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COMPREHENSIVE PROFILE IN HEAD AND NECK CANCER PATIENTS TREATED WITH IMMUNOTHERAPY

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COMPREHENSIVE PROFILE IN HEAD AND NECK CANCER PATIENTS TREATED WITH IMMUNOTHERAPY

Abstract

Background

Immunotherapy has a crucial role in the treatment of recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC). However, only a small percentage of patients achieve long-term benefit in terms of overall response and survival. It was shown that HNSCC has an immunosuppressive microenvironment due to high levels of regulatory T cells and immunosuppressive molecules, such as LAG3 and CD73.
The aim of our study was to investigate if the expression of CD73 by neoplastic and immune cells could affect the efficacy of anti-PD-1 immunotherapy and we evaluated the role of circulating CD137+ T cells in (R/M) HNSCC patients undergoing pembrolizumab treatment.

Methods

We reviewed data from 50 patients with R/M HNSCC receiving first line immunotherapy with or without chemotherapy based on a combined positive score (CPS). CD73 expression by cancer and immune cells was evaluated on pre-treatment and the percentage of stained cells was recorded. We analysed the association between CD73 expression on neoplastic and immune cells and early progression (EP), defined as progression occurring within 3 months.

PBMCs obtained from 40 (R/M) HNSCC patients with a PD-L1 combined positive score (CPS) ≥1 were analysed at baseline via cytofluorimetry for the expression of CD137, and it was found that the percentage of CD3+CD137+ cells is correlated with the clinical benefit rate (CBR), PFS, and OS.

Results

In 88% of patients the primary tumour site was in the oral cavity or larynx. All patients received pembrolizumab associated in 40% of cases to chemotherapy. CD73 was positive in 82% and 96% of cases on neoplastic and immune cells, respectively. The median value of CD73 was 32% for neoplastic cells and 10% for the immune ones. We observed a significant association between CD73 expression over the median

value and EP disease. We didn't record a correlation between the expression of CD73 on immune cells and early progression.

The results show that levels of circulating CD137+ T cells are significantly higher in responder patients than in non-responders ($p = 0.03$). Moreover, patients with CD3+CD137+ percentage ≥1.65% had prolonged OS ($p = 0.02$) and PFS ($p = 0.02$). Multivariate analysis, on a combination of biological and clinical parameters, showed that high levels of CD3+CD137+ cells (≥1.65%) and performance status (PS) = 0 are independent prognostic factors of PFS (CD137+ T cells, $p = 0.007$; PS, $p = 0.002$) and OS (CD137+ T cells, $p = 0.006$; PS, $p = 0.001$).

Conclusions

Our findings suggest that higher expression of CD73 on neoplastic cells could predict resistance to immunotherapy in patients with CPS positive R/M HNSCC. The addition of this biomarker to routine evaluation of CPS could help to select the patients primary resistant to anti-PD-1 immunotherapy.

Furthermore our results suggest that levels of circulating CD137+ T cells could serve as biomarkers for predicting the response of (R/M) HNSCC patients to pembrolizumab treatment, thus contributing to the success of anti-cancer treatment.

BACKGROUND

Head and neck squamous cell carcinomas (HNSCC) are a heterogeneous group of tumors that affect different anatomical sites, including skin, oral cavity, oropharynx, nasopharynx, hypopharynx, larynx, paranasal sinuses and salivary glands.

Squamous cell carcinoma, the most frequent histotype, makes up more than 90% of the total HNC, defined in this case as head and neck squamous cell carcinoma (HNSCC). They originate from the squamous epithelium that lines the mucous membranes of the aero-digestive tract.

These neoplasms have always aroused interest in oncology due to their high morbidity and strong psycho-social impact: they are destructive and disfiguring pathologies, which impact on common daily life activities, such as speaking, chewing and swallowing, and which for these reasons require aggressive treatments, themselves a cause of morbidity. They require multidisciplinary treatment including surgery, radiotherapy and oncology, which are variously associated with each other, and the results of the Keynote-048 study paved the way for immunotherapy treatment with Immune Checkpoint Inhibitors (ICIs). These drugs inhibit immune checkpoints, molecules that act as suppressants of the immune system, and stimulate lymphocytes to recognize and eliminate cancer cells.

The emerging data from recent clinical trials in different solid tumours showed that only a relatively small subset of patients really benefits from ICIs, underlining the crucial role of patients selection in the choice of

the best therapeutic strategy. New robust data are required to develop and validate molecular and genetic predictive biomarkers able to define immunologically cold and hot tumor allowing to detect responders or no responders patients in clinical practice. The advent of immunotherapy in clinical practice has led to the urgent need to implement a dynamic and personalized approach to the cancer patient in order to adapt the therapeutic strategy to the peculiar and specific state of the immune system characterizing both patients and tumor microenvironment.

The last effort on the identification of biomarkers failed to demonstrate that a single biomarker can optimally select patients resistant/responder to immunotherapy.

It's reasonable to imagine that a comprehensive profile, rather than a single biomarker, could be necessary to better select patient.

1. Epidemiology and risk factors

Head and neck neoplasms are the seventh most frequent neoplasm: 931,000 new cases diagnosed in 2020 and an annual mortality of 467,000 patients worldwide [1]. They predominantly affect the elderly and men, both of whom are considered risk factors for the disease. In addition, several studies report a ratio of incidence of these neoplasms between the two sexes M:F of 10:1 in the larynx and 4:1 in the hypopharynx.[2]

The association with risk factors such as smoking and alcohol is well understood, which have a synergistic effect in increasing the risk of the onset of these neoplasms (up to 80 times greater), leading to the accumulation of mutations that induce, moreover, greater resistance to therapy than HPV-related tumors (see below): smoking, for example, induces an increase in EGFR (Epidermal Growth Factor Receptor) which correlates with a reduced response to chemotherapy.[3]

HPV infection is present in about 35% of head and neck neoplasms, more specifically in oropharyngeal neoplasms, while lower fractions, <10% and 2.5%, are estimated for oral cavity and larynx cancers, respectively.[4-5]

The most frequent genotype of Papillomavirus associated with head and neck cancers is HPV16, found in up to 90% of HPV-associated head and neck cancers; less frequent is the finding of the other subtypes considered to be at high risk (found in 2.5% of each HNSCC).[6]

HPV positivity is strongly correlated with a better outcome, which means that these types of cancer must have a multidisciplinary approach that is different from non-HPV-related cancers. In fact, they show a better response to chemotherapy, concomitant chemoradiation treatment and Target Therapy. There is also an increase in Overall Survival (OS).[7-8]

The Epstein Barr Virus (EBV) is also an oncogenic virus that is a risk factor in HNCs, particularly for nasopharyngeal cancer (NPC), mainly represented in Southeast Asia. More precisely, the closest association with EBV infection is observed in undifferentiated nasopharyngeal carcinoma. The exact correlation and sequence of events describing this correlation is currently being investigated but not fully understood.[9]

Other risk factors derive from air inhalants and this phenomenon explains the increased incidence in highly urbanized and highly polluted countries such as China or India [10-11]. In addition, certain occupational activities, such as leather and wood processing, are associated with an increased risk of carcinomas of nasal guinea pigs and sinuses[12]. An inverse association with the risk of HNSCC has been reported with regard to the consumption of fruits and vegetables, being linked to the higher intake of vitamins C, E, carotenoids (vit. A) and folate (vit. B9) [13].

1.2 Pathogenesis

The pathogenesis of HNCs is multifactorial: genetic and environmental factors initially cause the mutation of single genes, which in turn lead to the dysregulation of metabolic-biomolecular processes. Information about the molecular biology of HNCs is still partial and lacunar, and several points need to be clarified for a better understanding of the mechanisms underlying oncogenesis.

The most common mutations in HNSCC result in inactivation and loss of function of tumor suppressor genes TP53 (72% of tumors) and CDKN2A (22%). Other less frequent mutations are in the PTEN (8%), BRCA1 (6%), and BRCA2 (7–9%) genes.

Among the oncogenic signaling pathways, mutations were found predominantly in the PI3K-AKT-mTOR pathway (in 30.5% of cases), in the JAK/STAT pathway (9.3%) and in the MAPK pathway (8%). PIK3CA was found to be the most frequently altered oncogene, through mutations or amplification, in both HPV-related and HPV-negative cancers (56% and 34%, respectively) [14, 15].

HPV also contributes to oncogenesis especially in oropharyngeal carcinomas, in younger patients and in patients with risky sexual behaviors [16]. In cancer cells, viral DNA can be present both in episomal form and in integrated form with the genome of the host cell. Two genes, which code for the homonymous proteins, are implicated in the dysregulation of the cell cycle: E6, which leads to poly-ubiquitination and subsequent degradation by the proteasome of the tumor suppressor p53 (defined as the guardian of the genome because of its crucial importance in DNA repair processes); E7, which leads to the degradation of pRb

(Retinoblastoma-associated protein) by separating it from the complex with the growth factor E2F and promoting cell cycle progression.

In particular, in high-risk HPV genotypes, the high expression of E6 and E7 proteins promotes the integration of viral episomes. In most cases, supplementation occurs by breaking down genetic material at the level of the E2 gene, which is critical for regulating cellular levels of E6 and E7 proteins. Therefore, they are overexpressed, favoring the transformation of the cell [17-18].

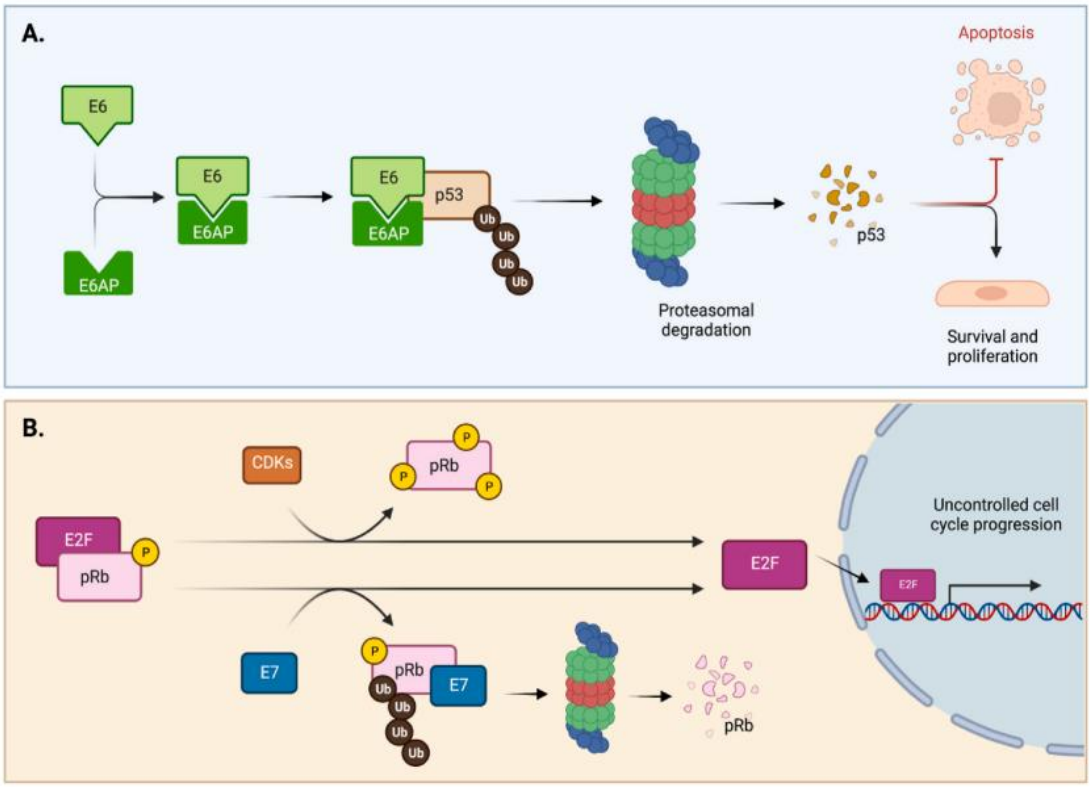


Fig.1 Role of HPV in head and neck cancer.

E6 and E7 HPV oncoproteins degrade p53 and pRb, respectively. A. E6-induced p53 degradation through the formation of a ternary complex. Binding of E6 to p53 induces its ubiquitylation and proteasomal mediated degradation, via the ubiquitin E3 ligase E6-associated protein (E6AP). B. Degradation of pRb, overexpression of CDKs and inactivation of CDK inhibitors all contribute to E2F overexpression and uncontrolled cell-cycle progression in cancers. Whereas hypo-phosphorylation of pRb prevents effects of E2F and renders cells quiescent, CDK/Dcyclin association causes its phosphorylation and release from E2F. Binding of E7 also causes release of E2F through ubiquitylation and proteasomal degradation of pRb. Galati L et al. Tumour Virus Res. 2022 Dec; 14:200245. doi: 10.1016/j.tvr.2022.200245.

HNCs originate from the epithelial cells of the mucosa of the oral cavity, larynx, pharynx and sinuses. Histologically, progression to invasive HNSCC follows an ordered series of events that begin with epithelial hyperplasia, progress to epithelial dysplasia, carcinoma in situ, or invasive carcinoma.

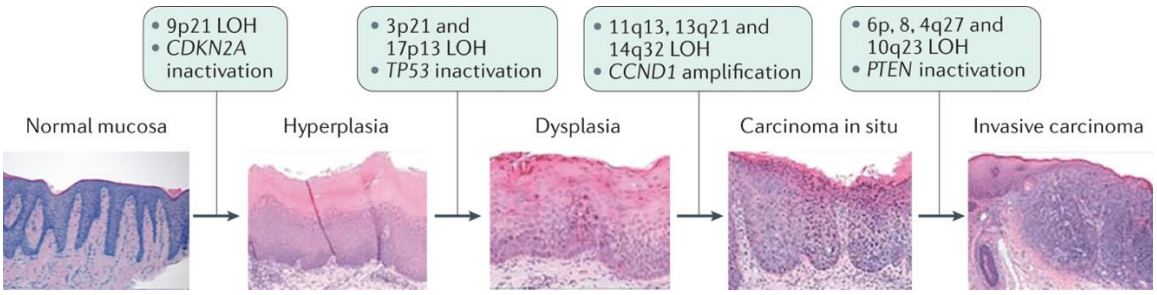


Fig.2 The image illustrates the different inactivated genes, loss of function (LOH) and amplification of genes that, in concert, lead to the development of invasive carcinoma. Johnson DE, Burtness B, Leemans CR, Lui VWY, Bauman JE, Grandis JR. Head and neck squamous cell carcinoma. Nat Rev Dis Primers. 2020 Nov 26; 6(1):92.

It should be noted that most patients with diagnosed carcinoma do not report a history of pre-malignant epithelial lesions [19].

A characteristic phenomenon of these neoplasms is field cancerization. It is defined as the presence of one or more mucosal areas containing clonal units that have genetic or epigenetic alterations associated with tumorigenesis. Each "risk field" has a monoclonal origin and does not have metastases or invasiveness, as it is preneoplastic by definition; Therefore, it may or may not present histological aberrations and mutations characteristic of dysplasia. They are normally undetectable on clinical inspection, but can sometimes take the form of leukoplastic lesions [20]. They can develop into carcinoma at a constant rate of 2-3% per year, implying that, despite radical surgical resection of the macroscopically visible neoplastic lesion, they may be the source of local recurrences or second primary tumors [21]. Both situations can be distinguished in the following way: recurrence is defined as a tumor that arose at a distance of less than 2 cm from the primary neoplasm or within 3 years of previous treatment, vice versa it is a second primary tumor.

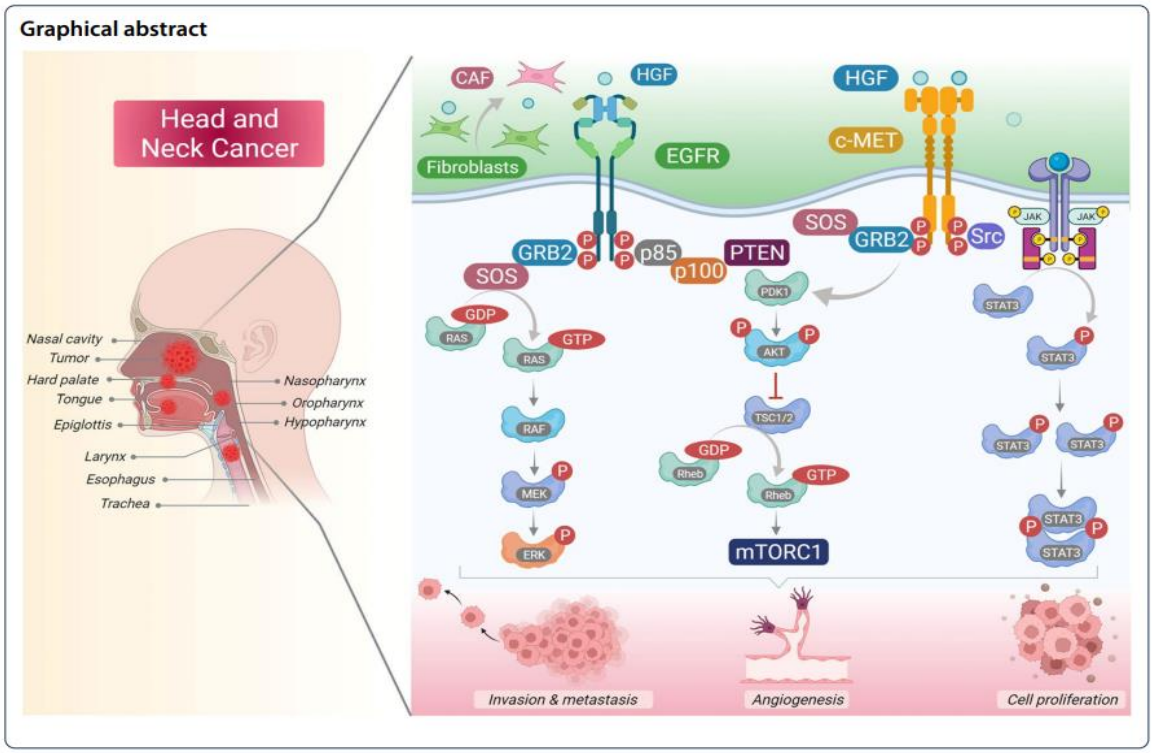


Fig. 3 Molecular biology of head and neck cancer. Raj S et al. Mol Cancer. 2022 Jan 26;21(1):31. doi: 10.1186/s12943-022-01503-1.

1.3 Clinical and pathological staging

The staging of tumors uses the criteria of the TNM classification, in which T (Tumor) describes the extent of the primary tumor; N (lymph Node) refers to the absence or presence and possible extent of regional lymph node involvement; finally, M (Metastasis) refers to the presence or absence of metastases [22].

The staging currently in force is that of the eighth edition of the NWT (UICC/AJCC 2017). With this publication, significant changes were introduced, summarized by the 2021 AIOM guidelines:

1) In oral cancers, the concept of invasion of the extrinsic muscles of the tongue has been eliminated as a criterion for diagnosing T4 and at the same time the concept of DOI (deep of invasion) has been inserted to define T. DOI is classified as superficial (<5 mm), medium (5–10 mm), and deep (>10 mm). Every 5 mm of depth increases the degree of categorization T of a level (up to 10 mm or more depth).

2) Extranodal extension, considered an unfavorable prognostic parameter for all subsites, is no longer so for HPV+ oropharyngeal neoplasms.

3) Extracapsular extension (ENE+) is considered only when the microscopic diffusion is more than 2 mm away from the lymph node capsule, i.e. when there is a clear spread to the surrounding tissues; a clinical diagnosis of ENE+ is made possible in the presence of irrefutable clinical data (cutaneous or cranial nerve

invasion with dysfunction of the same, multiple confluent lymph nodes, etc.); This diagnosis is also supported, not sufficiently if considered individually, by radiological data extremely suggestive of extranodal invasion. The presence of pathological ENE+ increases the categorization of lymph node status by one level compared to the clinical classification. In the presence of ambiguous situations, it is a general rule to adopt the lower (prognostically more favorable) staging.

4) There has also been an increase in the interest of other parameters (tumor invasion pattern; perineural invasion; lymphovascular invasion; comorbidities, etc.) that at present, although considered important from a prognostic point of view, are not considered such as to have to modify the TNM classification of the individual patient.

5) Pathologic staging, i.e. after surgery, adds information regarding the prognosis and is important for the choice of post-operative treatment. With regard to lymph node diffusion, pathological information should define: size, number and level of the lymph nodes involved, possible capsular infiltration, resection margins (infiltration and adequacy), the presence of peritumoral vascular invasion, lymphatic embolization and perineural involvement. With reference to the glottic larynx, margins of less than 5 mm are also considered adequate. Only pathological staging can therefore provide information about the oncological radicality (R0) of the intervention.

Even more important is the distinction between HPV 16+ oropharyngeal carcinomas (OPCs) and smoking/HPV-associated OPCs, for which a new staging system is essential. This distinction, in order to better stratify the risk and prognosis of the patient, is fundamental by virtue of the lower aggressiveness of HPV+ OPCs. In fact, through the use of the seventh edition of the TNM, patients with these tumors were overstaged, consequently they inappropriately received more intense treatments because they were equated with the other, much more aggressive pathological entity [23].

Table 1

T Category[a]	Criteria
T0	No primary tumor identified
T1	Tumor size ≤ 2 cm in greatest dimension
T2	Tumor size > 2 cm but ≤ 4 cm in greatest dimension
T3	Tumor size > 4 cm in greatest dimension or extension to lingual surface of epiglottis
T4	Moderately advanced tumor invading larynx, extrinsic tongue muscles, medial pterygoid, hard palate, or mandible or beyond
Clinical N Category	**Criteria**
Nx	Regional nodes cannot be assessed
N0	No regional nodal metastasis
N1	Metastasis to one or more ipsilateral nodes, ≤ 6 cm
N2	Metastasis to contralateral or bilateral lymph nodes, ≤ 6 cm
N3	Metastasis in any cervical lymph node > 6 cm
Pathologic N Category	**Criteria**
Nx	Regional nodes cannot be assessed
pN0	No regional nodal metastasis identified
pN1	Metastasis to 4 or fewer lymph nodes
pN2	Metastasis to 5 or more lymph nodes
M Category	**Criteria**
M0	Absence of distant metastasis
M1	Presence of distant metastasis

a

T Category	N Category	M Category	Stage Group
T0, T1, or T2	N0 or N1	M0	I
T0, T1, or T2	N2	M0	II
T3	N0, N1, or N2	M0	II
T0, T1, T2, T3, or T4	N3	M0	III
T4	N0, N1, N2, or N3	M0	III
Any T	Any N	M1	IV

b

T Category	N Category	M Category	Stage Group
T0, T1, or T2	N0, N1	M0	I
T0, T1, or T2	N2	M0	II
T3 or T4	N0, N1	M0	II
T3 or T4	N2	M0	III
Any T	Any N	M1	IV

c

Table 2

T Category[a]	Criteria
Tx	Primary tumor cannot be assessed
Tis	Carcinoma in situ
T1	Tumor size ≤ 2 cm in greatest dimension
T2	Tumor size > 2 cm but ≤ 4 cm in greatest dimension
T3	Tumor size > 4 cm in greatest dimension or extension to lingual surface of epiglottis
T4	Moderately advanced or very advanced tumor
T4a	Moderately advanced tumor invading larynx, extrinsic tongue muscles, medial pterygoid, hard palate, or mandible
T4b	Very advanced tumor invading lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encasement of the carotid artery
Clinical N Category	**Criteria**
Nx	Regional nodes cannot be assessed
N0	No regional nodal metastasis
N1	Metastasis to single ipsilateral node, ≤ 3 cm and ENE-negative
N2	Metastasis in a single ipsilateral lymph node > 3 cm but ≤ 6 cm in greatest dimension and ENE-negative *or* metastases in multiple ipsilateral lymph nodes, ≤ 6 cm in greatest dimension and ENE-negative *or* metastases in bilateral or contralateral lymph nodes, ≤ 6 cm in greatest dimension and ENE-negative
N2a	Metastasis in a single ipsilateral lymph node > 3 cm but ≤ 6 cm in greatest dimension and ENE-negative
N2b	Metastases in multiple ipsilateral lymph nodes, ≤ 6 cm in greatest dimension and ENE-negative
N2c	Metastases in bilateral or contralateral lymph nodes, ≤ 6 cm in greatest dimension and ENE-negative
N3	Metastasis in a lymph node > 6 cm in greatest dimension and ENE-negative *or* metastasis in any lymph node(s) and clinically overt ENE-positive
N3a	Metastasis in a lymph node > 6 cm in greatest dimension and ENE-negative
N3b	Metastasis in any lymph node(s) and clinically overt ENE-positive
Pathologic N Category	**Criteria**
Nx	Regional nodes cannot be assessed
N0	No regional nodal metastasis
N1	Metastasis to single ipsilateral node, ≤ 3 cm and ENE-negative
N2	Metastasis to single ipsilateral node, ≤ 3 cm and ENE-positive *or* metastasis in a single ipsilateral lymph node > 3 cm but ≤ 6 cm in greatest dimension and ENE-negative *or* metastases in multiple ipsilateral lymph nodes, ≤ 6 cm in greatest dimension and ENE-negative *or* metastases in bilateral or contralateral lymph nodes, ≤ 6 cm in greatest dimension and ENE-negative
N2a	Metastasis to single ipsilateral node, ≤ 3 cm and ENE-positive *or* metastasis in a single ipsilateral lymph node > 3 cm but ≤ 6 cm in greatest dimension and ENE-negative
N2b	Metastases in multiple ipsilateral lymph nodes, ≤ 6 cm in greatest dimension and ENE-negative
N2c	Metastases in bilateral or contralateral lymph nodes, ≤ 6 cm in greatest dimension and ENE-negative
N3	Metastasis in a lymph node > 6 cm in greatest dimension and ENE-negative *or* metastasis in a single ipsilateral lymph node > 3 cm in greatest dimension and ENE-positive *or* metastases in multiple ipsilateral, contralateral, or bilateral lymph nodes, with any ENE-positive
N3a	Metastasis in a lymph node > 6 cm in greatest dimension and ENE-negative
N3b	Metastasis in a single ipsilateral lymph node > 3 cm in greatest dimension and ENE-positive *or* metastases in multiple ipsilateral, contralateral, or bilateral lymph nodes, with any ENE-positive *or* a single contralateral node ≤ 3 cm and ENE-positive
M Category	**Criteria**
M0	Absence of distant metastasis
M1	Presence of distant metastasis

a

T Category	N Category	M Category	Stage Group
Tis	N0	M0	0
T1	N0	M0	I
T2	N0	M0	II
T3	N0	M0	III
T1, T2, T3	N1	M0	III
T4a	N0, N1	M0	IVA
T1, T2, T3, T4a	N2	M0	IVA
Any T	N3	M0	IVB
T4b	Any N	M0	IVB
Any T	Any N	M1	IVC

b

Table 1 Classification AJCC TNM (VIII ed.) of OPSCC HPV-related (p16+). Categories and definitions (a), clinical staging (b) and pathological (c). AJCC Cancer Staging Manual (8th. Edition).

Table 2 Classification AJCC TNM (VIII ed.) of OPSCC HPV-negative (p16-). Categories and definitions (a). Staging in groups with prognostic value (b). AJCC Cancer Staging Manual (8th. Edition).

1.4 Treatment of HNSCC

1.4.1 General aspects of HNSCC management

The treatment of head and neck tumors, considering the complexity of the tumors and the anatomical regions involved, is normally multidisciplinary: various professionals are involved such as oncologist, interventional radiologist, radiation oncologist, pathologist, maxillofacial surgeon, plastic surgeon, otolaryngologist, dentist and other professionals in the field.

The characteristics of the tumor, the patient's general state of health (in terms of Performance Status, patient age and comorbidities), the development of any toxicity, the localization and possible surgical attackability,

the stage, the constant balance of the risk-benefit ratio and the expected outcome [24-25] will be evaluated on a case-by-case basis.

1.4.2 Treatment of local disease

In early-stage disease (cT1-2, N0, M0) the treatment of choice is surgery or curative radiotherapy, often as an exclusive treatment for radical purposes. Surgery has two objectives: excision with R0 curative intent (without residual disease) and preservation of the function of the organ involved. Radiotherapy can be used either exclusively, at the same time as or after surgical therapy, with adjuvant intent to the latter. The total dose in radical-intent radiotherapy treatments is 66–72 Gy with conventional fractionation (1.8–2 Gy per day for 5 days per week), or brachytherapy treatment may be used.

In the early stages of oral cancer, (surgery is the treatment of choice; radiotherapy can only be used in an adjuvant setting because sensitivity to chemoradiation treatments in squamous oral carcinomas (OSCC) is minimal) in T1 cases, surgery and radiotherapy (preferably brachytherapy) may be used as an alternative or in combination, as the chances of cure are comparable for the two treatments, although surgical treatment is preferable, where a transoral resection can be performed. In intermediate extension (T2) malignancies, surgery is the treatment of choice [26].

Treatment of the early stages of carcinomas of the nasal cavities and sinuses is exclusively surgical, usually by endoscopic technique. Radiotherapy can be used but, particularly for tumors that relate to the base of the skull, the use of intensity-modulated technique, such as IMRT, is recommended. This technique allows a more selective and focused irradiation to the neoplastic target, limiting toxicity and complications and obtaining better oncological results.[27]

As far as the oropharynx is concerned, surgery or radiotherapy are indicated, alternatively, in T2N1 cases concomitant or consecutive chemoradiotherapy treatment can be opted for.[28]

In the nasopharynx, surgery is complicated both because of the complicated access to this site and because of the anatomical relationships between the nasopharynx itself and the endocranial structures, including the inner ear; consequently, as well as due to the high chemo-radiosensitivity of these neoplasms, the standard of care is represented by IMRT alone in the early stages, associated with induction or adjuvant chemotherapy or simultaneous chemotherapy in locally advanced disease.

Microsurgery and minimally invasive surgery are a valid therapeutic alternative, currently used in the second line for recurrences or residual disease; the endoscopic surgical approach is in any case the gold standard

compared to the open approach, which has been progressively abandoned. Chemotherapy in a neoadjuvant setting, with subsequent IMRT, is reserved for the most advanced stage of the disease.[29]

In hypopharyngeal carcinomas radiotherapy is preferable over surgery for the best functional outcomes, in particular IMRT increases locoregional control compared to RT-3D [30]. Tumors of the larynx, at an early stage, frequently of glottic or supraglottic origin, eligible in the first line for exclusive radiotherapy or microsurgery treatments, or microsurgery with radiotherapy (neoadjuvant) post-operative; the use of microsurgery is preferred to minimize any anatomical and functional iatrogenic damage, trying to fully preserve laryngeal functions; Partial or subtotal laryngectomies, laser resections or transoral robotic resections can be performed. Radiotherapy is used with hypofractionated regimens to achieve better locoregional disease control, better tolerance, and higher survival than canonical radiotherapy regimens. Demolition surgery is recommended in locally advanced disease (beyond pT2 and with N+) and is accompanied by tracheostomy or microsurgical reconstructions of the larynx; Alternatively, you can opt for radiotherapy with adjuvant chemotherapy, trying to preserve the organ and avoid demolition treatments that have a strong impact on quality of life.[31]

As far as treatments in an adjuvant setting are concerned, whether polychemotherapy or chemoradiotherapy, they are mandated in the presence of lymph node involvement (N+), or for suspicion of locoregional presence of occult metastases or micrometastases; This approach reduces the risk of recurrence and increases the survival rate.

Neoadjuvant polychemotherapy is used inductively before surgery to shrink tumor mass, decrease the risk of recurrence, and eliminate regional micrometastases. Treatment in an adjuvant setting is usually preferred, although the neoadjuvant setting is better tolerated. Both strategies can be used in combination, in patients with general health and performance status that allow it, to improve outcome.
Finally, lymph node resection is indicated in case of overt or potential locoregional lymph node involvement. The latter findings are typical of late-stage disease.[32]

1.4.3 Treatment of locally advanced disease

In locally advanced disease (T2-4b, N1-2, M0), a strategy based on radiotherapy and polychemotherapy in combination is indicated in the first line, if the primary tumor is unresectable or in the case of R≠0 resection margins or if the surgical approach does not allow a satisfactory outcome [33]. The chemoradiotherapy regimen of choice consists of *high-dose* cisplatin (100 mg/m2), administered (3 times per week) once every three

weeks for at least 3 cycles in combination with standard or accelerated fractionation concomitant radiotherapy.[34]

The combination of polychemotherapy and concomitant radiotherapy increases the survival of this group of patients: the drugs of choice are carboplatin in combination with 5-FU or cetuximab.[35]
Alternatively, regimens based on the combination of cisplatin (40 mg/m2), 1 time a week for at least 4 weeks, with concomitant boost radiotherapy, or cisplatin or hydroxyurea with 5-fluorouracil and radiotherapy, or carboplatin and paclitaxel with daily concomitant radiotherapy, are used.[36-37-38]

Chemotherapy drugs in adjuvant and neoadjuvant settings are used at this stage to consolidate the results of the primary intervention and to improve the outcome.
In the case of positive resection margins (R1), locoregional positive lymph nodes or other extranodal extension, better locoregional control of disease is desirable in view of the risk of recurrence and lower survival. Post-operative radiotherapy can therefore be opted for, in an adjuvant setting, with associated *high-dose* concomitant cisplatin.[39]

Neoadjuvant treatment, whose role in locally advanced disease is still under study due to the results still not conclusive, uses chemotherapy with consecutive radiotherapy, associated or not with target-therapy with cetuximab; alternatively, concomitant chemoradiotherapy is used [40]. Also in this case, induction strategies are used for organ preservation, in particular for primary laryngeal and hypopharyngeal carcinomas. TPF (taxol-platinum-fluorouracil) chemotherapy regimens that include cisplatin (75 or 100 mg/m2), docetaxel (75 mg/m2) and 5-FU (750 mg/m2 in continuous infusion for 5 days) are therefore used for induction, every 3 weeks for at least 3 cycles.[41-42]

1.4.4 Treatment of recurrent/metastatic disease

According to the eighth edition of AJCC TNM, stage four disease is defined as the presence of metastases (every T, every N, M1) and/or recurrence. The prognosis is severe, considering that current treatments do not allow a cure from the disease. The average survival is less than one year.
At the time of diagnosis, about two-thirds of patients are already at an advanced stage of disease, with positive locoregional lymph nodes: these patients are at risk of developing recurrences of locoregional disease and distant metastases. In fact, 10% of these patients have metastases already at this stage.[43]
Failure of therapy in early-stage or locally advanced malignancies leads to the evolution towards this stage, in which the disease is persistent or recurrent, ergo systemic rechallenge or re-surgical interventions are required; in the latter case, the intent is usually palliative rather than curative. In this case, therefore, the first

therapeutic option is systemic: radiotherapy is used in combination with polychemotherapy, immunotherapy and target-therapy regimens.

The goal of treatment is therefore to control the disease, limit progression, reduce any complications (including iatrogenic), always taking into account the patient's comorbidities, often in old age, and the performance status, often poor in these stages.

The management of advanced recurrent and/or metastatic disease has been, so far, represented by chemotherapy-based therapies or target-therapy agents. HNSCCs are chemosensitive tumors, which is why the most commonly used chemotherapeutic agents in monotherapy or combination are platinum derivatives (cisplatin and carboplatin), taxanes (paclitaxel and docetaxel), methotrexate, 5-FU and, more rarely, bleomycin and capecitabine.

In addition to these, there are biological drugs of the target therapy, among which the most widely used is cetuximab (anti-EGFR); other anti-EGFRs (panitumumab, zalutumumab, nimotuzumab) and tyrosine kinase inhibitors (TKI, erlotinib and gefitinib) have not yet demonstrated satisfactory results in terms of outcome in clinical trials, but are still being studied, as well as other agents. [44]

Initially, the first line of *standard-of-care* consisted of combination chemotherapy regimens based on platinum derivatives. Subsequently, the EXTREME regimen was approved in the United States in November 2011, which consisted of the combination of high-dose cisplatin or carboplatin with 5-FU and cetuximab, followed by cetuximab in maintenance; It became the go-to regimen for the treatment of metastatic cancer.[45] Alternatively, the TPEX regimen is used, a combination of cisplatin or carboplatin with docetaxel and cetuximab or other polychemotherapy combinations.[46]

Second-line, in non-platinum-sensitive or progressing cancers, taxane- or methotrexate-based regimens were opted for, although none of these demonstrated a clear survival benefit. In general, a platinum-based *approach is always preferred* for platinum-naïve patients, due to its significant superiority in terms of survival and outcome.[47]

The phase III Keynote-048 trial, which compared pembrolizumab monotherapy or pembrolizumab combined with chemotherapy (cisplatin or carboplatin and 5-fluorouracil) versus the EXTREME regimen, was the first study to suggest the use of immunotherapy in this type of cancer.[48]

As reported in the AIOM guidelines, the EMA has approved first-line pembrolizumab both as monotherapy and in combination with platinum derivatives and 5-fluorouracil only for patients with CPS ≥ 1 (Combined Positive Score). Therefore, Pembro+PF treatment in patients with PD-L1 CPS <1 has not been approved on

the basis of overall survival data derived from an exploratory analysis of approximately 80 patients, which showed a median survival of 11.3 months for Pembro+PF vs 10.7 months for EXTREME (HR 1.21, 95% CI 0.76-1.94, p=0.789). For patients with CPS $\geq$ 1, although the greatest objective responses are obtained with Pembro+PF, this regimen has a toxicity profile comparable to the EXTREME regimen, therefore it should be reserved for patients with good performance status. Instead, pembrolizumab monotherapy may be a preferred treatment option in patients with low disease burden, paucisymptomatic, and/or unfit for a combination treatment with chemotherapy. CPS is defined as the number of tumor cells, lymphocytes, and macrophages expressing PD-L1 divided by the total number of tumor cells, multiplied by 100.

Nivolumab is currently used as a second-line treatment in these patients, regardless of PD-L1 expression in immunohistochemistry, following evidence from the CHECKMATE-141 study, a phase III trial, involving 361 patients with refractory platinum recurrent or metastatic disease [49].

2. ROLE OF IMMUNOTHERAPY

2.1 Tumor immunoediting

Before immunotherapy can be described, it is necessary to establish the relationship between the immune system and carcinogenesis.

The immune system plays a focal role in the recognition and elimination of cancer cells, a process called "immunosurveillance", which, in fact, can be defined as the first step of a larger dynamic process, immunoediting. This is divided into three phases: elimination, balance, and escape.[50]

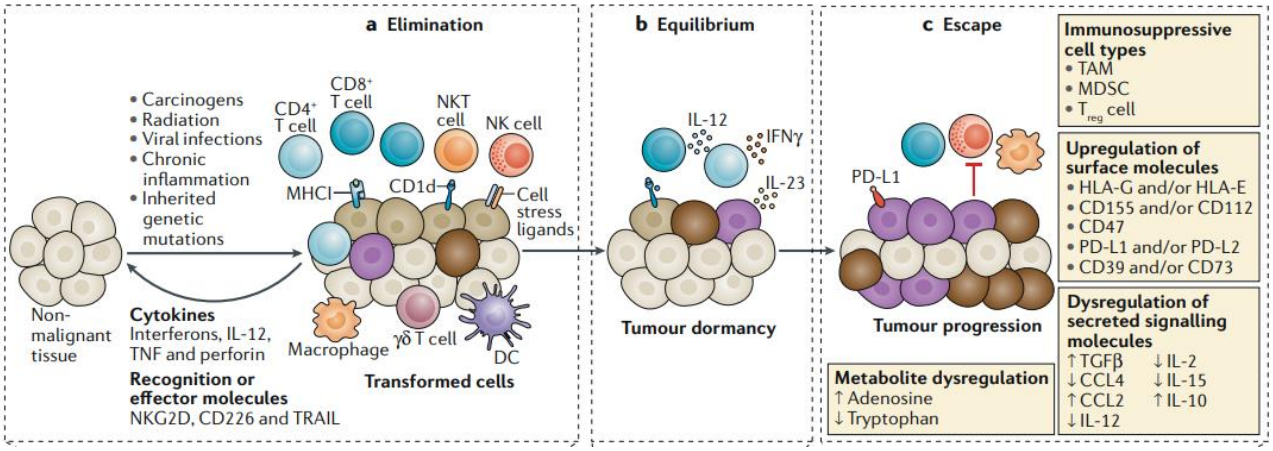


Figure 4: Cancer immunoediting and response to cancer immunotherapy. Cancer immunoediting proceeds through three phases: elimination, equilibrium and escape. a | During the elimination phase, the innate and adaptive immune systems cooperate to recognize transformed cells that have escaped intrinsic tumour suppression and to eliminate them before tumours become clinically detectable. If tumour cell destruction occurs, elimination constitutes the totality of the immunoediting process. b | Tumours capable of surviving the elimination phase can progress into the equilibrium phase, in which net growth is limited and cellular immunogenicity is edited by the adaptive immune system. c | Edited tumours can then enter into the escape phase, in which their growth is unrestrained — largely owing to the activation of immunosuppressive and/or immunoevasive pathways. Escaped tumours are those clinically detectable as visible tumours.

O'Donnell JS et al. Cancer immunoediting and resistance to T cell-based immunotherapy. Nat Rev Clin Oncol. 2019 Mar;16(3):151-167. doi: 10.1038/s41571-018-0142-8.

2.1.1 Elimination Phase:

Several studies have highlighted the crucial role of the immune system in the control of transformed cells. Among the various fundamental experiments was the one carried out on mice regarding the RAG-2 gene, which is selectively expressed in the cells of the lymphoid system. This gene is essential to allow the somatic rearrangement of the receptor of different SI cell populations: T lymphocytes, B lymphocytes and Natural Killer (NK) cells.

Following injection of the carcinogenic chemical 3-methylcholanthrene, homozygous RAG-2-deficient mice developed tumors more frequently and rapidly than mice with wild-type RAG-2.[51] These and other studies showed that T lymphocytes (both αβ and γδ), B lymphocytes and NK cells play a crucial role in immunosurveillance. Clinical and empirical evidence of this is the increased incidence of carcinomas in immunodeficient populations.[52]

2.1.2 Equilibrium and Escape Phase

The elimination phase of immunosurveillance can only eliminate a significant percentage of transformed cells, while some cells escape the enormous pressure and control exerted by the immune system. There is therefore a latency period between the end of the elimination phase and the actual escape phase in which the tumor occurs, which is the equilibrium phase [53]. In it, the tumour is in a dormant state. In a 2003 study, two patients each receiving a kidney from the same organ donor, treated 15 years earlier for melanoma and considered disease-free at the time of donation, died from the same cancer 1-2 years after transplantation. Probably, neoplastic cells were in a phase of equilibrium within the organ and post-transplant immunosuppressive therapy has eliminated the escape process.[54]

A tumor in the equilibrium phase can undergo three possible phenomena: 1) elimination mediated by SI; 2) permanence in this state thanks to immune control mechanisms; 3) completion of the immunoediting process and transition to the escape phase from the SI. A noteworthy consideration is that the first 2 steps of the immunoediting process could be therapeutic targets of immunotherapy in the future.

Among the mechanisms of tumor immunoevasion there may be: the reduced expression of HLA class 1 proteins, which are involved in the processes of antigen presentation to the SI; the production, by tumor cells, of galectin-1 and indoleamine 2,3 dioxygenase (IDO) that negatively regulate the activation and survival of T lymphocytes [55]; the production of immunosuppressive cytokines such as TGF-β or IL-10; the generation and activation of populations of T cells with immunosuppression functions such as CD4+CD25+ regulatory T lymphocytes (T regs). The latter are thought to play an important role in conferring protection against normal lymphocyte response against tumors.[56]

2.2. Immune Checkpoints

The term immune checkpoints refers to several molecules that have the ability to stimulate or inhibit the immune response through a receptor-binding mechanism. They are expressed in different immune cells, APCs and tumor cells and act mainly by mediating processes related to innate and acquired

immunity, in particular on T lymphocytes. These checkpoints include molecules such as PD-1, PDL-1, LAG3, B7-H3, TIM3, TIGIT [57]. These are molecules that are critical for the tolerance of self antigens and for modulating the intensity and duration of the immune response. Several tumors can express immune checkpoints to facilitate immune evasion, but a role has also been found with regard to metastasis, epithelium-mesenchymal transition, resistance to treatments, self-renewal and antiapoptosis. [58]

Activated T cells have inhibitory receptors such as CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and PD-1 (programmed death protein 1) on their surface, which, by binding their respective ligands, transmit an inhibitory signal that attenuates the activation and effector functions of these cells. Many cancers, for example, evade the immune response by taking advantage of the expression of PD-L1, which is recognized by lymphocyte PD-1.

2.3 Checkpoints Inhibitors

In 2011, the FDA approved the use of ipilimumab in the treatment of mestastatic melanoma. This drug is a monoclonal antibody that targets CTLA-4 and prevents it from interacting with its ligand. This was a fundamental step in cancer research, considering that at the time no other treatment improved the survival of patients with this cancer [59].

Another important family of ICIs is the one that blocks the interaction between PD-1 and PD-L1. Currently, it is believed that it may have a wider therapeutic range than CTLA-4 inhibitors.

In 2014, the FDA approved the use of 2 anti-PD-1 monoclonal antibodies for the treatment of melanoma: Nivolumab and Pembrolizumab. In 2015, nivolumab was also approved for non-small cell lung cancer [60]. They are currently used in the treatment of several other metastatic cancers, including first- and second-line head and neck cancers.

It is likely that these treatments will soon be extended to many other types of cancer, depending on the possible positive results of the hundreds of clinical trials currently conducted.

3. PREDICTIVE BIOMARKERS OF RESPONSE

Despite clear improvements in the treatment of HNCs, there is still a high number of patients who do not benefit from immunotherapy. The current interest of immuno-oncology is to find predictive biomarkers of response in order to correctly identify patients who are candidates or not for such therapies, in order to optimize therapeutic strategies. Many clinical trials are trying to identify innovative biomarathoners; currently, in fact, the only biomarker considered in clinical practice to discern patients who may respond to the use of ICI is PD-L1 through the CPS (Combined Positive Score) [61].

In reality, the goal of modern immuno-oncology is to find not only the predictive factors of response, but above all the mechanisms of resistance.

3.1. Tumor Microenvironment (TME)

The tumor microenvironment consists of molecules, cytokines, tissues and different cell subpopulations that, through complex signaling pathways, constantly interact with cancer cells. Different cells include T and B lymphocytes, NK lymphocytes, mast cells, tumor-associated fibroblasts (CAFs), neutrophils, macrophages, myeloid-derived suppressor cells (MDSCs). The combination of these cells, in head and neck tumors of non-responsive patients, contributes to immunosuppression against the antitumor response. Tumour-associated fibroblasts, for example, can promote the development of HNSCC by secreting inflammatory factors, remodeling the extracellular matrix, and stimulating angiogenesis [62]. Myeloid-derived suppressor cells mediate immunoevasion through the release of soluble factors, such as arginase, and cytokines, such as IL-10 and TGF-β, which inhibit T cell proliferation; They also promote tumor progression by inducing angiogenesis and epithelial-mesenchymal transition [63].

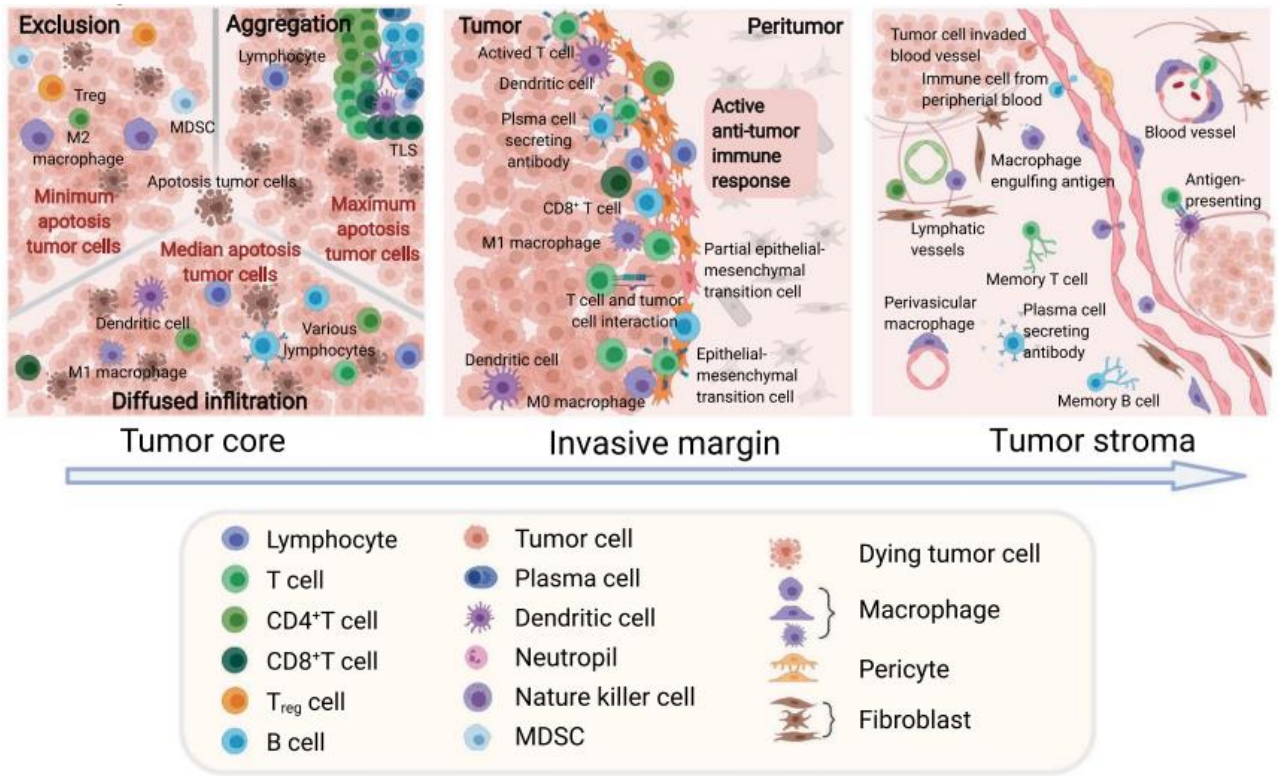


Figure 5: Representative spatial architecture of immune cells in the tumor microenvironment. Primary tumors are divided into the tumor core, tumor stroma, and invasion margin based on tumor compartments.

Fu T. et al. J Hematol Oncol. 2021 Jun 25;14(1):98. doi: 10.1186/s13045-021-01103-4.

As mentioned above, CD8+ lymphocytes play a crucial role by directly killing cancer cells via enzymes such as perforin and granzymes; similarly, CD4+ cells participate in this phenomenon by enhancing immune responses or recruiting other cells to suppress cancer cells. The density of these cells is an important prognostic factor, for example the quantification of CD8+, which constitutes the Immunoscore, can be considered a complementary factor to TNM in different types of tumors.[64]

It is important not only the number of lymphocytes present, but also the phenotype. There are 3 different patterns:

1) Immune-Inflamed, characterized by the presence of CD4+, CD8+ and M1 macrophages in the tumor parenchyma, in the vicinity of tumor cells; It should be noted that both immune cells and tumor cells express PD-1 and PD-L1 and other immune checkpoints, which testify to the tumor's attempt to escape in the face of pressure exerted by the immune system. These tumors have a better response to immunotherapy.

2) Immune-Excluded, in which immune cells infiltrate the stroma but not the parenchyma. A mechanism linked to TGF-β has been found: studies in preclinical models have established that by blocking this signaling pathway it is still possible to convert it to an Inflamed pattern.
3) Immune-Desert, in which there is the absence of lymphocytes in both the stroma and the parenchyma. In these patients, ICI therapy has very poor results [65].

In addition, in head and neck cancers, TILs can be dysfunctional, constituting the so-called "exhausted" phenotype. This is due to several mechanisms, including the increase of several immune checkpoints (PD-1, LAG.3, TIM-3 and CTLA-4) and the action of T-regulatory lymphocytes.
One of the mechanisms studied that causes CD8+ dysfunction is the high level of Regulatory T Lymphocytes (T-reg), both in circulation and infiltrating the tumor. They are a subpopulation of CD4+ lymphocytes, characterized by the $CD4^{+CD25+FOXP3+}$ immunophenotype, which, physiologically, play a crucial role in regulating and containing SI activity to avoid autoimmunity.
This phenomenon, however, is also exploited by several tumors to reduce the activity of SI against neoplastic cells: Tregs are recruited in TME, in which TGF-β, especially in the advanced stages of HNC, increases its immunosuppressive activity, increasing the levels of IL-10 with anti-inflammatory action and reducing the activity of TILs. Despite this, it has been found that high levels of FOXP3+ infiltrate are associated with a better prognosis, probably because, in this case, they are directly related to a massive lymphocyte response against the tumor.[66]

3.2 CD137+ and sCD137 T lymphocytes

Among the biomarkers of greatest interest in the context of immunotherapy is CD137 (4-1BB), a molecule belonging to the TNFR family (Tumor Necrosis Factor receptors), expressed by activated T lymphocytes, dendritic cells, monocytes, neutrophils, B lymphocytes and NK [67-68] cells. The binding between CD137 and its ligand (CD137L), expressed by antigen-presenting cells (APCs), induces bidirectional activation of intracellery signaling pathways. In T lymphocytes, this binding induces cell division and survival and increases the effector functions of both CD8+ T lymphocytes, promoting the demethylation of the main CD8 genes and promoting cytotoxic activity and cytokine release (IFNγ, TNFα, and IL2), and CD4+ T lymphocytes, inducing the production of Th1 cytokines. In APCs, it promotes maturation and survival and increases the ability to present antigen [69-70]. This biomarker also identifies tumor-specific T lymphocytes that develop naturally in mouse models and humans, highlighting the importance of the CD137+ T cell subpopulation in inducing the antitumor immune response. In fact, several studies have shown that high levels of infiltrating and circulating CD137+ T lymphocytes correlate with increased survival in patients with lung, ovarian and liver

cancer and with response to immunotherapy treatment [71-73]. Completely different is the function of the soluble form of CD137 (sCD137) which is released, as a result of alternative splicing, by overactivated immune cells and cancer cells. Its interaction with CD137L prevents dendritic cell maturation and T-cell activation, and elevated circulating levels of sCD137 were associated with minor PFS and OS. Moreover, in mouse models, the interaction between this molecule and CD137 agonist antibodies leads to both the reduction of sCD137 levels and the attenuation of the activating capacity of these antibodies, favoring the success of the treatment.

3.3 Ecto-5′-nucleotidase (NT5E), CD73

Ecto-5′-nucleotidase (NT5E), better known as CD73, is a GPI-anchored ecto-nucleotidase that plays a critical role in the establishment of an immunosuppressive tumour microenvironment by promoting the catabolism of extracellular ATP to adenosine [74]. The latter dampens the anti-tumour immune response by suppressing the effector cell functions and stabilizing immunosuppressive regulatory cells [75], essentially through A2a [76] and A2b receptors [77]. CD73 is expressed on stromal, tumour and infiltrating immune cells [78], and it is upregulated on regulatory T cells in response to adenosine signalling itself [79] and to hypoxia [80]. In addition to its enzymatic function, CD73 promotes cancer invasiveness and metastatic properties by regulating cell-matrix interactions [81, 82].
Previous studies have shown that CD73 is expressed in several cancer types, including breast, colorectal, non-small-cell lung cancer, glioblastoma, and melanoma [83]. High CD73 expression is associated with poor prognosis and decreased response to chemotherapy in different cancers [84, 85]. However, the possible role of this molecule in the specific setting of immunotherapy is still unknown.

4. Aims

Despite the multimodality treatment for early-stage tumours, disease recurrence and/or metastasis (R/M) are frequent and commonly associated with a poor prognosis [86]. The advent of immune checkpoint inhibitors (ICIs) has remarkably changed the management of R/M HNSCC. Immunohistochemical expression of PD-L1 with a CPS ≥1 is required for the administration of immunotherapy in this setting. However, for still unknown reasons, only a relatively small subset of patients (15-20%) really benefits from immunotherapy [87]. For this reason, the identification of novel biomarkers correlated to tumorigenesis and neoplastic progression is a possible strategy to stratify patients' prognosis in this therapeutic setting [88].

New robust data are required to develop and validate molecular and genetic predictive biomarkers able to define immunologically cold and hot tumor allowing to detect responders or no responders

patients in clinical practice. The advent of immunotherapy in clinical practice has led to the urgent need to implement a dynamic and personalized approach to the cancer patient in order to adapt the therapeutic strategy to the peculiar and specific state of the immune system characterizing both patients and tumor microenvironment.

The primary endpoint of our study is to identify the association between the expression of CD73 by neoplastic and/or immune cells and progression free survival (PFS), overall survival (OS), objective response rate (ORR) and early progression (EP) in patients with R/M HNSCC treated with anti-PD-1 immunotherapy.

The secondary endpoint of our study is to investigate the association between the levels of circulating CD137+ T cells and progression free survival (PFS), overall survival (OS), objective response rate (ORR) and early progression (EP) in patients with R/M HNSCC treated with anti-PD-1 immunotherapy.

5 Materials and Methods

Before treatment, patients were clinically staged with contrast enhanced computerized tomography (CT) scan and magnetic resonance imaging (MRI). All patients were discussed and judged as non-eligible for local/regional treatments by the multidisciplinary team of our hospital. The final version of the protocol was approved by the Institutional Ethics Committee (Ethical Committee no. 4421, "Sapienza University"). Anonymized data including age, sex, ECOG PS, comorbidities, history of tobacco smoking, alcohol abuse and primary tumour sites were collected. Based on PS, related symptoms, age, nutritional status and comorbidities, patients were judged as either frail or clinically fit and therefore scheduled for the two different treatments (either chemotherapy plus immunotherapy or immunotherapy alone).

According to the KEYNOTE 048 TRIAL [89], three weekly treatment sessions of cisplatin (100 mg/ m2 of body-surface area) on day 1, or carboplatin (at an area under the curve of 5 mg/ml/minute) on day 1, plus fluorouracil (1000 mg/m2 /day for 4 days), plus pembrolizumab at flat dose of 200 mg, were administered every 21 days for a maximum of 6 cycles to patients considered fit at the baseline clinical evaluation. Pembrolizumab monotherapy at flat dose 200 mg was administered intravenously

to those patients deemed frail and unfit for the combination regimen. Patients who achieved at least stable disease (SD) as their best response were maintained on Pembrolizumab.

Tumour response was assessed every 12 weeks using Immune Response Evaluation Criteria in Solid Tumors (iRECIST) guidelines and classified as complete response (CR), partial response (PR), SD, and progressive disease (PD). Early progression (EP) was defined as the progression of disease occurring in the first three months. Objective response rate (ORR) was defined as PR plus CR. Toxicities were recorded at day 1 of every cycle and classified according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.0). Progression-free survival (PFS) was defined as the time from the administration of treatment until the first progression or treatment death. The overall survival (OS) was defined as the time from patient registration to death from any cause.

5.1 Materials and Methods

From February 2021 to July 2022, 50 patients affected by R/M HNSCC with CPS ≥1 were treated with first line immunotherapy or immunotherapy in association with chemotherapy. Slides for PD-L1 evaluation were immunostained with SP263 clone on a Ventana BenchMark Ultra.

5.1.1 Evaluation of CD73 expression

Sections representative of the paraffin-embedded tissue from 32 core biopsies and 18 surgical samples were used for the evaluation of CD73 expression on neoplastic cells and immune cells (including lymphocytes, monocytes/macrophages and polymorphonuclear leukocytes). CD73 immunostaining was performed using the D7F94 clone (catalogue #13160, rabbit IgG, dilution 1:200, Cell Signaling Technology, Danvers, MA, USA Netherland). Positive controls (lung adenocarcinoma and squamous cell carcinoma) were used for each run of staining. Negative controls were obtained by omitting the primary antibody. All neoplastic and immune cells present in each sample were evaluated. The presence of at least 200 viable neoplastic cells was used as inclusion criteria. Each case was scored independently by 2 pathologists (BC and GDA) in a blind manner. Disagreements were resolved with the help of a third pathologist trained for PD-L1 evaluation.

As previously reported [84] positivity on neoplastic cells for CD73 was defined as the presence of membrane immunostaining, either strong or weak, with or without cytoplasmic staining. The positivity on immune cells was defined on the same line. The percentage of stained neoplastic and immune cells (0-100%) was recorded and the median value of CD73 expression was then calculated for each type of cell (neoplastic and immune cells).

The study samples were subgrouped respectively as: "low-neoplastic CD73" (L-nCD73) and "high-neoplastic CD73" (H-nCD73), and "low-immune CD73" (L-iCD73) and "high-immune CD73" (H-iCD73), based on CD73 expression levels below or equal and above the median value.

5.1.2 Statistical analysis

In the descriptive analysis, quantitative variables were described as mean and range, while qualitative variables as number and percentage. The association between CD73 expression and CPS or EP was evaluated using the $\chi 2$ test. To determine factors associated with EP, univariate and multivariate logistic regression models were used. Results of both univariate and multivariate analysis were expressed as Odds Ratios (OR) and 95% confidence intervals (CIs). All analyses were performed using IBM SPSS Statistics for Window Version 23.0 (Armonk, NY, USA) or GraphPad Prism (GraphPad, Inc, San Diego, CA, USA).

5.2 Materials and Methods

From March 2021 to March 2023 forty patients with recurrent or metastatic HNSCC were enrolled and the follow-up was monitored for 24 months. Patients were mainly treated with pembrolizumab or pembrolizumab plus chemotherapy using standard doses and schedules until disease progression or unacceptable toxicity. Toxicity was reported according to Common Terminology Criteria for Adverse Events (version 4.0) and was evaluated on day 1 of every cycle until the end of treatment.

Criteria for inclusion were age >18 years; histologically documented diagnosis of HNSCC of the oral cavity, oropharynx, larynx, salivary glands, and nasopharynx; and ECOG performance status (PS) scored between 0 and 2. Exclusion criteria were autoimmune disease, systemic immunosuppression,

and any significant comorbidity. PFS, OS, and CBR were evaluated. PFS was defined as the time immunotherapy began until the first documented tumour progression or death from any cause. OS was defined as the interval between the beginning of immunotherapy to death from any cause. The response was assessed every month until disease progression using immune-related Response Evaluation Criteria in Solid Tumours (i-RECIST) and classified as a complete or partial response and stable or progressive disease. The CBR was used to classify patients as responders (patients with a complete or partial response and stable disease) and non-responders (progressors) after 6 months of therapy. CPS was defined as the number of PD-L1-positive cells, including tumour cells, lymphocytes, and macrophages, divided by the total number of tumour cells x 100. The study was conducted following the Declaration of Helsinki and good clinical practice guidelines. All patients provided signed informed consent (RIF.CE: 4181).

5.2.1 PBMCs Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples of 40 HNSCC patients using Ficoll Hypaque (lympholyte-H, Cedarlane, Burlington, VT, Canada) before the immunotherapy began (T0). Cells were cryopreserved until use.

5.2.2 Cytofluorimetry

PBMC phenotyping was carried out via cytofluorimetry combining the following conjugated anti-human monoclonal antibodies (MoAbs): anti-CD3 BV510 (clone HIT3a), anti-CD8 APC-H7 (clone SK1), and anti-CD137 APC (clone 4B4-1). All antibodies were purchased from Becton Dickinson (San Diego, CA, USA). Live and dead cells were stained using a LIVE/DEAD fixable yellow dead cell staining kit (Invitrogen, Waltham, MA, USA).

The autofluorescence of the cells and fluorescence minus one (FMO) were used as negative controls for the expression of CD137. Samples were analysed using a FACSCanto II flow cytometer and analysed by FlowJo (version 10.8.8, Becton Dickinson) analysis software.

5.2.3 Statistical Analysis

Descriptive statistics (median, range, and percentages) of the clinical and biological characteristics of HNSCC patients were analysed. Student's t-test was used to compare two groups of data. The impacts of clinicopathological variables on OS and PFS were analysed via univariate followed by multivariate analyses (UVA and MVA, respectively).

With regards to UVA, HNSCC patients' OS and PFS were analysed using the Kaplan–Meier method and log-rank tests. The optimal cut-off values of CD137 for OS or PFS were those that corresponded to the minimum p-value for each endpoint among those calculated using Kaplan–Meier curves, varying the threshold of CD137 from the minimum to the maximum values determined in our cohort. The clinicopathological variables deemed of potential relevance in the univariate analysis (corresponding to a cut-off of $p < 0.10$) were included in the multivariate Cox proportional hazards regression analysis to identify the prognostic variables. The sample size calculation was performed assuming an level of 0.05 and a level of 0.20 (power 80%). With these assumptions, the required sample size was 17 cases in each group (i.e., a total of 34 cases) to detect a percentage difference of at least 1.5% between groups, and having standard deviations of 1% and 2%, in the groups with good and poor prognosis, respectively. The simple size was increased to 40 patients to take patient loss at follow-up into consideration.

6.1 Results

Clinical and pathological features of the study population are listed in *Table 3*. Briefly, thirty-seven patients were male (74%), 13 female (26%) and median age was 67.5 years (range 38-87). Pre-treatment ECOG PS was 0 in 10 patients, 1 in 24 patients and 2 in 16 patients. Smoking habit as well as previous alcohol abuse were reported in 38 (76%) and 33 (66%) of patients, respectively.

The primary tumour site was the oral cavity in 31 patients (62%), the larynx in 13 patients (26%), nasal cavity in 3 cases (6%), hypopharynx in 2 patients (4%) and oropharynx in the remaining 1 patient (2%). Thirty-one patients (62%) had visceral metastases. Thirty patients (60%) received

pembrolizumab in monotherapy while 20 (40%) patients received the association of immunotherapy with chemotherapy (*Table 3*). Early progression (EP) disease occurred in 26 patients (52 %).

In all cases the histological type was squamous cell carcinoma, most frequently with a moderate (37/50: 74%) or poor (12/50 24%) degree of differentiation.

Tissue samples were obtained from the primary tumour in 39 cases (78%), being the oral cavity the most common site (n= 31, 62%) and from metastatic lesions in the remaining 11 (22%). In particular, the examined metastatic sites included lung (n=5; 45%), lymph nodes (n=4; 36%) and subcutaneous tissue (n= 2; 18%).

Human papilloma virus (HPV) status was evaluated in 19 out of the 31 oral cavity tumours with a positivity in only 1 patient.

All cases were scored as CPS ≥1. According to clinically relevant cut-off, we further stratified our study sample in two groups: "low-CPS" (L-CPS), and "high-CPS" (H-CPS), with CPS expression ≤ 20 or >20, respectively. Based on this criterion, 21 patients (42%) were classified as L-CPS and 29 cases (58%) as H-CPS.

Thirty patients (60%) received Pembrolizumab in monotherapy while 20 (40%) received the association of immunotherapy with chemotherapy.

The median PFS was 3 months in the overall population; median PFS was 3 and 4 months in the monotherapy and combo group, respectively. On the other hand, median OS was 6 months in both groups; in particular, median OS was 6 and 5.5 months in the monotherapy and combo group, respectively.

Table 3

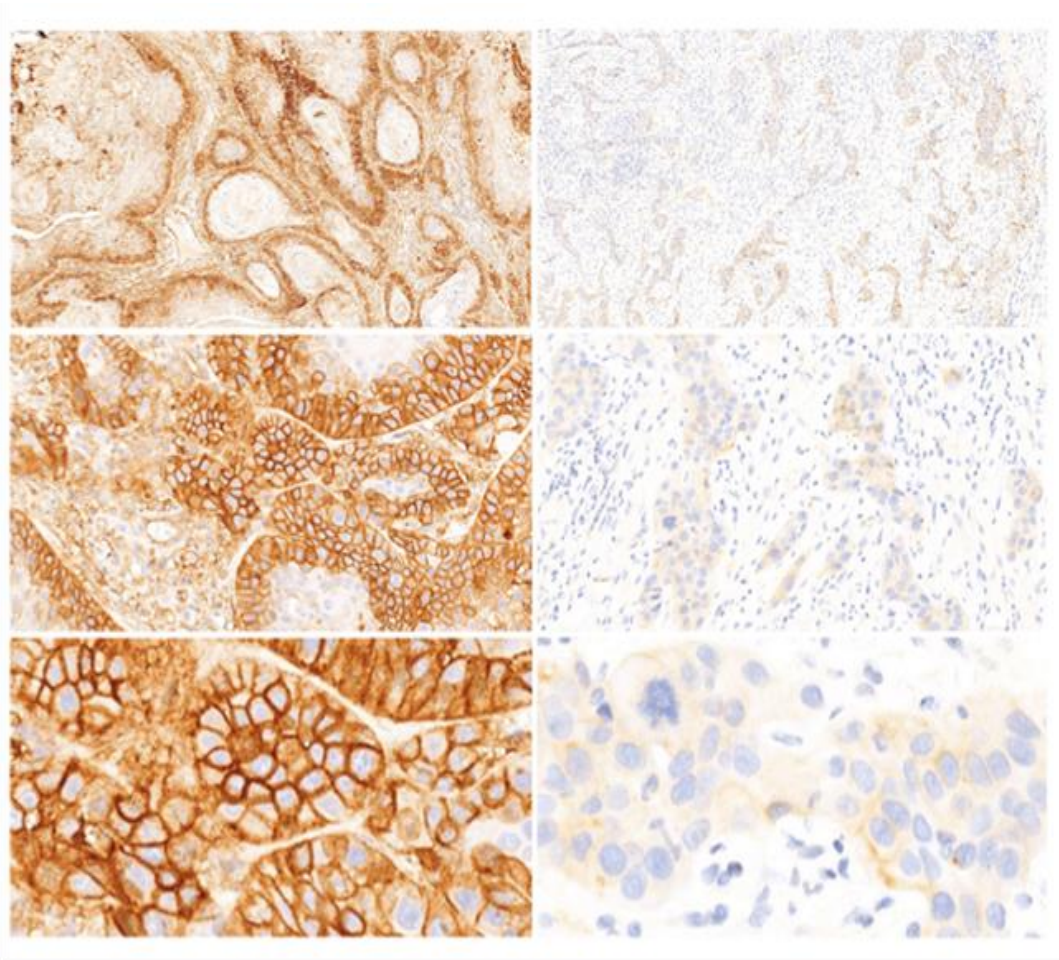
Clinico-pathological features of the study population

Characteristic	N (%)
Sex	
Male	37 (74)
Female	13 (26)
Age – yr	
Median (range)	67.5 (38-87)
<65 yr	*22* (44)
65-75 yr	14 (28)
>75 yr	14 (28)
ECOG Performance Status	
0	10 (20)
1	24 (48)
2	16 (32)
Primary tumor site	
Oral Cavity	31 (62)
Larynx	13 (26)
Nasal cavity	3 (6)
Hypopharynx	2 (4)
Oropharynx	1 (2)
Nuclear grading	
G1	1 (2)
G2	37 (74)
G3	12 (24)
Primary/Metastatic tumor	
Primary	39 (78)
Metastasis	11 (22)
Smoking habits	
Yes	38 (76)
No	12 (24)
Previous alcohol abuse	
Yes	33 (66)
No	17 (34)
Therapy for metastatic disease	
CT + Pembrolizumab	20 (40)
Pembrolizumab	30 (60)
Visceral metastases	
No	19 (38)
Yes	31 (62)
Early Progression	
No	24 (48)
Yes	26 (52)
nCD73	
*≤ median v*alue	26 (52)
> median value	24 (48)
iCD73	
≤ median value	29 (58)
> median value	21 (42)
CPS	
≤ 20	21 (42)
> 20	29 (58)

6.1.1 Expression of CD73 on neoplastic cells

The median expression value of CD73 on neoplastic cells was 32% (range 0–80%). The level of CD73 expression was substantially homogeneous in the surgical specimens as compared with biopsy samples. Twenty-six cases (52%) were below the median value and were classified as L-nCD73 and 24 (48%) were in the H-nCD73 group. Absence of CD73 immunostaining was recorded in 9/26 (34.6%) of the L-nCD73 cases. Representative histological images of L-nCD73 and H-nCD73 are shown in *Figure 6*. There was no significant correlation between the immunohistochemical expression of CD73 on neoplastic cells and the CPS value (*Table 4A*), the degree of tumor differentiation and the site of the lesions (primary/metastasis). No statistically significant differences were found between CD73 expression and specific subsites.

Figure 6



Staining intensity of CD73 on neoplastic cells in HNSCC samples. Representative images of High-CD73 (4X, 10X, 20X original magnification) and Low-CD73 (4X, 10X, 20X original magnification).

Table 4A

Relation between CD73 expression on neoplastic cells and CPS value

	L-CPS N (%)	**H-CPS N (%)**
L-nCD73 (N=26)	9 (34,6)	17 (65,4)
H-nCD73 (N=24)	12 (50)	12 (50)

6.1.2 Expression of CD73 on immune cells

The median expression value of CD73 on immune cells was 10% (range 0–80%). Twenty-nine cases (58%) were below and 21 (42%) were above the median value. Absence of CD73 immunostaining was recorded in 2 out of the 29 patients of the L-iCD73 group. There was no significant correlation between the immunohistochemical expression of CD73 on immune cells and the CPS value *(Table 4B)*, the degree of tumor differentiation and the site of the lesions (primary/metastasis) and the different subsite of the lesion.

Table 4B

Relation between CD73 expression on immune cells and CPS value

	L-CPS N (%)	**H-CPS N (%)**
L-iCD73 (N=29)	11 (37,9)	18 (62,1)
H-iCD73 (N=21)	10 (47,6)	11 (52,4)

6.1.3 Association of CD73 with Early Progression Disease

Early progression was recorded in 26 patients (52%). In the H-nCD73 group the majority of patients (66.6%) experienced an early progression as compared with 38,5% in the Low-nCD73 category (p=0.043) (*Table 5A*).

The expression of CD73 on immune cells was not significantly associated with EP (*Table 5B*).

On both univariate and multivariate analysis only the expression of CD73 on neoplastic cells was correlated with evidence of early disease progression (p = 0.049; p = 0.033 respectively) (*Table 6*).

No significant association was recorded between early progression and the CPS value.

Table 5A

Relation between CD73 expression on neoplastic cells and early progression (EP)

	EP No N (%)	**EP Yes N (%)**
L-nCD73 (N=26)	16 (61,5) *	10 (38,5)
H-nCD73 (N=24)	8 (33,3)	16 (66,6)

**p*=0.043 (Test del Chi-Quadro/Fisher)

Table 5B

Relation between CD73 expression on immune cells and early progression (EP)

	EP No N (%)	**EP Yes N (%)**
L-iCD73 (N=29)	17 (58,6)	12 (44,8)
H-iCD73 (N=21)	7 (33,3)	14 (66,6)

Table 6

Univariate and multivariate analysis of factors associated with early progression (EP)

	p value	Odds ratio (95% CI)	*p* value	Odds ratio (95% CI)
Age <50 50+	0.707	0.7 (0.11-1.57)	0.723	1.46 (0.18-11.99)
Nuclear grading G1 G2 G3	0.434	1.64 (0.48-5.61)	0.37	0.53 (0.13-2.11)
Treatment Pembrolizumab CT+Pembrolizumab	0.420	1.6 (0.51-4.99)	0.363	0.56 (0.16-1.96)
CD73 on neoplastic cells ≤ median value > median value	0.049*	3.2 (1-10.2)	0.033*	0.26 (0.08-0.89)
CPS ≤ 20% > 20%	0.598	1.35 (0.44-4.17)	0.466	0.63 (0.18-2.2)

*$p<0.05$ statistically significant values

6.2 Results

Forty patients with recurrent or metastatic HNSCC were enrolled (*Table 7*).

All patients had CPS ≥ 1 and underwent pembrolizumab as monotherapy (67%) or pembrolizumab plus chemotherapy (33%). Before starting anti-PD1 treatment, 38% of patients were scored as performance status (PS) = 0 and 42% as PS = 1. Only eight patients (20%) were defined as PS = 2. The oral cavity was the primary tumour site in most patients (60%), followed by the larynx (20%), oropharynx (10%), salivary glands (8%), and nasopharynx (2%). CPS ≥ 20 was observed in 58% of tumour samples. Most patients were HPV-negative (95%) and current or former smokers (75%); approximately half of the patients (58%) denied alcohol consumption. The clinical benefit rate (CBR) was used to classify responder (R) (38%) and not-responder (NR) (62%) patients to an anti-PD-1 treatment. A total of 25 patients suffered disease progression 6 months after beginning immunotherapy, while 5 and 10 patients showed a stable or partial response, respectively.

The median PFS and OS were 3.5 and 10 months, respectively.

Table 7

Patients' characteristics

	n° (%) 40 (100)
Sex:	
Male	31 (78)
Female	9 (22)
Age:	
Median range	71 (42–87)
< 65	17 (43)
>65	23 (57)
EOCG Performance Status:	
0	15 (38)
1	17 (42)
2	8 (20)
Primary tumour site:	
Oral cavity	24 (60)
Larynx	8 (20)
Oropharynx	4 (10)
Salivary glands	3 (8)
Nasopharynx	1 (2)
CPS:	
>1	40 (100)
>20	23 (58)
Therapy for metastatic disease:	
Pembrolizumab	27 (67)
CT+ Pembrolizumab	13 (33)
HPV status:	
negative	38 (95)
positive	2 (5)
Smoking status:	
smoker (current/former)	30 (75)
non-smoker	10 (25)
Alcohol consumption:	
yes (moderate/high)	17 (42)
no	23 (58)
Response to treatment:	
yes	15 (38)
no	25 (62)

CPS: PD-L1 combined positive score; CT: chemotherapy.

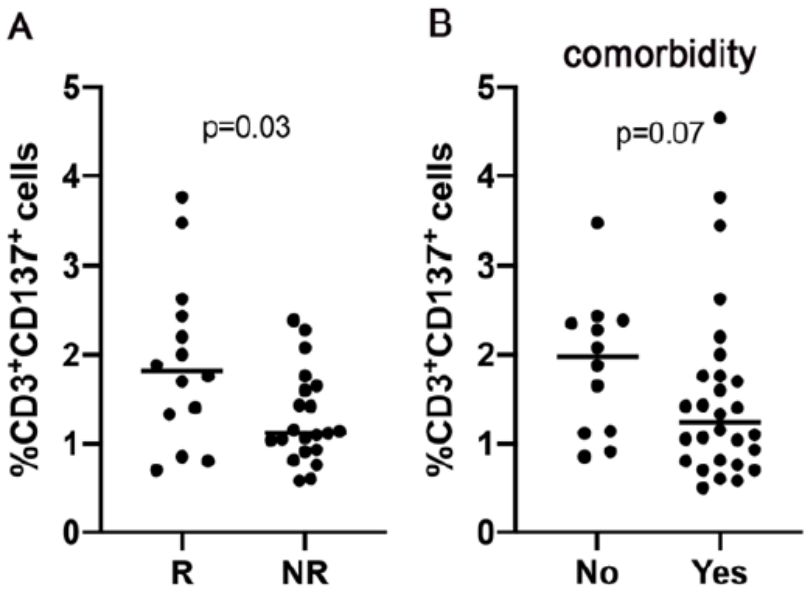
6.2.1 High Circulating Levels of CD137+ T Cells Are Associated with Response to Pembrolizumab Treatment

To understand the role of circulating CD137+ T cells in (R/M) HNSCC patients undergoing pembrolizumab treatment, we evaluated the levels of CD137+ T cells derived from the peripheral blood of the 40 cancer patients before beginning immunotherapy.

The results demonstrate that high levels of circulating CD137+ T cells are correlated with (R/M) HNSCC patients' response to pembrolizumab treatment ($p = 0.03$) (*Figure 6A*). Indeed, responding patients showed a significantly higher percentage of CD3+CD137+ than non-responders (R vs. NR; 1.9 ± 0.24 vs. 1.2 ± 0.1, $p = 0.03$). This difference could not be ascribed to CD8+CD137+ or CD4+CD137+ T cells, which did not show any significant association with clinical response (CD8+CD137+: R vs. NR: 0.9 ± 0.1 vs. 0.78 ± 0.1, $p = 0.5$; CD4+CD137+: R vs. NR: 0.9 ± 0.1 vs. 0.73 ± 0.1, $p = 0.3$). Moreover, no difference in the percentage of CD137+ T cells was found when patients were analysed according to treatment (pembrolizumab alone vs. pembrolizumab plus chemotherapy, $p = 0.3$).

The levels of CD137+ T cells were also found to be correlated with several clinical parameters, i.e., PS, presence of visceral metastasis, CPS ≥ 1, CPS ≥ 20, comorbidities, toxicity, and previous therapies. No significant correlation was found between the number of CD137+ T cells and any of these parameters; however, the patients with no comorbidity tended to have higher levels of CD137+ T than those with worse clinical status (*Figure 6B*) (no comorbidity vs. comorbidity: 1.9 ± 0.2 vs. 1.2 ± 0.2, $p = 0.07$). All these data suggest that the subset of CD137+ T cells could be considered as a potential biomarker for the response of (R/M) HNSCC patients to pembrolizumab treatment and serve as a useful tool to identify patients for whom pembrolizumab treatment would improve clinical status.

Figure 6A *Figure 6B*



CD137+ T cells are correlated with response to anti–PD–1 treatment and patient survival**.**
Figure 6A The scattered dot plot shows the values of CD3+CD137+ cells in responder (R) and non–responder (NR) patients ± standard deviation (SD). The horizontal lines correspond to the median values of CD3+CD137+ lymphocytes of the two groups.
Figure 6B The scattered dot plot represents the values of CD3+CD137+ cells in patients with (Yes) or without (No) comorbidities. The horizontal lines correspond to the median values of CD3+CD137+ lymphocytes of the two groups.

6.2.2 CD137+ T Cells as a Predictive and Prognostic Factor of PFS and OS in (R/M) HNSCC Patients

The levels of circulating CD137+ T cells and several clinical parameters, such as age, sex, CPS value, PS, and presence of visceral metastasis, were further examined by univariate analysis to predict survival *(Tables 8 and 9).* Concerning the analysis of CD3+CD137+ cells, a cut-off of 1.65% was found. The identified cut-off values for OS and PFS correspond to the minimum p-values (OS, $p = 0.02$; PFS, $p = 0.02$). The numbers of patients having CD137 values ≥ or <1.65 were 24 (60%) and 16 (40%), respectively. Patients with a percentage of CD137+ T cells ≥ 1.65% showed an increase in PFS (CD137 ≥ 1.65% vs. CD137 < 1.65: median survival was not reached vs. 2.5 months, $p = 0.02$) and OS (CD137 ≥ 1.65% vs. CD137 < 1.65: median survival was not reached vs. 3.5 months, $p = 0.02$) (Figure 7A). Moreover, univariate analysis of clinical parameters showed that PS was the only

parameter associated with prolonged PFS and OS. Patients scored as PS = 0 had a longer PFS (PS = 0 vs. PS = 1, 2: median survival was not reached vs. 2 months, p = 0.003) and OS (PS = 0 vs. PS = 1, 2: median survival was not reached vs. 3 months, p = 0.0006) compared to patients with PS = 1, 2 (Figure 7B). Multivariate analysis revealed that PS = 0 and the percentage of CD137+ T cells ≥ 1.65% are two independent prognostic factors of PFS and OS (Tables 2 and 3).

Table 8

Predictive and prognostic factors for progression free survival

Variables	Univariate Analysis		Multivariate Analysis	
	HR (95%CI)	*p* Value	HR (95%CI)	*p* Value
Age (<71 vs. ≥71)	0.65 (0.24 to 1.44)	0.25		
Sex (Male vs. Female)	0.53 (0.13 to 1.19)	0.1		
CPS ≥ 1	3.18 (0.89 to 222.3)	0.06		
CPS ≥ 20	0.99 (0.4 to 2.43)	0.99		
Metastasis (Yes vs. No)	1.18 (0.5 to 3.03)	0.65		
PS (0 vs. 1, 2)	0.28 (0.1 to 0.64)	**0.003**	2.2 (1.3 to 3.8)	**0.002**
$CD3^+CD137^+ \geq 1.65$	2.46 (1.1 to 6.3)	**0.02**	0.28 (0.1 to 0.7)	**0.007**

CPS: PDL1 combined positive score; PS: performance status.

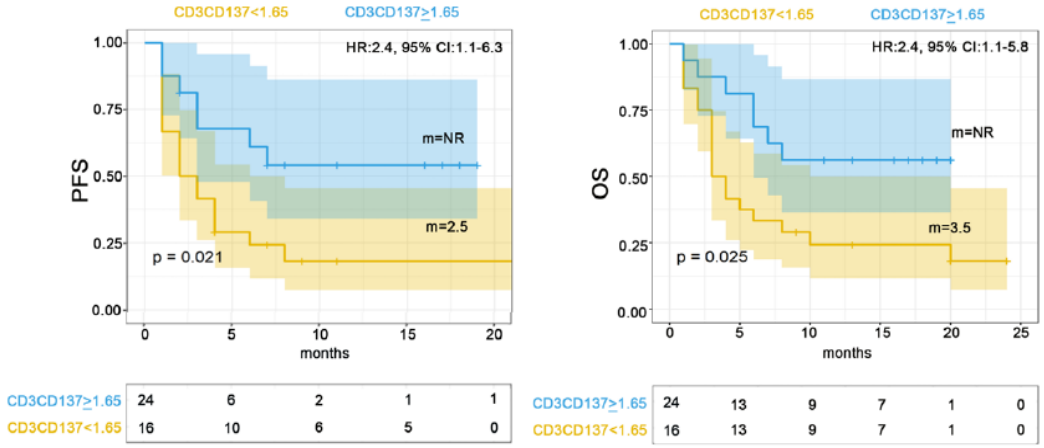
Table 9

Predictive and prognostic factors for overall survival.

Variables	Univariate Analysis		Multivariate Analysis	
	HR (95%CI)	*p* Value	HR (95%CI)	*p* Value
Age (<71 vs. ≥71)	0.58 (0.24 to 1.29)	0.17		
Sex (Male vs. Female)	0.488 (0.14 to 1.13)	0.08		
CPS ≥ 1	3.66 (0.94 to 186.8)	0.05		
CPS ≥ 20	1.01 (0.44 to 2.31)	0.9		
Metastasis (Yes vs. No)	1.16 (0.51 to 2.7)	0.7		
PS (0 vs. 1, 2)	0.23 (0.1 to 0.53)	**0.0006**	2.29 (1.39 to 3.78)	**0.001**
$CD3^+CD137^+ \geq 1.65$	2.24 (1.12 to 5.8)	**0.02**	0.28 (0.11 to 0.69)	**0.006**

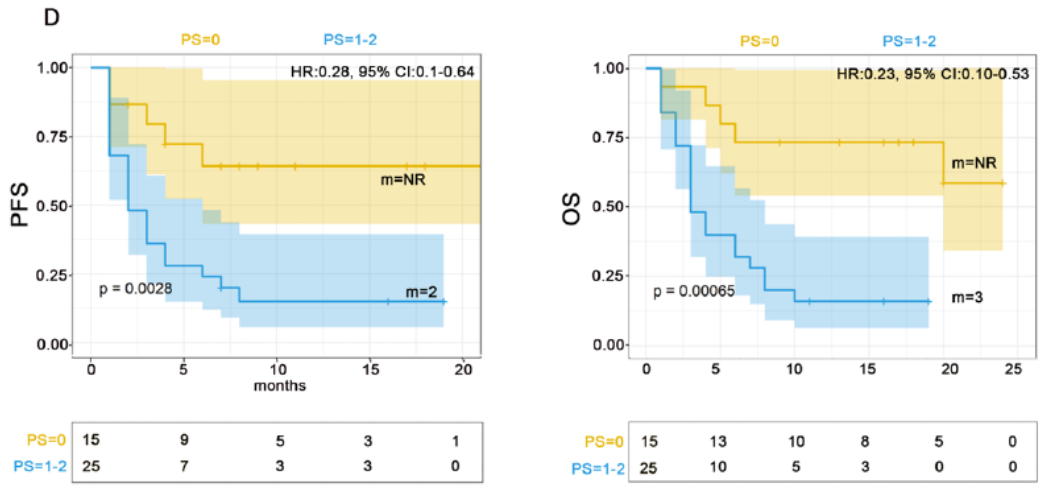
CPS: PDL1 combined positive score; PS: performance status.

Figure 7A



Kaplan-Meier curves for PFS and OS were used to determine 1.65% as the cut-off of circulating CD3+CD137+ cells wherein patients with a percentage ≥ 1.65% showed prolonged survival

Figure 7B



Kaplan–Meier curves for PFS and OS considering the score related to performance status (PS) (PS = 0 vs. PS1 = 1, 2). Patients with PS = 0 showed a longer PFS and OS than those with PS = 1, 2. m = Months; NR = not yet reached.

7. Discussion

In the last few years, expression of CD73 by neoplastic cells has been correlated with tumor invasiveness and metastatic potential in several cancer types [90].

Importantly, CD73 is also expressed on Tregs, NK cells, and macrophages, promoting an immunosuppressive tumor microenvironment [91]. For this reason, it is regarded as a promising target for immunotherapy, either with an anti-CD73 single agent or in combination with chemotherapy, radiotherapy, and other immunotherapeutic agents. Several anti-CD73 monoclonal antibodies are currently tested in clinical trials [92-94].

The role of this promising biomarker has been also investigated in the setting of HNSCC [95,96].

This is a very aggressive neoplasia, with a poor response to conventional treatment regimens and a 40% rate of deaths at 5 years [97]. Recurrent or metastatic HNSCC is even more challenging, and immunotherapy has an emerging role as therapeutic tool, however only 15-20% of patients will benefit from immune checkpoint blockade, either in monotherapy or in association with chemotherapy. Thus, the identification of responder or resistant patients is an urgent clinical need. In view of that, novel tissue biomarkers are envisaged to identify patients more prone to disease progression during immunotherapy. In this setting, CD73 represents a promising molecule, actively investigated in distinct cancer types [98-101]. Our results show the association between high CD73 expression by neoplastic cells and poor prognosis, in line with previous reports in HNSCC [102].

CD73 has also been implicated in drug resistance, although the mechanisms underlying this phenomenon are still unclear. Due to its immunosuppressive activity, CD73 could affect patients' response to immune checkpoint inhibitors. In our study, we tested this hypothesis by correlating the immunohistochemical expression of this biomarker with early progression of recurrent/metastatic HNSCC under immunotherapy. We observed CD73 expression by neoplastic cells in most tumors (41/50, 82%). However, there was a variability in the percentage of tumor cells expressing this biomarker, with 24 cases (48%) showing expression rates above the median value of 32%. Indeed, there was a significant correlation between early disease progression and CD73 expression levels. In

particular, 66% of H-CD73 patients experienced progression within three months of immunotherapy, irrespective of the degree of tumor differentiation, disease status (locally advanced vs metastatic) and CPS value. This result expands our previous observation on TNBC, providing additional evidence that the effect of CD73 in promoting tumor immune resistance could be influenced by the number of neoplastic cells expressing this biomarker. We propose the median value of CD73 expression as a threshold for stratification of R/M HNSCC patients under immunotherapy, based on the observation that this is the only variable significantly correlated with EP, both in univariate and multivariate analysis. Our results provide evidence in support of previous hypothesis on the possible role of CD73 in dampening the response to ICIs therapy.

However, the cut-off value of 32% needs to be validated in a larger cohort of patients.

According to our results, the expression of CD73 by immune cells has no prognostic value in this specific clinic-therapeutic scenario. Perhaps, a deep characterization of immune infiltrate with cell-specific immune markers could be an additional tool to integrate the expression of CD73 on neoplastic cells.

With regard to this study, we propose that the levels of CD137+ T cells prior to immunotherapy, as a biomarker of immune activation, is able to predict the response and clinical outcome of (R/M) HNSCC patients to pembrolizumab treatment. Moreover, we identify the CD137+ T cell subset as an independent prognostic factor of survival, suggesting that the presence of this immune population represents a crucial point for successful anti-PD-1 immunotherapy administered as first-line treatment. Interestingly, by combining the levels of CD137+ T cells and PS, it is possible to better define the profile of (R/M) HNSCC patients with longer survival.

In line with our results, several other studies have identified circulating CD137+ T cells as predictive biomarkers in different cancer settings. In advanced renal carcinoma, CD137+ lymphocytes are a predictor of the clinical response to tyrosine kinase inhibitors [103]. In non-small cell lung carcinoma, patients with early progression show decreased levels of circulating CD137+ T cells, together with

high levels of the IgM-rheumatoid factor [104]. In melanoma patients, CD137+CD8+ T cells are associated with a disease-free status [105]. In addition, we recently demonstrated and validated, in a cohort of 109 patients with different metastatic solid tumours, that high levels of circulating CD137+ T cells (cut-off: 1.2%) are a prognostic factor for PFS and OS, and that CD8+CD137+ T cells are a prognostic factor for PFS. We also showed that patients with a complete response to anti-PD-1 immunotherapy had high levels of CD137+ cells in the tumour microenvironment within tertiary lymphoid structures surrounding the tumour mass [106]. In the present study, the critical cut-off value of CD137+ T cells for indicating survival was identified as 1.65%. Moreover, the analysis carried out on CD8+CD137+ and CD4+CD137+ T cells revealed that these cellular subsets, examined as single populations, do not seem to have a particular impact on the induction of an anti-tumour immune response. These data disagree with those of several authors' suggesting a primarily role for CD137 in CD8 T cells [107,108]. However, several others demonstrated that both CD8+CD137+ and CD4+CD137+ T cells contribute, with similar efficacy, in responses against tumours. Indeed, triggering the CD137 pathway promotes the development of cytotoxic activity in CD8+ T cells and the induction of a Th1 response in CD4 T cells, which boosts effector anti-tumour functions [109-111]. All this evidence suggests that CD137+ T cell levels and the prevalence of a specific CD137+ T cell subset can vary according to both the tumour's histotype and the TME. In particular, in HNSCC, high levels of activated tumour-specific T cells are required to overcome the immune suppression induced by tumour mass, and the synergistic involvement of both CD8+CD137+ and CD4+CD137+ T cells is particularly important in this type of tumour, which is characteristic of a highly immunosuppressive milieu [112].

Moreover, most patients (95%) analysed in this study were HPV-negative. These patients had worse prognoses, and were characterised by a TME with a low number of TILs and high infiltration of immunosuppressive cells, confirming the need to strongly activate the immune response to obtain an efficacious immune response. In addition, the cut-off value of 1.65% for CD137+ T cells was used to analyse the survival rates of patients treated with pembrolizumab and pembrolizumab plus

chemotherapy CD137+ T cells seemed to distinguish patients with a longer OS only in the pembrolizumab group.

However, the difference in the pembrolizumab plus chemotherapy patients was not found to be statistically significant due to the limited number of patients included in this group.

The fundamental role of the CD137+ T cell subset in the induction of anti-tumour immunity was further demonstrated in earlier studies of mice models in which agonistic anti-CD137 antibodies were employed. In those studies, antibodies were demonstrated to increase the levels of anti-tumour-specific memory T cells, induce a long-lasting immune response [113], and reduce immunosuppression by decreasing the amount of regulatory Tregs and MDSCs [114]. In addition, clinical studies have demonstrated that the agonistic monoclonal antibody anti-CD137, urelumab, triggers the activation of IFN signalling and pro-inflammatory cytokines [115]. All this evidence highlights the importance of CD137+ T cells as key contributors to the anti-tumour immune response and as a novel protagonist of immune-based approaches.

To date, PD-L1 CPS is the most widely used biomarker for guiding the selection of (R/M) HNSCC patients for treatment based on predicted response, although with contradictory evidence. Its role as a biomarker has been demonstrated in different phase III clinical trials (KEYNOTE 040 and KEYNOTE 048) [116-89], in which it was observed that PD-L1+ patients showed increased survival. However, several other studies have shown similar therapeutic benefits for both PD-L1positive and PD-L1 negative patients, with no significant difference in overall survival, suggesting that PD-L1 alone does not adequately distinguish which patients will benefit from treatment [117]. In our study, all patients were PD-L1 CPS-positive and underwent immunotherapy. However, most patients (62%) were nonresponders with poor survival, confirming that in our cohort of patients, the expression of PD-L1 alone was not sufficient for optimal patient selection.

Discordant results across studies could be ascribed to several factors. The most relevant is linked to the absence of uniformity in the assays and variability in the threshold [118].

Different PD-L1 antibodies have been used in the numerous assays with significant variation in the percentage of positive immune and tumour cells [119]. Moreover, the current guidelines do not specify the timing of the analysis, the location of biopsy, or the volume of tumour samples associated with the incorrect classification of PD-L1 expression in tumour tissue. In addition, PD-L1 signalling can be regulated by several pathways, such as PI3K, Akt/PKB, and MAPK; these are frequently altered in HNSCC patients, leading to PD-L1 being subjected to extreme temporal variation and spatial heterogeneity [120]. This heterogeneity could also be dependent on any previous therapy, such as chemotherapy, that increases PD-L1 levels [121].

Beyond PD-L1 CPS, most potential biomarkers examined in HNSCC are derived from tumour tissue analysis [122]. The major limitation of these approaches is the availability of material that cannot be analysed over time. Instead, the analysis of circulating CD137+ T cells overcomes all of these critical issues and provides information regarding the activation state of a patient's immune system in real time. Moreover, consecutive blood withdrawals could be used to predict the efficacy of therapy and to monitor disease development. This analysis can be also standardised and easily employed in a hospital setting to monitor the immune fitness of cancer patients during anti-tumour therapy.

In conclusion, in this study, we identified CD137+ T cells as a potential biomarker to predict the success of pembrolizumab treatment and longer survival in (R/M) HNSCC patients. Although the number of patients was limited, these results are particularly impor tant in this patient group, in which PD-L1 CPS does not represent a reliable immunological parameter for patient selection. We believe that the predominance of this cellular subset could be used by oncologists to monitor the response to pembrolizumab treatment as upfront therapy. Further studies with large cohorts that include nivolumab-treated patients will be conducted to validate these data.

Conclusions

In the novel scenario of precision immune-oncology our findings are also relevant in view of the increasing importance of CD73 as a target for specific monoclonal antibodies, either as single therapy or in combination with a PD-1 inhibitor in patients with locally advanced or metastatic solid tumors, including HNSCC. In this setting, CD73 expression levels by neoplastic cells could help to identify patients more prone to respond to these target therapies.

With regard to CD137+ T cells, we identified as a potential biomarker to predict the success of pembrolizumab treatment and longer survival in (R/M) HNSCC patients. Although the number of patients was limited, these results are particularly important in this patient group, in which PD-L1 CPS does not represent a reliable immunological parameter for patient selection. We believe that the predominance of this cellular subset could be used by oncologists to monitor the response to pembrolizumab treatment as upfront therapy. Further studies with large cohorts that include nivolumab-treated patients will be conducted to validate these data.

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