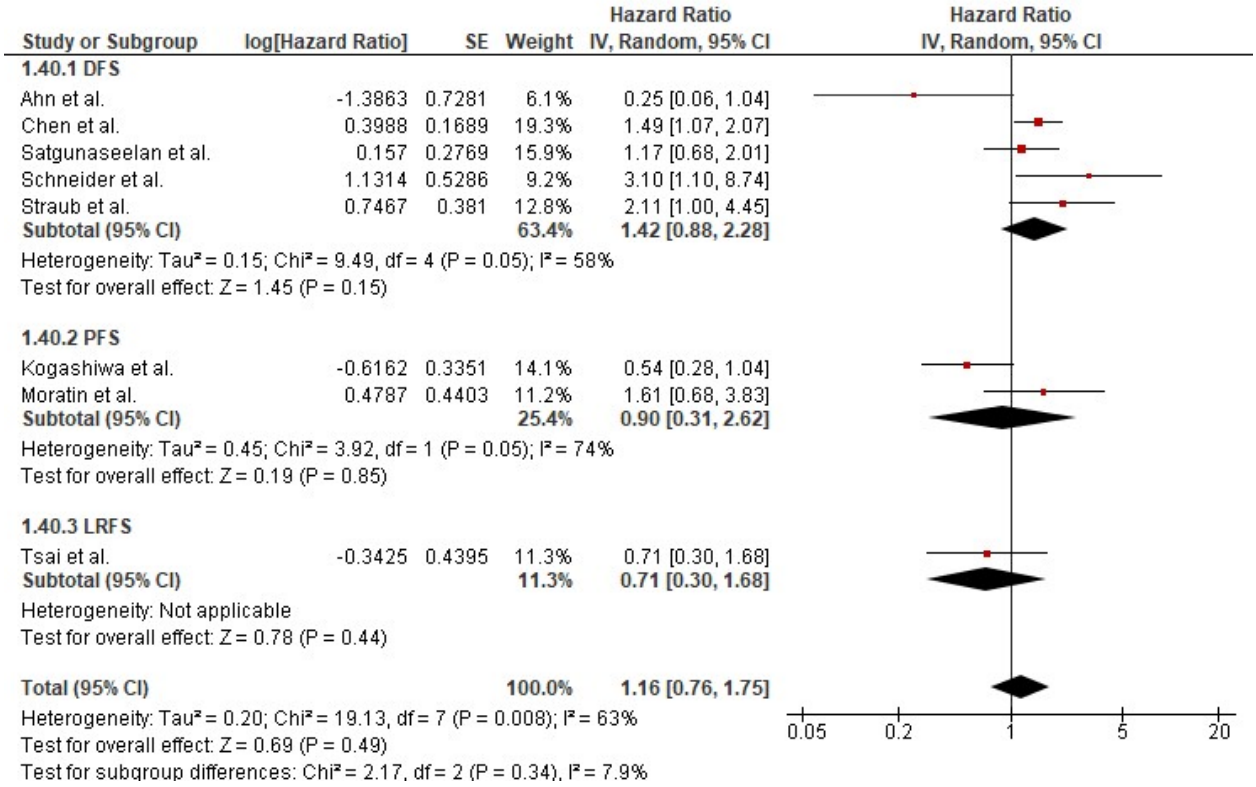


Figure 9. Forest plot for *Disease-free Survival (DFS)/Progression-free Survival(PFS)/Local-Regional Progression-free Survival (LRFS)*



Subgroup analyses

Subgroup analysis was carried out to determine sources of heterogeneity or confounding factors with the *a priori* determined variables: continent, tumour subsite, cut off value, antibody used and immunostaining pattern.

Continent/Anatomic subsite/Multivariate analysis

Continent sub-analysis was done for Asia vs. non-Asia as this gave the greatest number of studies to compare and this did not have any significant results. Only three studies had survival parameters specific to an anatomic subsite (OSCC of the tongue) [200,244,259] but these studies reported separate survival parameters and could not be combined. No multivariate studies were available for DFS. Four multivariate studies were available for OS and three for DSS and neither subgroup showed a statistically significant result **Figure 10.**

Cut-off value

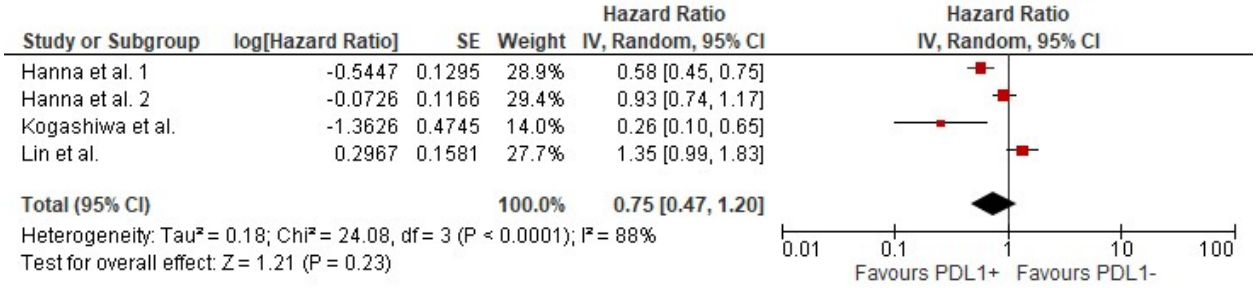
Survival parameters were sub-grouped by the cut-off percent of positive cells (1%, 5%, 10%) used for deciding if a tumour is overexpressing PD-L1. OS and DSS did not show any statistically significant results when sub-grouped by cut-off point. DFS showed a statistically significant result at a 5% cut-off (HR=1.56, 95% CI= 1.16-2.09, p=0.003) but this was not robust to sensitivity analysis nor was it statistically significant when DFS was combined with PFS/LRFS **Figure 11**.

Antibody

Only two antibodies were used in more than one study that reported the same outcome measurement (antibodies E1L3N & 5H1). 5H1 was used in two studies assessing OS and showed a worse prognosis (HR=2.63, 95% CI=1.15-6.02, p=0.02). E1L3N was used in two studies of DFS and did not produce a significant result. E1L3N was also used in four studies of DSS representing 543 patients and showed a statistically significant result for worse prognosis (HR=2.19, 95% CI= 1.41-3.39, p=0.0004) **Figure 12**.

Figure 10. Forest plot for multivariate analysis in OS and DFS

Overall Survival



Disease Specific Survival

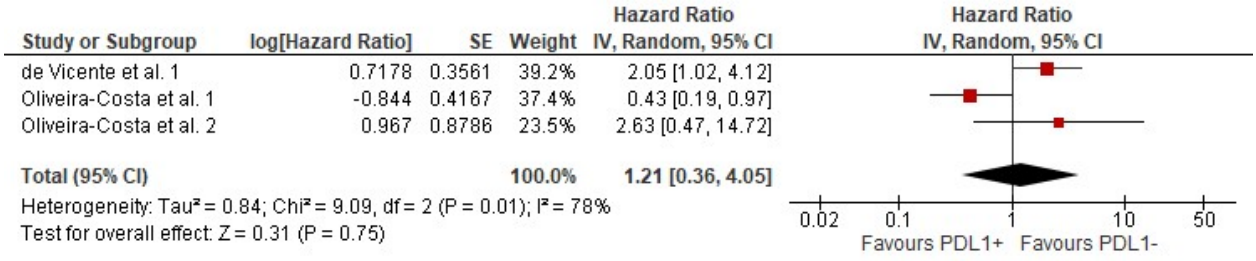


Figure 11. Forest Plot for Survival by PD-L1 cut-off point in Disease Free Survival

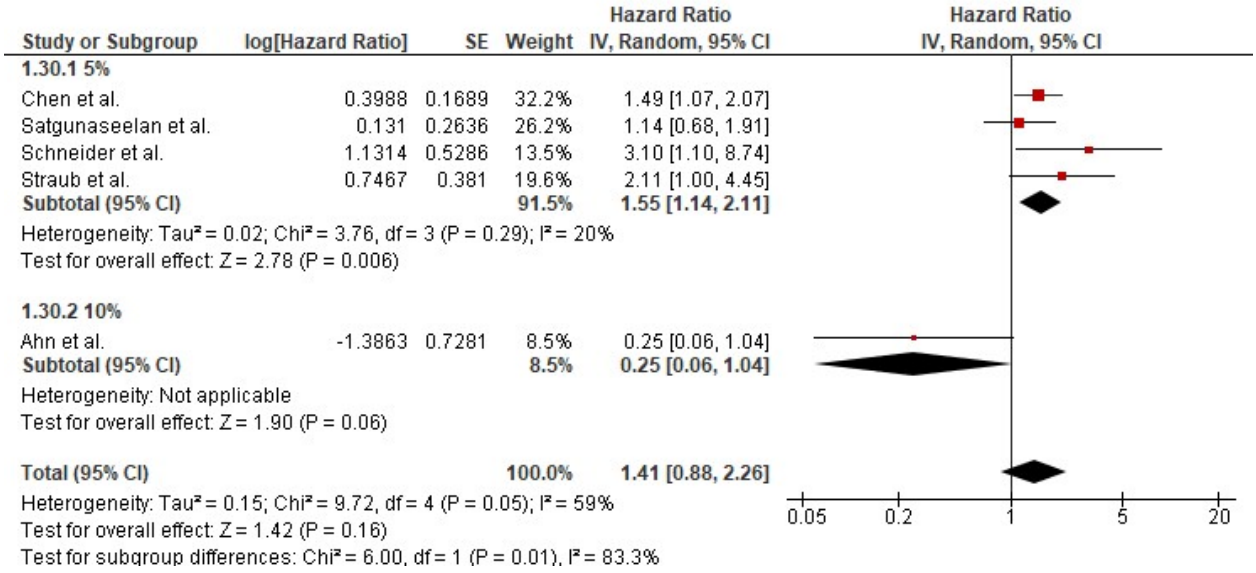
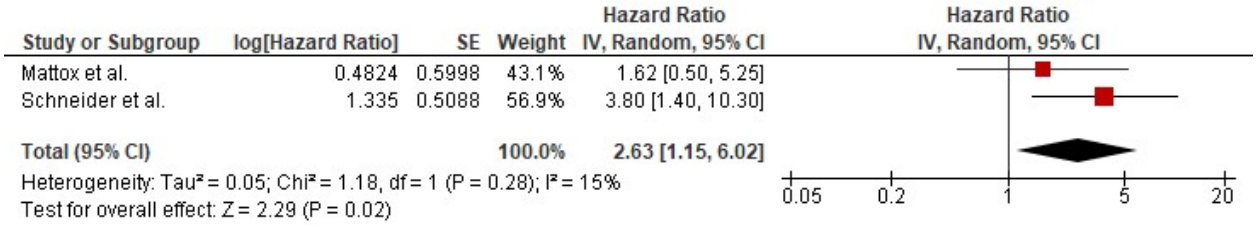
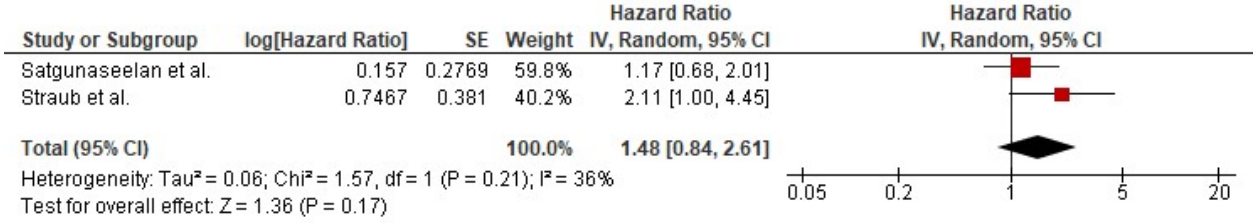


Figure 12. Forest Plot of Studies Grouped by Antibody Used

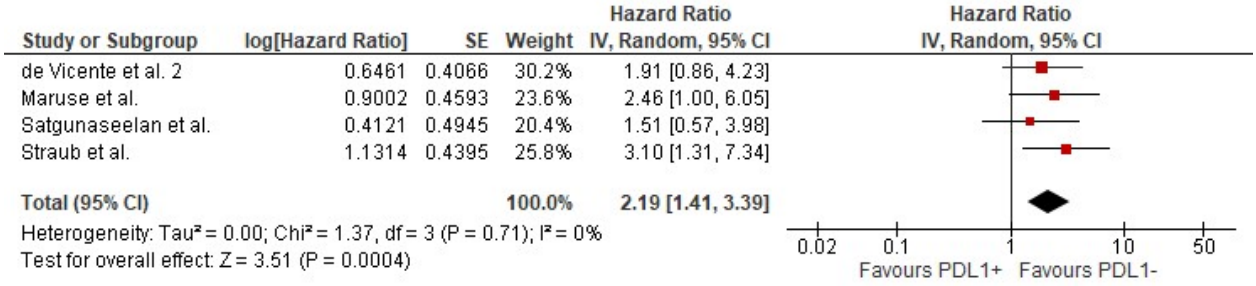
5H1 Antibody – Overall Survival



E1L3N – DFS



E1L3N – DSS

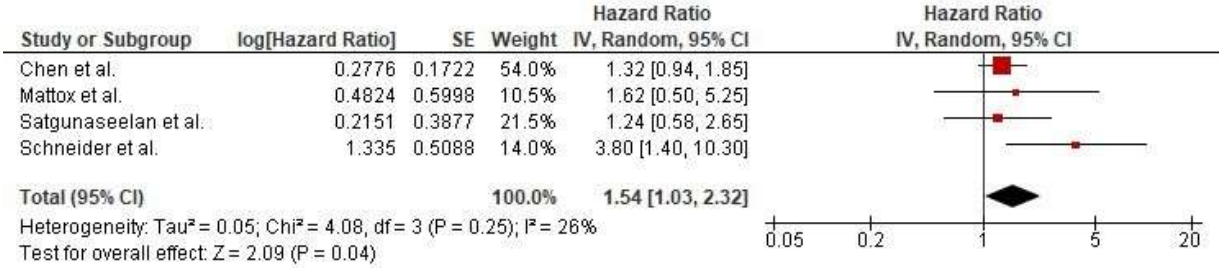


Staining Pattern

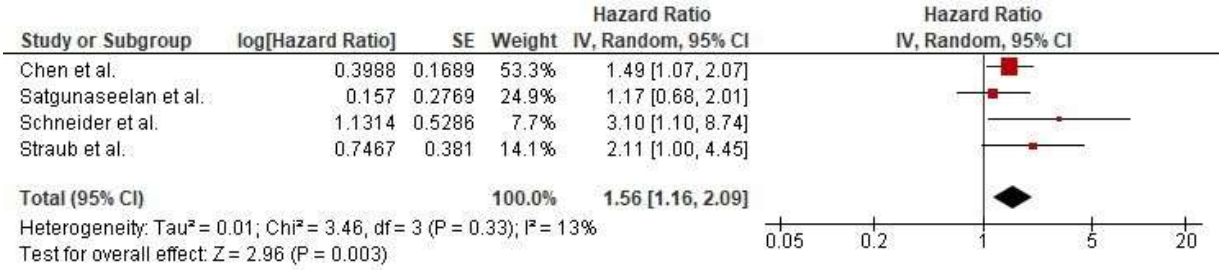
Studies used different definitions of PD-L1-positivity based on the location of staining. The methods used included: membranous-only staining (10 studies)[163,186,188,190,195,199,200,259,261,262], cytoplasmic or membranous staining (7 studies)[184,192,194,236,243,257,260], cytoplasmic or nuclear staining (2 studies)[195,244]. Some studies considered more than one staining pattern and eight studies did not specify their methodology[189,235,237,245,256,258,263,264]. Subgroup analysis of staining pattern and OS was not significant (HR=1.28, 95%CI=0.69-2.35, p=0.43) but after excluding small studies[190] (1 study with 23 patients) the result was significant (HR=1.54, 95% CI=1.03-2.32, p=0.04). DFS showed a worse prognosis when measuring membranous staining (HR=1.56, 95% CI=1.16-2.09, p=0.003). This was unchanged when PFS/LRFS was added as these studies either measured both cytoplasmic and membranous staining or did not state their methodology. The DSS subgroup was also significant when grouped by membranous staining (HR=1.74, 95% CI=1.14-2.66, p=0.01) **Figure 13**.

Figure 13. Forest plots for survival when measuring PD-L1 only in the cell membrane

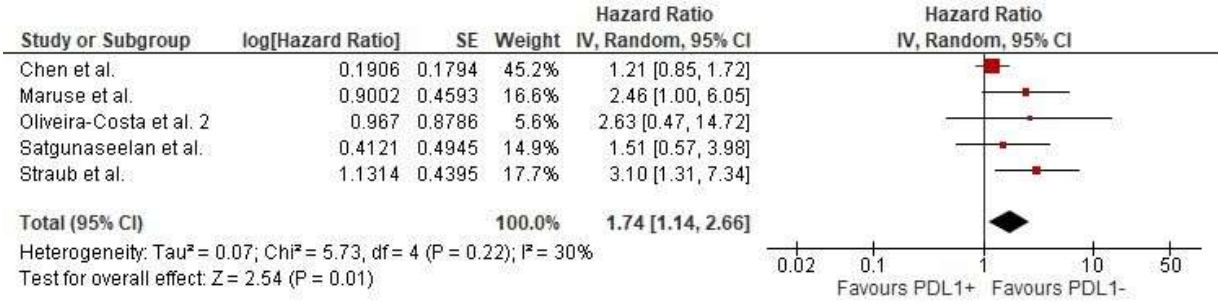
Overall Survival – Membrane only studies, excluding small studies (1 study with 23 patients)



Disease Free Survival – Membrane only studies



Disease Specific Survival - Membrane only studies

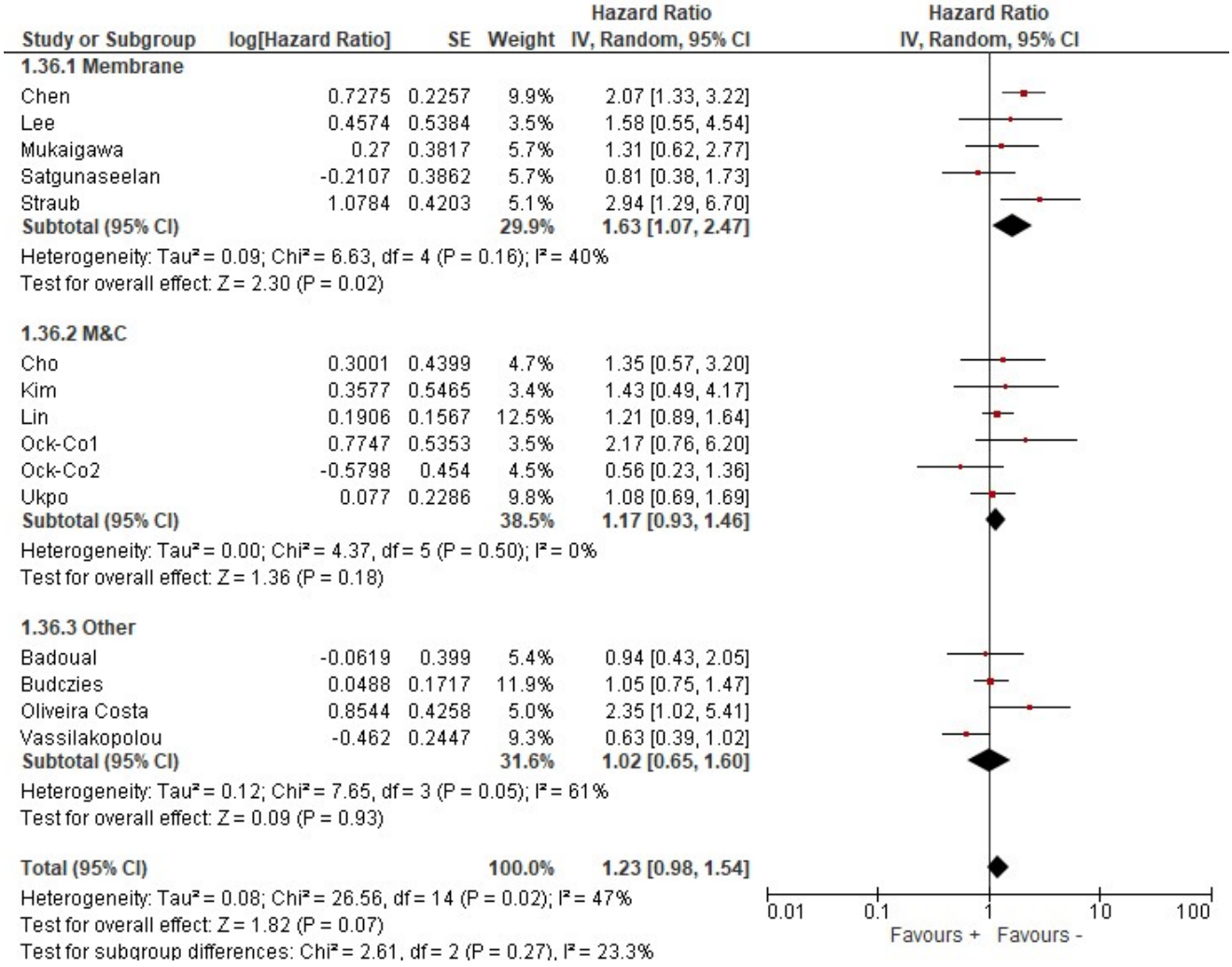


Clinicopathological associations with PD-L1 overexpression

No significant association was found between PD-L1 overexpression and the risk of recurrence, T status (T1/T2 vs T3/T4), M status, tumour grade, or age (>56, >60, >65).

Meta-analysis of PD-L1 overexpression in N0 patients and N+ patients (when PD-L1 is measured in the primary tumour and not the lymph node) included 15 studies with 1,707 patients. The result was close to significant for increased odds of being N+ if PD-L1 positive (OR=1.35, 95% CI=0.97-1.88, p=0.07). Heterogeneity was moderate (I^2=47%, p=0.02) and significance could be achieved by exclusion of small studies (exclusion of studies with <25 patients, OR=1.40, 95% CI=1.02-1.93, p=0.04; <50 patients, OR=1.51, 95% CI=1.08-2.11, p=0.02). The <50 patients sub-group includes 12 studies with 1,598 patients and remains significant when put to *leave-one-out* sensitivity analysis **Figure 14**.

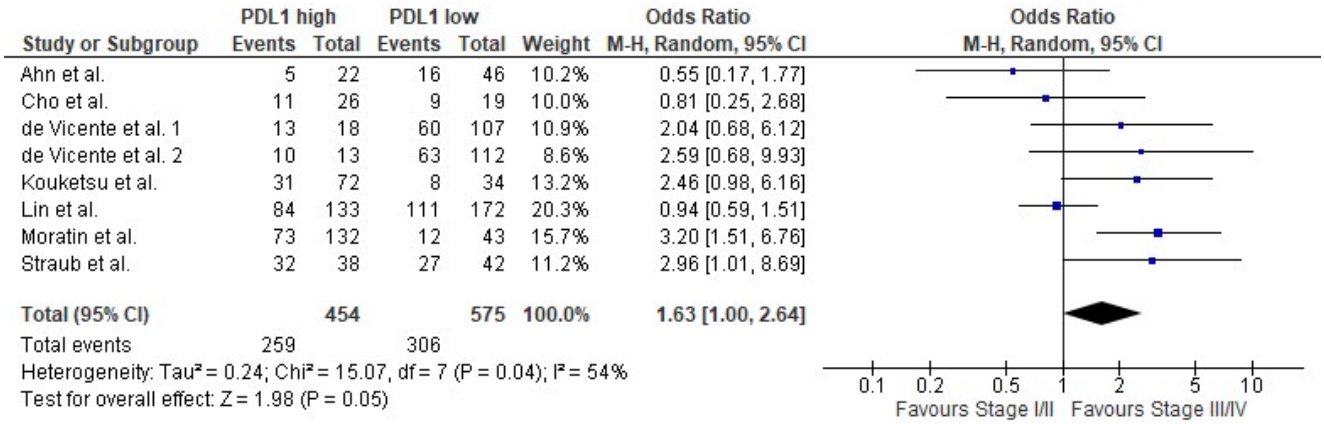
Figure 14. PD-L1 expression in N0 vs N+ (grouped by study size)



Stage

Meta-analysis of cancer stage was carried out including 7 studies with 1,029 patients using data comparing PD-L1 expression in stage I/II versus stage III/IV. A significant association was found between PD-L1 overexpression and advanced stage OSCC (OR= 1.63, 95% CI=1.00-2.64, p=0.05). Heterogeneity was significant (I^2 =54%, p=0.04) and the significance was not robust to *leave-one-out* sensitivity analysis **Figure 15**.

Figure 15. PD-L1 expression in Stage I/II vs Stage III/IV (event)



Gender

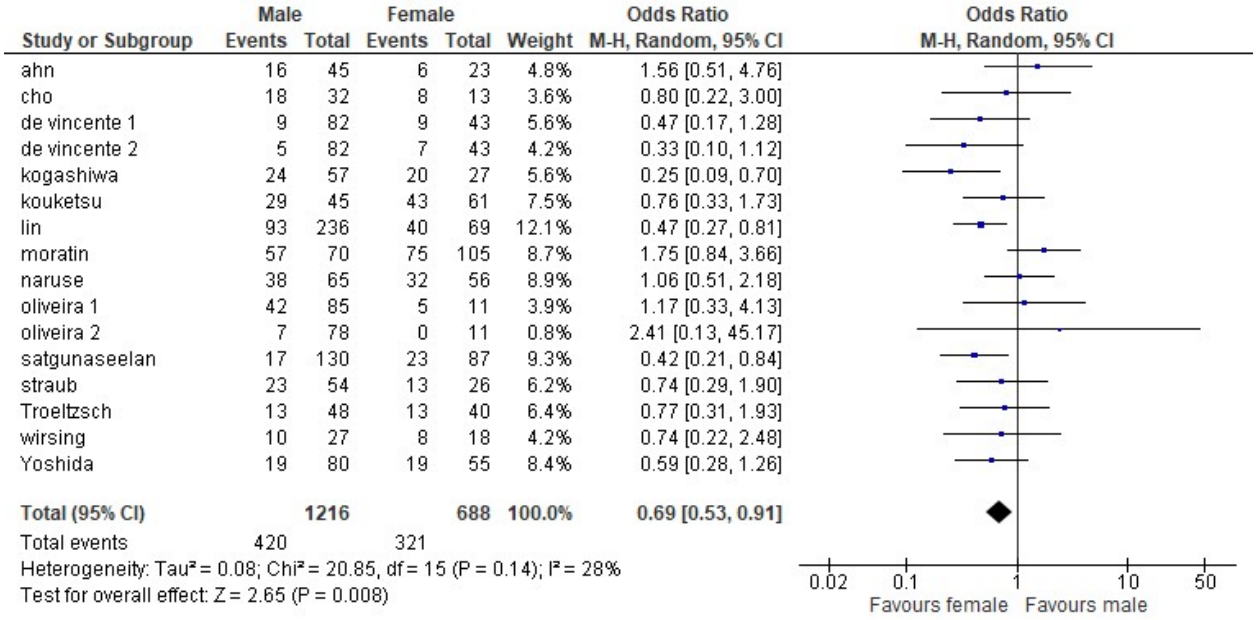
PD-L1 overexpression by gender was assessed in 14 studies of 1,683 patients. Meta-analysis showed an increased incidence of high PD-L1 overexpression in female patients (OR=0.69, 95% CI=0.53-0.91, p=0.008) **Figure 16**.

Smoking and Alcohol

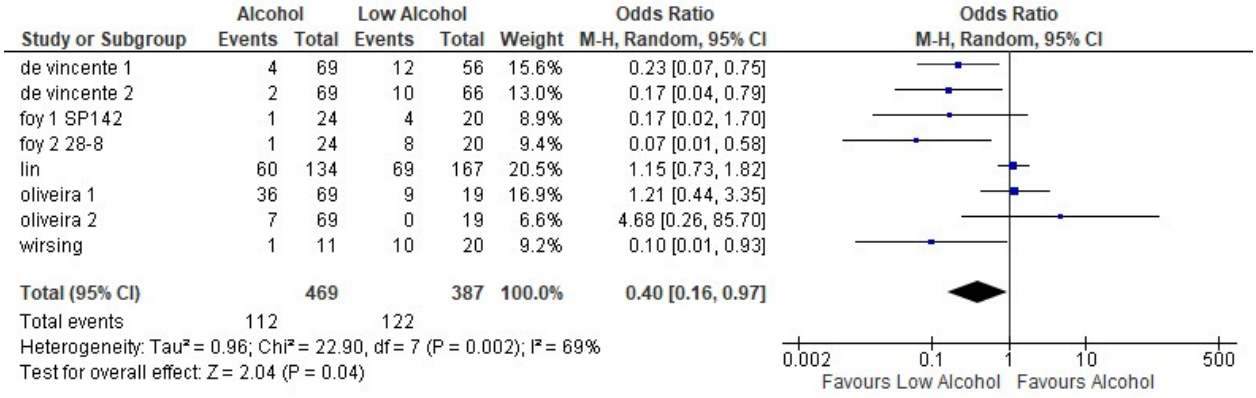
Six studies assessed PD-L1 overexpression in smokers compared to non-smokers in 676 patients. Meta-analysis showed increased PD-L1 overexpression in non-smokers (OR=0.45, 95% CI=0.27-0.75, p=0.002). This was not robust to *leave-one-out* sensitivity analysis. Five studies assessed PD-L1 overexpression in alcohol drinkers versus non-drinkers in 591 patients. A significant result was found with higher PD-L1 overexpression in non-drinkers (OR=0.40, 95% CI=0.16-0.97, p=0.04). Heterogeneity was significant (I^2=69%, p=0.002) and the significance was not robust to sensitivity analysis **Figure 16**.

Figure 16. Correlation between gender, alcohol and tobacco and PD-L1 expression

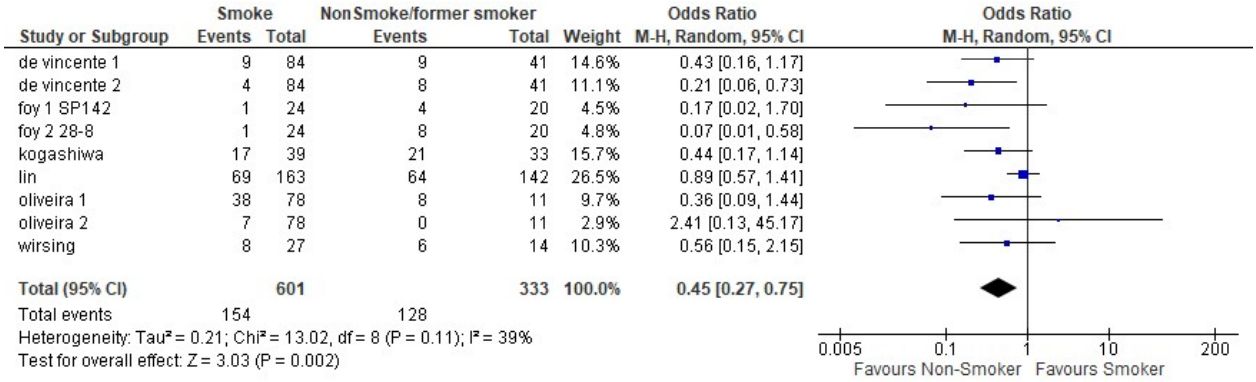
Gender



Alcohol



Smoking



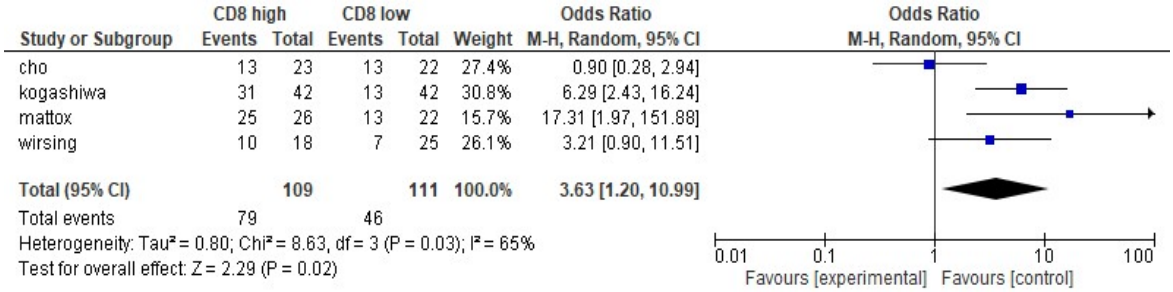
Tumour Infiltrating Lymphocytes (CD8, CD4, PD-1)

A positive correlation was found between PD-L1 overexpression and CD8, CD4 and PD-1 expression in the tumour microenvironment. Four studies assessed PD-L1 overexpression and CD8 levels in 220 patients and found a positive association between PD-L1 overexpression and a high CD8 count (OR=3.63, 95% CI=1.20-10.99, p=0.02). Three studies with 132 patients assessed PD-L1 levels and CD4 levels and again found a positive association between PD-L1 overexpression and a high CD4 count (OR=3.25, 95% CI=1.36-7.76, p=0.008). Finally, two studies assessed PD-1 overexpression in relation to PD-L1 in 194 patients. A significant association was found between PD-1 overexpression and PD-L1 overexpression. (OR=33.36, 95% CI=1.88-591.69, p=0.02) **Figure 17**.

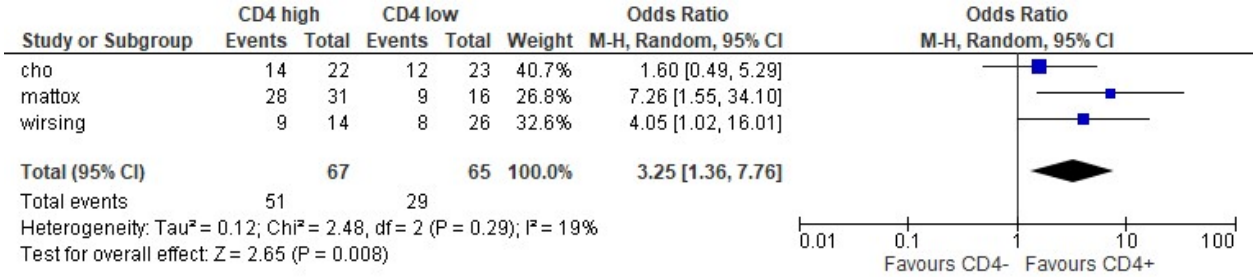
For all meta-analyses funnel plots were made and Egger's test was carried out. None showed evidence of publication bias **Annex 1**.

Figure 17. PD-L1 expression and tumour infiltrating lymphocytes (Event is PD-L1 overexpression)

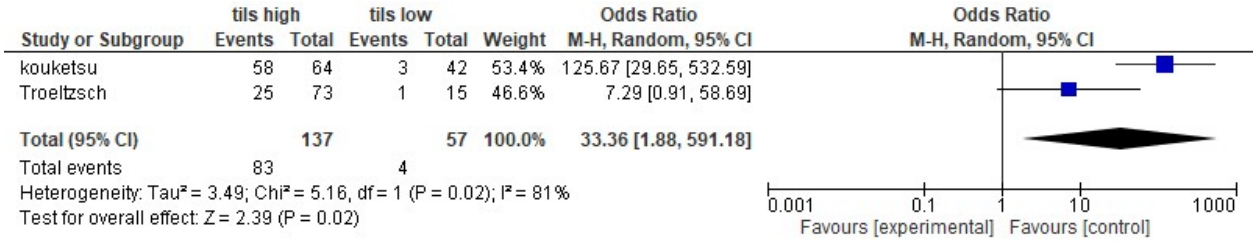
Tils – CD8



Tils – CD4



Tils- PD-1



PD-L1 prevalence in OSCC

To investigate sources of heterogeneity a meta-analysis of the proportion of PD-L1 positive cases in each study was undertaken. This showed that PD-L1 positive cases ranged from 7% to 87% with confidence intervals ranging from 1% to 98%, **Figure 18.**

Five studies used different PD-L1 scoring methods on the same patients. This allowed us to assess the effect of each method on the proportion of PD-L1 positive patients as summarized in **Table 9.** The change in the proportion of PD-L1 positive cases is expressed as a percent difference between the two methods. For example, using different anti-PD-L1 antibodies in the same tumours led to an increase in the proportion of PD-L1 cases by 11% in Foy et al.[256] and 5% in de Vicente et al.[263]. Two studies assessed PD-L1 staining by intensity. The number of PD-L1 positive cases increased by 13% in the Straub et al. study[163] and by 34% in the Ahn et al. study[257] when staining at any intensity was counted (and not just the moderately or strongly staining cases). By measuring PD-L1 staining in the cytoplasm and not the cell membrane Oliveira-Costa et al.[195] increased the proportion of PD-L1 expressing patients by 42%.

Tissue Microarrarys (TMA) may also be a source of heterogeneity. PD-L1 has a non-homogenous staining pattern within a tumour and the selection of a small area of the tumour may lead to both false negatives and false positives. Three studies assessed the validity of their TMA studies by repeating staining in a subset of whole tumour sections [188,259,262]. Yoshida reported nearly 9% of cases to be incorrect superficially but had 100% agreement in the deep tissue of the tumour. Schneider et al. found a 10% false positive rate. Satgunaseelan et al. reported 5 of 14 cases to be false positives and 4 of 16 cases to be false negatives.

Figure 18. Meta-analysis of the proportion of PD-L1 positive cases per study

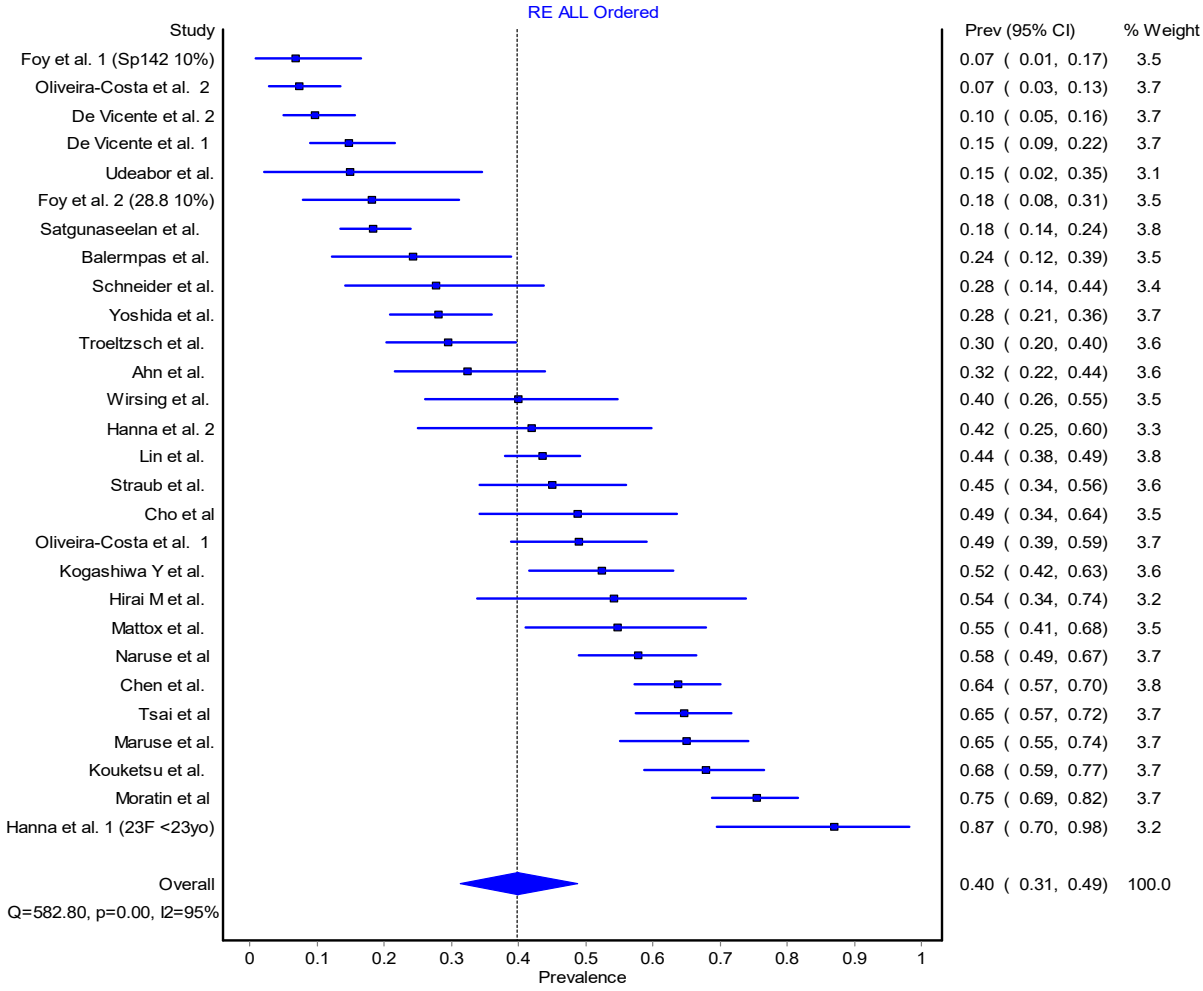


Table 9. – A comparison of IHC methods and the percent change in PD-L1 positive cases that they cause

Method 1	%	Method 2	%	Percent Difference %
Antibody effect				
Antibody 22C3 – de Vicente et al. (10%)	15	Antibody E1L3N – de Vicente et al. (10%)	10	**-5**
Antibody 28.8 – Foy et al. (10% cut-off)	18	Antibody SP142 – Foy at al. (10% cut-off)	7	**-11**
Intensity Effect				
Straub et al. 1+,2+,3+ intensities	45	Straub et al. 2+,3+ intensities	32	**-13**
Ahn et al. 1+,2+ intensities	66	Ahn et al. 2+ intensity	32	**-34**
Cut-off effect				
Antibody E1L3N – de Vicente et al. (1%)	24	Antibody E1L3N – de Vicente et al. (10%)	10	**-14**
Antibody 22C3 – de Vicente et al. (1%)	36	Antibody 22C3 – de Vicente et al. (10%)	15	**-21**
Antibody SP142 – Foy at al. (1% cut-off)	23	Antibody SP142 – Foy at al. (10% cut-off)	7	**-16**
Antibody 28.8 – Foy et al. (1% cut-off)	48	Antibody 28.8 – Foy et al. (10% cut-off)	18	**-30**
Cytoplasmic vs. Membranous staining effect				
Oliveira-Costa et al. Cytoplasmic staining	49	Oliveira-Costa et al. Membranous staining	7	**-42**

II. Immunohistochemistry study

We assessed 55 OSCCs from 55 patients, of which 32 (58%) expressed PD-L1 at a 5 % cut-off (**Figure 19**). We compared PD-L1 expression between clinical and histopathological variables (**Table 10)**. The study included 14 females and 41 males. No significant difference was seen between genders for TPS (Mann-Whitney, p=0.79) or at a 5% cut-off point (Chi-squared, p=0.93). The study included 9 patients with multiple tumours. The patients with multiple tumours were not more likely to express PD-L1 (Fisher's exact test, p=0.14) or have a higher TPS (Mann-Whitney U, p=0.30). Tobacco consumption information was available for 32 patients. The TPS was significantly higher in non-smokers and ex-smokers compared to smokers (Mann-Whitney U, p=0.006), but not significant when grouped at a cut off of 5% PD-L1 expression (Fisher's exact test, p=0.14). There was a significant correlation between female gender and non-smoker/ex-smoker status, and this may represent a confounding factor (Fisher's exact test, p=0.03). Alcohol consumption information was available for 23 patients. A higher TPS was seen in non or previous drinkers *vs* in those who were active drinkers (Mann-Whitney U, p=0.047), but when grouped at a 5% cut-off, no significant difference was seen (Fisher's exact test, p=0.21). Alcohol and Tobacco consumption were significantly correlated. In this study all drinkers were smokers, and most smokers also drank (Fisher's, p=0.01). Full information was available for 15 patients for both alcohol and tobacco habits. A higher TPS was seen in those who neither smoked nor drank (never or previous users) compared to those who actively both smoked and drank (Mann-Whitney U, p=0.02), and when grouped at a 5% cut-off non/ex-smoker and drinkers were significant more likely to be PD-L1 positive (Fisher's exact test, p=0.03).

OSCCs of the tongue showed higher PD-L1 expression than all other locations grouped together, both by TPS (Mann-Whitney U, p=0.04) and by proportion, at a 5% cut-off (Chi-squared, p=0.04). Gender and smoking status were also significantly related to location. Tumours of the tongue were more common in women (13/14 women had tongue OSCCs, Fisher's exact test p=0.004), as well as being more common in non-smokers (Fisher's exact test, p=0.02).

Neither T, N or M status showed any significant association with PD-L1 proportion or TPS, nor did stage, grade, differentiation, clinical appearance or the presence of multiple tumours (p-values can be found in **Table 10**).

PD-L1 expression in tumour cells was higher when the adjacent epithelium expressed PD-L1, both by proportion and TPS (Fisher's exact test p=0.001, Mann-Whitney U p=0.001). PD-L1 expression in the adjacent epithelium was also found to be higher in non/ex-smokers compared to smokers (Fisher's exact test, p=0.02) but was not found to be associated with the number of tumours developed nor any other clinicopathological characteristics. Distant epithelium was present in 10 tumours and was only positive for PD-L1 in 1 tumour, and this failed to show any significant associations.

A high intensity of tumour infiltrating lymphocytes (TILs) was associated with PD-L1 positive tumours, and with an increased TPS (Chi-squared, p=0.04; Mann-Whitney U, p=0.01). TILs were more likely to express PD-L1 if tumour cells also expressed PD-L1 (Fisher's, p=0.03). A constitutive staining pattern was present in 19 patients and an induced staining pattern was seen in 13 patients. This failed to correlate with any clinicopathological characteristics or survival benefit.

Patients follow-up ranged from 2-132 months (median 56 months) with 14 cancer specific deaths. Tumour PD-L1 expression at a 5% cut-point was not significant for disease-specific survival (DSS) (Univariate analysis, Log-rank test, p=0.36) (**Figure 20A**). A significantly worse DSS was found for N+ status (N0 vs N+, p=0.03) and for not having surgical treatment (Surgery vs No Surgery, p=0.001). A worse DSS prognosis was seen as the level of TILs increased but failed to reach the level of significance (Mild vs moderate vs intense, p=0.13; Mild and moderate vs intense, p=0.06) (**Figure 20B**).

Disease free survival (DFS) was only significant for lack of surgical treatment (p=0.006). Tumour PD-L1 staining was not significant for DFS (p=0.44) or OS (29 patients, p=0.45).

Cox regression survival analysis was undertaken for PD-L1, as well as any variables with a p-value <0.1 from the Kaplan-Meier analysis. Neither univariate or multivariate analysis showed PD-L1 to be significant for DSS, DFS or OS (**Table 11**). Having not had surgery and a positive N status were both significant for a shorter DSS in univariate analysis but not in multivariate analysis. Only not having surgery was significant for a shorter DFS in univariate and multivariate analyses. No factor was significant for OS.

Figure 19. (a) PD-L1 positive tumour with PD-L1 staining in tumour cells and in the epithelium adjacent to the invading tumour. 5×. (b) Tumour PD-L1 constitutive staining with intense TIL infiltrate and PD-L1 negative adjacent epithelium. 100×. (c) PD-L1 positive tumour with constitutive staining pattern. 200×. (d) PD-L1 positive tumour with induced staining pattern. 200×. (e) PD-L1 positive tumour with intense immune infiltrate. 100×. (f) PD-L1 negative tumour with mild immune infiltrate. 200×

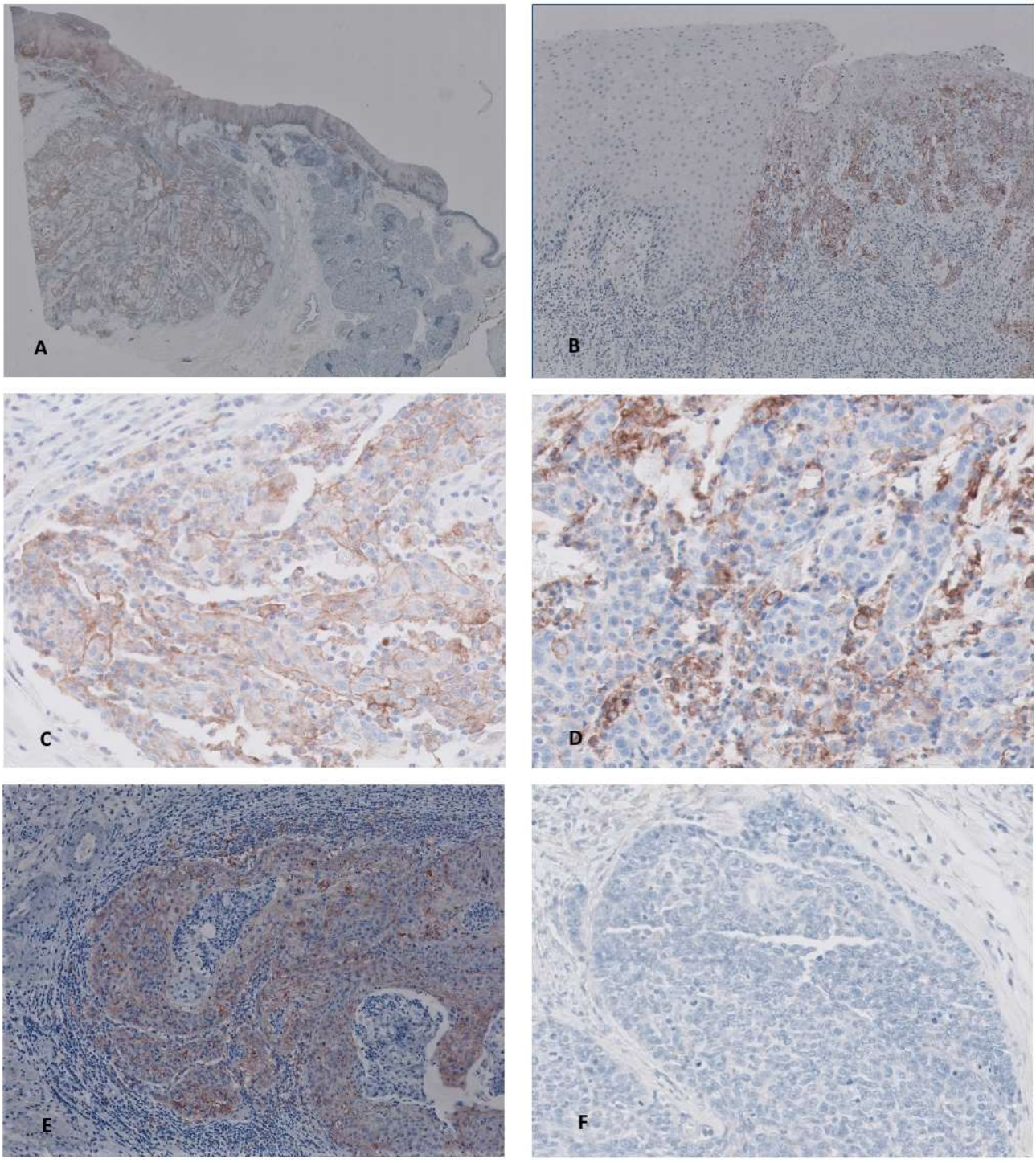


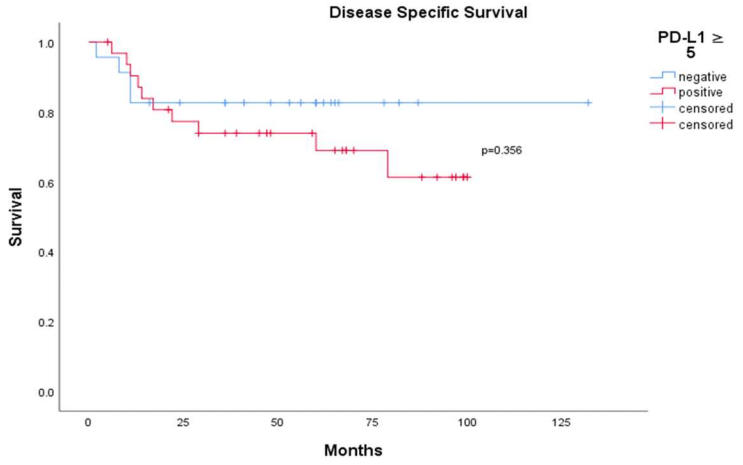
Table 10 – Clinicopathological variables by PD-L1 level

		PD-L1 TPS < 5% (n)	PD-L1 TPS ≥ 5% (n)	p-value *	Mean TPS (SD)	p-value **
Gender	Female	6	8	0.93	20 (21)	0.79
	Male	17	24		19 (25)	
Number of tumours	1 tumour	17	29	0.14	22 (25)	0.30
	Multiple	6	3		9 (16)	
Smoking status	Non or Ex-Smoker	3	8	0.14	27 (28)	0.006***
	Smoker	13	8		5 (9)	
Alcohol	Non or Ex-Drinker	5	7	0.21	18 (26)	0.047
	Drinker	8	3		6 (15)	
Alcohol and Smoking	Non-Smoker & Non-Drinker	3	5	0.026***	23 (31)	0.009***
	Smoker & Drinker	7	0		0 (0)	
Location	Non-Tongue[A]	10	7	0.036***	12 (22)	0.038***
	Tongue	9	23		24 (24)	
Clinical Appearance	Ulcerated	15	21	0.59	22 (25)	0.42
	Not Ulcerated	7	7		13 (21)	
T status	T1	8	10	0.076	20 (25)	0.329
	T2	5	17		24 (25)	
	T3	2	0		0 (0)	
	T4	6	5		16 (25)	
N status	N0	14	17	0.23	22 (27)	0.66
	N+	6	15		19 (20)	
M status	M0	19	26	1.00	20 (25)	0.261
	M1	0	1		54	
Stage	I	8	7	0.37	16 (25)	0.55
	II	2	8		32 (30)	
	III	3	3		12 (19)	
	IV	8	14		20 (22)	
Grade of differentiation	Well or Moderate	18	22	0.44	21 (26)	0.58
	Poor	5	10		16 (19)	
PD-L1 in adjacent epithelium	PD-L1 in epithelium	3	17	0.001***	31 (26)	0.001***
	No PD-L1 in epithelium	18	11		13 (21)	
TILs infiltrate	Intense	9	22	0.029***	27 (27)	0.013***
	Mild/Moderate	14	10		10 (16)	
TILs PD-L1 staining	High	0	7	0.034***	33 (30)	0.063
	Low	23	25		18 (23)	

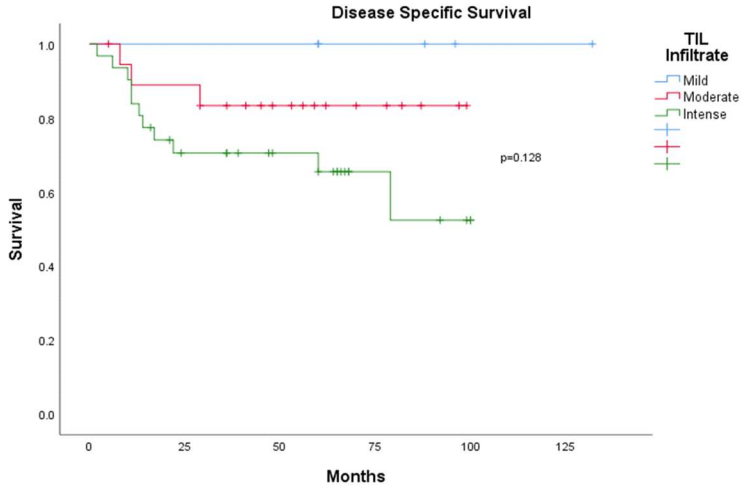
*Chi-squared test if all rows are greater than or equal to 5, if not Fisher's exact test is applied, **Mann-Whitney U test for two variables or Kruskal-Wallis H test for three or more variables, Full information for all variables was not available for all samples, ***p-value < 0.05; [A]Non-Tongue tumours included 11 floor of mouth, 3 buccal mucosa, 1 retromolar trigone, and 1 hard palate. Six tumours overlapped between tongue and floor of mouth and were excluded from analysis (when these 6 were included with tongue tumours the result was still significant.)

Figure 20 - Kaplan Meier curves for disease-specific survival. No significant difference in survival was found between (a) PD-L1 positive and negative tumours, (b) mild, moderate or intense tumour infiltrating lymphocytes or (c) mild and moderate versus intense tumour infiltrating lymphocytes

A) PD-L1 ≥ 5%



B) TIL Intensity mild vs moderate vs intense



C) TIL Intensity mild or moderate vs intense

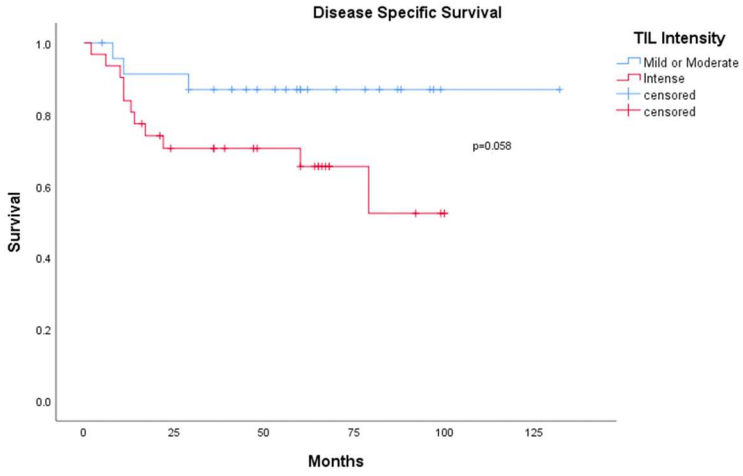


Table 11 - Univariate Cox Analysis

Disease Specific Survival				
Parameter	**Categories**	**HR**	**95% CI**	**p-value**
PD-L1	≥ 5%/ <5%	1.71	0.54-5.48	0.364
Surgery	No Surgery/Surgery	7.39	1.95-28.05	0.014
N status	N+/N0	3.25	1.08-9.77	0.036
TILs	Intense/Mild & Moderate	3.22	0.89-11.57	0.074
Disease Free Survival				
Parameter	**Categories**	**HR**	**95% CI**	**p-value**
PD-L1	≥ 5%/ <5%	1.15	0.48-2.73	0.756
Surgery	No Surgery/Surgery	6.91	1.85-25.8	0.004
Overall Survival (29 samples)				
Parameter	**Categories**	**HR**	**95% CI**	**p-value**
PD-L1	≥ 5%/ <5%	0.58	0.14-2.45	0.459

Discussion

I. Discussion of the Systemic review and meta-analysis

Our meta-analysis has identifying interesting correlations which can guide future studies and understand which patients may be likely to express PD-L1. As the biological function of PD-L1 protein comes from its action in the membrane it would be logical for studies to consider the presence of PD-L1 exclusively in the cell membrane. As PD-L1 enhances immune evasion, its presence would be expected to confer a negative prognosis. In this sense, our meta-analysis considering the expression of PD-L1 exclusively in the cell membrane has shown a significant relationship between PD-L1 overexpression and disease-free survival (HR=1.56, 95% CI=1.16-2.09, p=0.003), disease-specific survival (HR=1.74, 95% CI=1.14-2.66, p=0.01) and overall survival (HR=1.54, 95% CI=1.03-2.32, p=0.04). The importance of cell membrane PD-L1 is reinforced by the lack of prognostic influence using combined PD-L1 expression – in the membrane and cytoplasm – on disease free survival (DFS) (HR= 1.42, 95% CI=0.88-2.28, p=0.15), OS (HR=1.00, 95% CI=0.75-1.35, p=0.98), and the combined progression-free survival (PFS)/local-regional progression free survival (LRFS)/DFS (HR=1.16 95% CI=0.76-1.75, p=0.49). Therefore, our results indicate that the evaluation of PD-L1 expression, for the purpose of prognostic assessment, should be limited to membranous expression of the protein. Further investigations are required to clarify the function of PD-L1 in the cytoplasm, as there are examples of important oncogenic proteins with different biological functions depending on its location, such as β-catenin. Our meta-analysis also reveals other interesting associations with important clinicopathological features. A significant correlation was found with advanced stage OSCCs (OR= 1.63, 95% CI=1.00-2.64, p=0.05) as well as a close to significant correlation with N+ status (OR=1.35, 95% CI=0.97-1.88, p=0.07). In this last case, when a small study with only 24 patients is removed, we observe a positive association with PD-L1 expression and N+ status (HR=1.40, 95% CI=1.02-1.93, p=0.04). Taken together, all the previous results seem to identify PD-L1 overexpression in OSCC as a negative prognostic marker.

We have also identified both non-smokers and non-drinkers to be more likely to have high levels of PD-L1 expression (OR=0.45, 95% CI=0.27-0.75, p=0.05 and OR=0.40, 95% CI=0.16-0.97, p=0.04, respectively). This is of interest as tobacco and alcohol are the main risk factors for developing OSCC. Foy et al.[256] has shown that OSCCs in patients who neither smoke nor drink have enrichment of pathways inducing T-cell activation, interferon-gamma signalling and PD-1 signalling, as well as increased CD8 T-cell infiltration in the tumour microenvironment. These results indicate that OSCCs that have developed in patients who do not smoke or drink could represent a singular subgroup with an increase in both the expression of PD-L1 and in the ability to evade the immune response. This is relevant as it has been reported that anti-PD-1/PD-L1 therapy may have a higher clinical benefit in OSCC patients who do not smoke or drink[256], and this is reasonable as therapy targeting a specific pathway should be more effective in tumours with that pathway activated. Our results also show an increased expression of PD-L1 in women (OR=0.69, 95% CI=0.53-0.91, p=0.008). This association is poorly understood but it has been reported that the expression and function of PD-L1 can be upregulated by oestrogen[265–267]. We have also found an association between PD-L1 overexpression with a high tumour infiltrating lymphocytes count, as measured by CD4 (OR=3.25, 95% CI=1.36-7.76, p=0.008), CD8 (OR=3.63, 95% CI=1.20-10.99, p=0.02) and PD-1 (OR=33.36, 95% CI=1.88-591.69, p=0.02). This correlation may be explained by the capacity of tumour infiltrating lymphocytes to release factors, like interferon-gamma, which can induce the expression of PD-L1 in cancer cells. This finding represents an example of how neoplastic cells can exploit the immune response for their own benefit. There is only one meta-analysis by Troiano et al.[202] of the prognostic value of PD-L1 in OSCCs which did not find any significant result, except the association of PD-L1 with female gender. Nonetheless, we should highlight

that our search-strategy and inclusion criteria allowed us to include more than double the number of patients and studies than the meta-analysis by Troiano et al. [202] (26 studies/2,532 patients *vs* 11 studies/ 1,060 patients), making, in our opinion, our results more robust.

To evaluate the quality of the included studies we used the QUIPS tool, which showed that not all studies were conducted with the same rigor. The "Statistical Analysis and Reporting" section showed high and moderate biases in several studies as they had selective reporting of survival parameters. The largest source of bias was found in "prognostic factor measurement" with studies lacking a clear or reliable methodology. As a result, our proportion meta-analysis showed a wide spread of patients classified as having PD-L1 positive OSCCs (7%-87%) **Figure 18**. Furthermore, we identified four aspects of IHC (cut-off point, antibody choice, staining intensity, and staining location) that influenced the percentage of cases considered positive by between 5% and 42% **Table 9.** We therefore believe that future studies measuring PD-L1 should use clear and reproducible methods and present their data in such a way that it can be compared to the available results in the literature. The choice of a cut-off point is arbitrary but most studies set a cut-off of ≥5% of tumour cells (20 of the 26 studies). Data used to evaluate prognostic influences can also be presented at different cut-off points, as was done by Foy et al.[256], or as individual patient data as was done by Straub et al.[163]. In the literature, there is no gold standard for the choice of a specific antibody for IHC although the majority of studies (15 of 26) used one of the following: 22C3, 28-8, E1L3N, SP142, SP263, 5H1. Some of these antibodies (22C3, 28-8, SP142) are also FDA approved as diagnostic tests in the prescription of anti-PD-L1/PD-1 treatment, and therefore have a clinical application[268].

We have identified potential limitations in our study. First, we only searched databases favouring articles written in English, which may have led to missing publications in other languages. However, all non-English articles that were retrieved were excluded for reasons other than their publication language. Second, as previously mentioned, high levels of heterogeneity were present in the assessed studies. We accounted for this using a random effects model in all meta-analyses. Third, we consider that the use of tissue microarrays (TMAs) in 11 of the 26 studies may represent a limitation as the expression of PD-L1 within tumours is heterogeneous and may generate false-positive or false-negative results. Therefore, measuring PD-L1 in whole tissue sections may be more appropriate.

II. Discussion of the Immunohistochemistry study

Our study of PD-L1 expression in 55 patients with OSCC showed 58% had positive tumours. For our analysis, we used a cut-off of 5% of tumour cells staining as this was the most frequently used in the literature[253]. The choice of 5% is otherwise arbitrary and so we also analysed PD-L1 expression as a continuous variable using a tumour proportion score (TPS). TPS is also the scoring system used to decide whether a patient is a candidate for treatment with pembrolizumab (an anti-PD-1 antibody) in recurrent/metastatic HNSCC[218]. An alternative scoring system known as Combined Positive Score (CPS) measures the proportion of PD-L1 in both tumour and immune cells. It has been used to predict response to pembrolizumab when its level is above 1%[218]. The scoring used in this study was TPS as this was the system most commonly used at the start of the study and that would make our results easiest to compare to previous investigations. As both TIL and tumour PD-L1 staining were highly correlated, it is unlikely that the choice between these two systems would have a large effect on the results.

In agreement with our systematic review, we found TPS was significantly higher in both non-smokers and ex-smokers compared to smokers (mean TPS 27% *vs 5%,* p=0.02*)* as well as non-drinkers or ex-drinkers compared to heavy drinkers (18% *vs* 6%, p=0.047). Analysing the combined use of both alcohol and tobacco showed an even greater difference in those who neither smoked nor drank compared to smoker-drinkers (23% *vs* 0%, p=0.009). This suggests that the combined effect of alcohol and tobacco has a greater ability to limit PD-L1 expression then either habit on its own. The mechanism by which tobacco or alcohol may alter PD-L1 expression is not clear. Foy et al.[256] studied PD-L1 expression in HPV-negative OSCCs and found a higher expression in non-smoker/drinkers compared to smoker/drinkers. They also showed an increased activation of pathways involving T-cell activation and interferon-γ (IFN-γ) response[256]. Similarly, in HNSCC, Mandal et al. found that smoking (as measured by a mutational signature and not self-reported tobacco use) was associated with a lower T-cell infiltrate and lower IFN-γ signalling[269]. De la Iglesia et al.[270] studied HSNCC and also found lower levels of PD-L1 positive tumour cells in active smokers, as well as reduced IFN-γ and IFN-α pathways. As IFN-γ is a potent inducer of PD-L1[271] this may explain the increase in PD-L1 in non-smokers with HNSCC. It is worth noting, PD-L1 expression is higher in non-smokers in almost every study of PD-L1 in OSCC[184,194,195,256,263,272], while the opposite relation has been seen in other tumours, namely lung SCC (LUSCC). A systemic review has shown higher PD-L1 levels in current smokers with LUSCC as well as a better response to anti-PD-1 treatments[273]. Wang et al.[274] showed that cigarette smoke can induce PD-L1 in lung epithelium both *in vitro* and *in vivo*. Desrichard et al[275]. studied the contradictory relation between smoking and PD-L1 expression in HNSCC and LUSCC. In both, they found a higher mutational load in smokers *vs* non-smokers, but HNSCC-smokers had lower levels of immune infiltrate and IFN-γ signalling compared to HNSCC non-smokers. The opposite was found in LUSCC-smokers, which showed higher levels of immune infiltration and IFN-γ signalling. This may also be relevant for treatment as LUSCC-smokers seem to have better responses to PD-1 targeted treatments than non-smokers. Again the inverse is true in HNSCC, where it is the non-smokers that seem to have a better response to anti-PD-1 treatment[275].

The relation of PD-L1 expression and tobacco-associated protein expression is less clear. For example, P53 expression is frequently altered in OSCC, and has been associated with tobacco use[276,277]. However, overexpression of p53 has been found to be significantly related to PD-L1 expression in OSCC in one study which did not provided any information on tobacco use[278]. As p53 mutations are common in OSCC, further research into the relation with PD-L1 expression is justified.

In our previous meta-analysis, PD-L1 positive OSCC tumours have been shown to occur more often in females with an odds ratio of 1.44[253]. While, this study failed to find a significant difference in PD-L1 expression between genders, it did fall within the confidence intervals of that meta-analysis. The association between gender and PD-L1 may be confounded by tobacco use as the females in this study were less likely to smoke than men (14% versus 80% were smokers). Gender differences in immune response have also been described, with women showing stronger immune responses to infections[279]

and increased incidence of autoimmune diseases[280]. This may be due to increased expression of X-linked immune genes (such as *TLR7* and *TLR8*), the direct effects of hormones (such as oestrogen, progesterone and testosterone), and the influence of the gut microbiome on immune competency[281].

PD-L1 positive tumours were significantly more likely to appear in the tongue (OTSCC) compared to all other oral locations (p=0.04) and have a higher TPS (p=0.04). PD-L1 expression and location within the oral cavity has been described in 6 previous studies, with 3 finding an increased frequency of PD-L1 positive tumours in the tongue(Satgunaseelan et al., 2016; Troeltzsch et al., 2017; Wirsing et al., 2018). However, our recent meta-analysis failed to find a significant association between PD-L1 expression and location[253]. OTSCC may have a distinct epidemiological profile to other neoplasms of the oral cavity[282]. OTSCC has been increasing in incidence, particularly in both younger and female patients, and the reason why is not clear, as tobacco and alcohol use have decreased in these populations[283,284]. Studies of HNSCC have shown that non-smoker/non-drinkers predominately develop OTSCC [285], particularly in younger patients[286]. Our study had a strong association between gender and location (13 of the 14 female patients had OTSCC) as well as an increased, but not significant, number of OTSCC in non-drinkers and non-smokers (p=0.10). PD-L1 expression in OTSCC may therefore be an oncogenic factor in female, non-smoker, and non-drinker patients.

Patients with multiple tumours did not show any significant clinical differences to patients with a single tumour. Our study looked at the first tumour in 8 of 9 patients with multiple tumours (the ninth had 2 primary tumours and one was chosen at random). Following treatment, the phenotype and molecular markers of a cancer may change. While PD-L1 levels have been shown to increase in response to chemotherapy in cancer cell lines[145], this effect was not noted *in vivo*[146].

No significant relationship was found with T, N or M status. Our meta-analysis of PD-L1 in OSCC did not show an association with T or M status, however it did find an association with higher PD-L1 levels in N+ tumours, but only when assessing studies with >50 patients[253]. While the present study did not find a significant association with N status (p=0.23), when our data is added to the meta-analysis the result remains significant (OR=1.53, 95%CI=1.11-2.10, p=0.009). The presence of PD-L1 therefore may facilitate nodal metastasis through immune evasion, but further research and large sample sizes are needed to definitively state this observation.

This study found that PD-L1 positive tumours were more likely to express PD-L1 in the adjacent non-tumoral epithelium (p=0.001). The presence of PD-L1 in the adjacent epithelium suggests it may have been present before invasion, and could have played a role in cancer development and progression. PD-L1 expression has been studied in oral potentially malignant disorders, with increased PD-L1 in epithelial dysplasia compared to controls[210,238,243,287]. Sieviläinen et al.[288] did not find PD-L1 in the epithelium but rather in the lamina propria of oral dysplasia samples and seldom in healthy controls. Given that immune evasion is considered a hallmark of cancer these findings justify further research into immune checkpoint function in oral potentially malignant lesions.

Tumours with an intense lymphocytic infiltrate were more likely to have high PD-L1 expression in tumour cells (p=0.03). This can seem paradoxical as PD-L1 is thought to inhibit T cell activation and cause T cell apoptosis[81]. However, intense lymphocyte infiltration is a common finding in PD-L1 positive tumours, and increased levels of CD8+ and CD4+ T cells in PD-L1 positive tumours has been shown in our previous metanalysis[253]. The appearance of both a high TIL infiltration and PD-L1 positive tumour cells has been called adaptive immune resistance[289], as the presence of PD-L1 may have been induced by lymphocytes releasing inflammatory cytokines, such as IFN-γ. This induced expression may be visualised

with IHC, with PD-L1 staining seen along the periphery of tumour nests, and it has been theorized that these tumours may respond more favourably to anti-PD-1 treatment[290]. This study classified all PD-L1 positive tumours as having either an induced or constitutive pattern but found no correlation between clinicopathological characteristics or survival. The patients in our sample were not treated with immune checkpoint inhibitors and it may be interesting to investigate whether these expression patterns are useful in determining who may benefit from anti-PD-1 treatments. TILs were also scored for their PD-L1 expression and were categorized into low or high PD-L1 expression, with high TIL staining only being present in patients with high tumour cells expressing PD-L1. The presence of PD-L1 in TILs may therefore be induced in the same way as in tumour cells. An improved prognosis has been reported in HNSCCs with PD-L1 expression in immune cells and not tumour cells[291]. Further research is needed to determine the effects of PD-L1 expression in immune cells.

As PD-L1 does not act alone and future studies should consider the expression of its receptor, PD-1. Past studies have found that the measurement of both PD-L1 and PD-1 was a better predictor of survival than either factor alone[261]. PD-L1 is also known to be expressed on tumour associated macrophages (TAMs), where it may encourage a M2-phenotype, which is thought to be immunosuppressive[292]. Shifting TAMs from a M2 to a M1 phenotype (proinflammatory) may be a therapeutic option in cancers, and the PD-1/PD-L1 pathway may be a potential target for this[292].

PD-L1 at a 5% cut-off was not significantly associated with improved survival outcomes (DSS HR=1.71 95% CI=0.54-5.48, DFS HR=1.15, 95% CI=0.48-2.73, p=0.76, OS HR=0.58, 95% CI=0.14-2.45, p=0.46). Our recent metanalysis found a significantly worse DSS for PD-L1 positive tumours (HR= 1.54, 95% CI=1.03-2.28, p=0.03) but this had significant heterogeneity (I^2=58%, p=0.01) and only included 8 studies [253]. Our finding falls within the confidence interval of the meta-analysis and when added does not change the final result (New HR=1.55, 95% CI=1.07-2.24, p=0.02). Four patients did not have surgery and this was a significant factor for DSS and DFS and likely represented patients with advanced disease who were not candidates for curative treatment. When evaluating TILs, the worst survival was seen in tumours with an intense infiltration, followed by moderate and finally mild inflammation, although the difference was not significant (log rank p=0.13).

The findings, particularly for survival, are limited by the sample size. To detect a hazard ratio of 1.54, (as we found in our metanalysis of DSS and PD-L1) with a power of 80%, α of 5%, and 58% of cases positive for PD-L1, we calculate 173 events would need to be observed. This would then require a sample size of approximately 360. Achieving this size may not be feasible due to a lack of patients, however we believe that the information we, or other studies with small sample sizes, have provided is nonetheless useful for prognostic studies as they can be combined in future meta-analyses. We also note that our study excludes microinvasive/carcinomas-in-situ tumours, which ensures more reliable PD-L1 measurement, but may limit the extrapolation of our findings to these groups. These early tumours may be of interest to study for PD-L1, as our study shows PD-L1 staining is often present in the adjacent tumour epithelium. Only one section was stained for IHC per tumour, and this could miss any heterogeneity in PD-L1 expression within the same tumour. However, PD-L1 expression is known to be heterogenous even within the same tumour section. Our method of analysing the whole tumour section, as opposed to randomly chosen fields within a section, allows us to better capture the overall PD-L1 expression. Finally, TILs were measured by the intensity of their infiltrate however this lacks information on whether TILs are functional or not. Future studies should consider assessing TILs for markers of apoptosis (such as by using a TUNEL assay or caspase-3 immunohistochemistry expression)."

Conclusions

Through our meta-analysis we present evidence that the overexpression of PD-L1 in the cell membrane of OSCC tumour cells has a negative impact on survival, especially of DSS and DFS. Furthermore, we have identified that there appears to be a subgroup of OSCC in females, non-smokers, and non-drinkers with an increased expression of PD-L1, which may allow a greater capacity to evade the anti-tumour immune response. We found that IHC methodology can have a significant effect on PD-L1 measurement. We can therefore recommend good practices for future studies including measuring PD-L1 exclusively in the membrane, using a cut-off of ≥5%, using a validated antibody, and avoiding tissue microarrays.
Our IHC study of PD-L1 expression in 55 patients with OSCC agreed with our meta-analysis finding that PD-L1 expression is significantly increased in non-smokers and non-drinkers and this association is greater when both risk factors are absent. This association may be important for understanding the aetiology of oral cancer in patients with no known risk factors. We have also shown PD-L1 expression to be significantly associated with tongue OSCC, although further research is needed to confirm this association. We have shown PD-L1 to be found in the adjacent epithelium, further suggesting that its presence in normal or dysplastic epithelium could be involved in early oncogenesis. PD-L1 expression in tumour cells may be induced as a reaction to the inflammatory infiltrate, although we did not find a clinical difference when PD-L1 expression appeared to be induced or constitutive.

In summary, this thesis adds to the understanding of PD-L1 expression in OSCC. Specifically, that PD-L1 expression is increased in non-smokers, non-drinkers and potentially in women as well. That PD-L1 expression is increased in tumours with an intense inflammatory infiltrate. That PD-L1 expression is increased in the epithelium adjacent to the tumour and this may play a role in oncogenesis. Finally, we have shown evidence that the presence of PD-L1 expression in the tumour cell membrane may predict a worse likelihood of survival.