

# Immunohistochemical expression of Podoplanin and P16-INK in Oral Squamous Cell Carcinomas

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**UNIVERSITY OF SPLIT  
SCHOOL OF MEDICINE**

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**IMMUNOHISTOCHEMICAL EXPRESSION OF PODOPLANIN AND P16-INK IN  
ORAL SQUAMOUS CELL CARCINOMAS**

**Diploma thesis**

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## List of Abbreviations

AJCC	American Joint Committee on Cancer
CLEC-2	C-type lectin-like receptor 2
CT	Computerized-Tomography
DOI	Depth of invasion
EBV	Ebstein-Barr virus
ERM	Ezrin, Radixin, Moesin
HNC	Head and neck cancer
HPV	Human Papillomavirus
MRI	Magnetic-Resonance-Imaging
OSCC	Oral squamous cell cancer
PET	Positron-Emission-Tomography
PDPN	Podoplanin
SCC	Squamous cell cancer
SSE	Surface squamous epithelium

## **1. INTRODUCTION**

## 1.1. Epidemiology of Oral Squamous Cell Carcinoma

Head and neck cancers (HNC) are among the ten most common types of cancers worldwide (1). Around 500,000 individuals are affected per year (2). The oral cavity, pharynx and larynx are the most common locations for HNC. Other locations include the nasal cavity, paranasal sinuses and the trachea.

HNC most commonly develops from squamous cells of the mucosal epithelium, i.e., squamous cell carcinomas (SCC). Non-squamous cell carcinomas develop in the salivary gland, paranasal sinuses, nerves, blood vessels, lymphatic vessels, lymphatic tissue, connective tissue, cartilaginous and osseous structures. Cancers of the skin, eye, ears, esophagus, thyroid gland and the brain are usually excluded in the classifications and epidemiological considerations of HNC.

Oral squamous cell carcinomas (OSCC) account for more than 90% of all HNC (3). Around 60% of them occur in developing countries (4). They tend to arise more in men and the peak incidence is around 60 years of age (5).

## 1.2. Etiology of Oral Squamous Cell Carcinoma

OSCC can be divided anatomically into 'oral cavity-OSCC' anteriorly and 'oropharyngeal-OSCC' posteriorly.

Oral cavity-OSCC are commonly associated with tobacco and alcohol consumption. They are most often located on the lips, anterior tongue, gingivae, floor of mouth and palate. Tobacco and alcohol induce direct damage to the cellular DNA leading to carcinogenesis (6).

Oropharyngeal-OSCC on the other hand are primarily associated with human papillomavirus (HPV) infection. They are most often found in the oropharynx and surrounding structures, i.e., base of tongue, tonsils and the Waldeyer's lymphatic ring (7).

Other risk factors include areca/betel nut chewing (prevalent in Asian countries), poor oral hygiene, chronic mechanical irritation, Epstein-Barr virus (EBV) infection as well as hematopoietic stem cell transplantation (8).

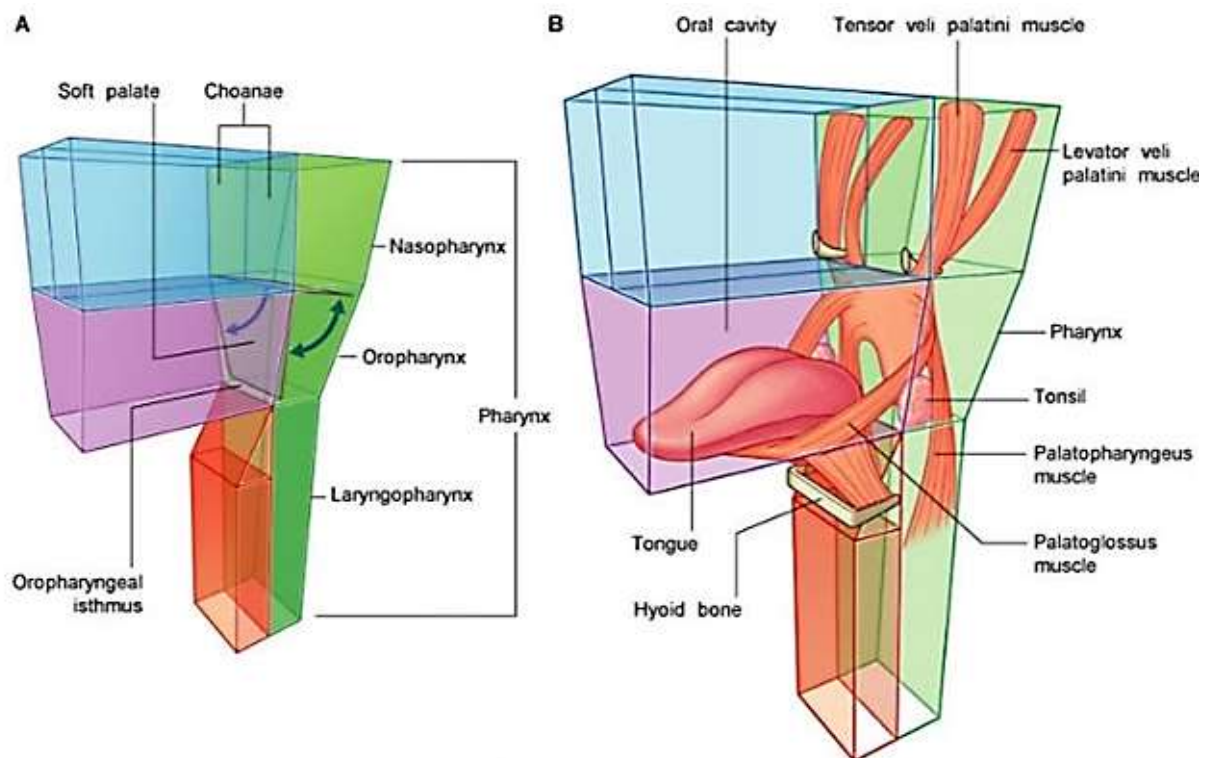
OSCC may arise de novo or from preexisting premalignant lesions. The most common premalignant lesion is oral leukoplakia (whitish non-scrapable patch) which has a 20-30% increased risk for malignant transformation (9-11).

Other premalignant lesions include oral erythroplakia (easily bleeding reddish patch), submucous fibrosis and lichenoid dysplastic lesions (12, 13).

### 1.3. Anatomy and Histology of Oral Cavity

The oral cavity is a shared passage into the aerodigestive tract, i.e., respiratory tract and gastrointestinal tract (Figure 1). Its multiple functions are related to both tracts. However, participation in facial expression is also one of its functions. Articulation and ventilation can be linked to the respiratory tract. Its functions in regard to the gastrointestinal tract are food ingestion, -mastication, -sensation, -gustation, -enzymatic digestion and -deglutition. There are three large paired salivary glands, as well as over hundred small salivary glands secreting saliva into the oral cavity, thereby contributing to digestion and protection of the teeth and mucosa.

The borders of the oral cavity are the lips (anteriorly), buccae/cheeks (laterally), oropharynx (posteriorly), hard- and soft palate (superiorly) and oral diaphragm formed by mylohyoid muscle (inferiorly) (14, 15).



**Figure 1.** Oral cavity shown as a purple box, oropharynx is shown as a green box (2005 by Elsevier, Inc.)

The oral cavity can be divided anatomically into a minor space anteriorly and major space posteriorly (Figure 2-4).

The minor space is between the lips and the teeth and is also known as ‘oral vestibule’ (Figure 2). The oral vestibule can further be subdivided into three regions based upon the anatomic structure its mucosa is facing: the buccal-, alveolar- and gingival mucosa. The buccal mucosa is mobile and covers the lateral walls of the oral vestibule (cheeks) and the inner side of the lips.

The alveolar mucosa is also mobile and it covers parts of the alveolar surfaces of the maxilla and mandible that are distant from the teeth.

Finally, the gingival mucosa is non-mobile and attached to parts of the alveolar surface of the maxilla and mandible that are close to the teeth, therefore also covering the neck and root of teeth.



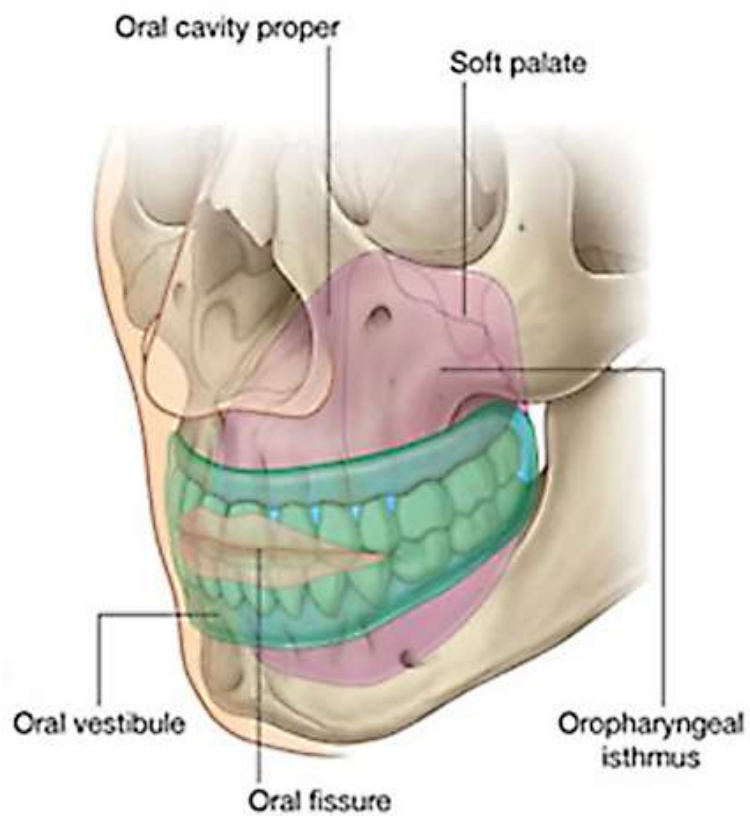
**Figure 2.** Oral vestibule, subdivided in three regions: Buccal-, Alveolar- and Gingival mucosa (by Mohamed Hamze, MD., October 2006, Link: [https://commons.wikimedia.org/wiki/File:Healthy\\_gingiva.jpg](https://commons.wikimedia.org/wiki/File:Healthy_gingiva.jpg), accessed 09th July 2023)

The major space is between the teeth and the oropharyngeal inlet and is known as the ‘oral cavity proper’. It harbors the tongue and it is continuous with the oropharynx (or mesopharynx) posteriorly, which in turn freely communicates with the nasopharynx (or epipharynx) superiorly and the laryngopharynx (or hypopharynx) inferiorly.





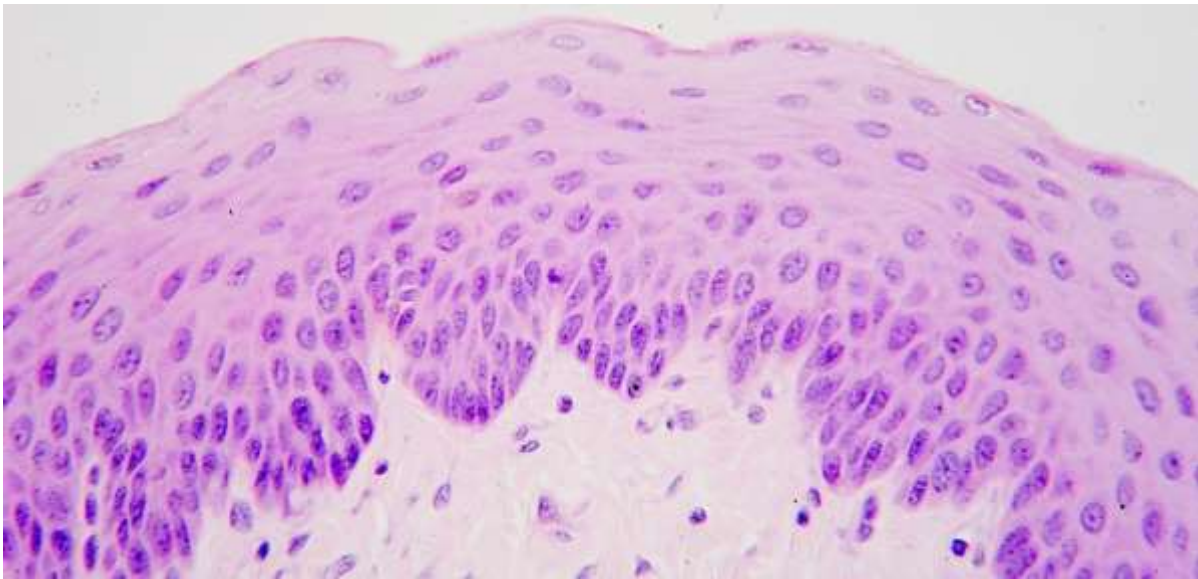
**Figure 3.** Left: Oral vestibule (colored in green), Right: Oral cavity proper (colored in green) (2023 by KenHub)



**Figure 4.** Schematic drawing of the oral vestibule (colored in green) and oral cavity proper (colored in purple) (2005 by Elsevier, Inc.)

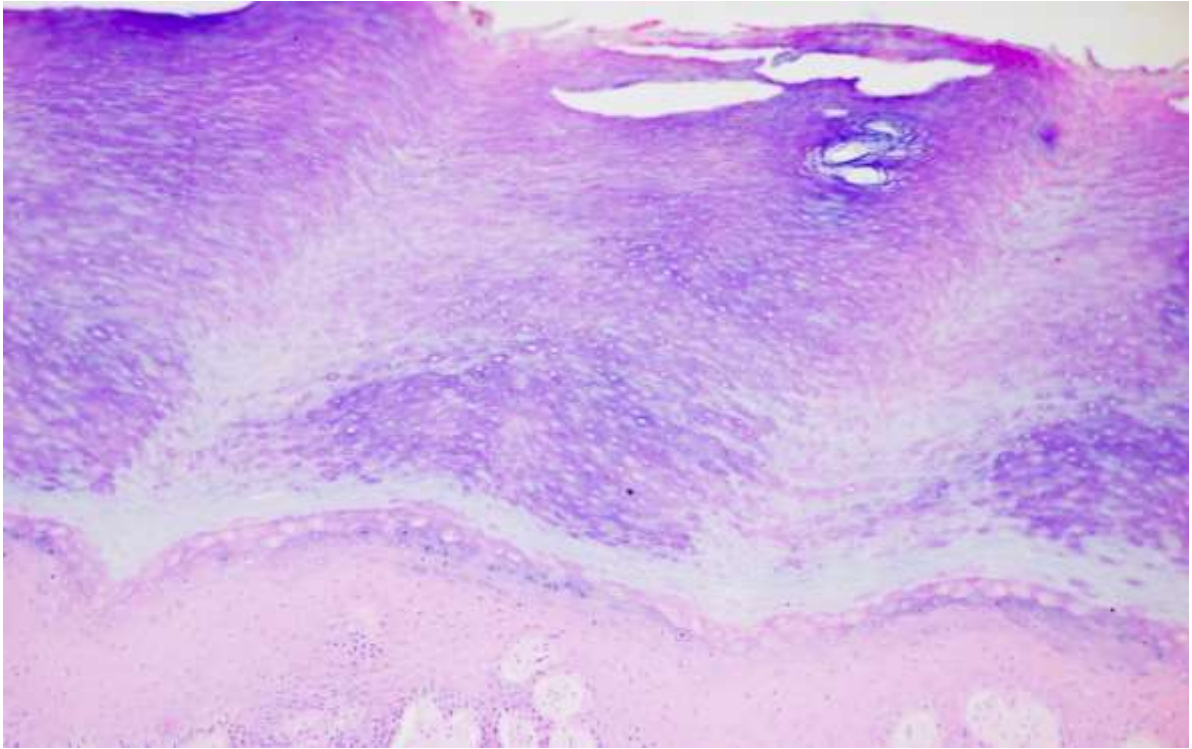
The epithelial lining of the entire oral mucosa is composed of stratified squamous epithelium. However, the degree of keratinization correlates to the mechanical demands of the mucosa, which varies according to the location and mechanical needs of the tissue (16–18). Three histological types of oral mucosa can be described:

First, ‘lining or movable mucosa’ which is attached to movable anatomical structures. It is found in the oral vestibule, namely in the buccal mucosa, alveolar mucosa and inner lips, as well as in parts of the oral cavity proper, i.e., soft palate and oral diaphragm. This movable mucosa has a non-keratinized stratified squamous epithelium (Figure 5).



**Figure 5.** Microscopic view of non-keratinized stratified squamous epithelium (19)

Second, ‘masticatory or rigid mucosa’ which is firmly attached to the underlying bone. It is found in the gingival mucosa and hard palate. This type has a keratinized stratified squamous epithelium to cope with higher mechanical demands (Figure 6).



**Figure 6.** Microscopic view of keratinized stratified squamous epithelium (20)

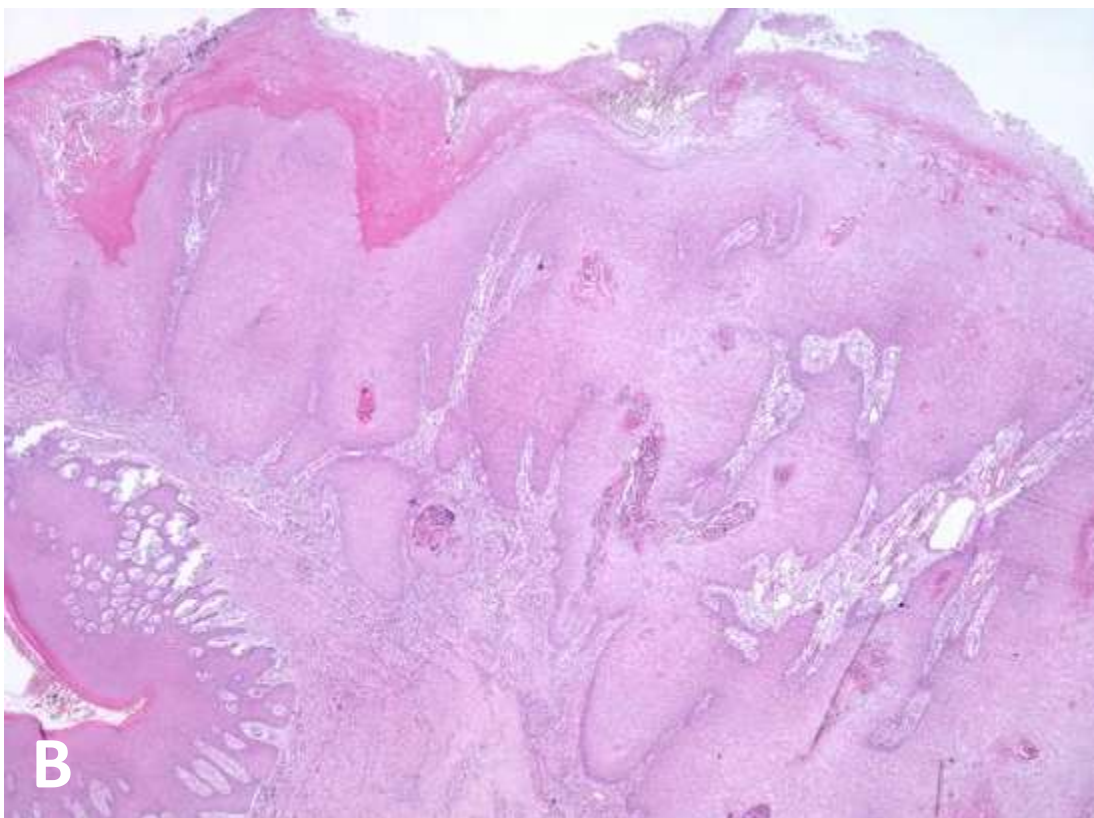
Third, ‘specialized mucosa’ which is found on the dorsum of the tongue. It is partially keratinized and non-keratinized, permitting endurance against mechanical stress of mastication and simultaneously allowing full functionality of the taste buds.

#### 1.4. Histology of Oral Squamous Cell Carcinoma

The most common histological type of oral cancer is squamous cell cancer (SCC), i.e., oral squamous cell cancer (OSCC) which arises from the mucosal epithelium. There are five histologic OSCC variants:

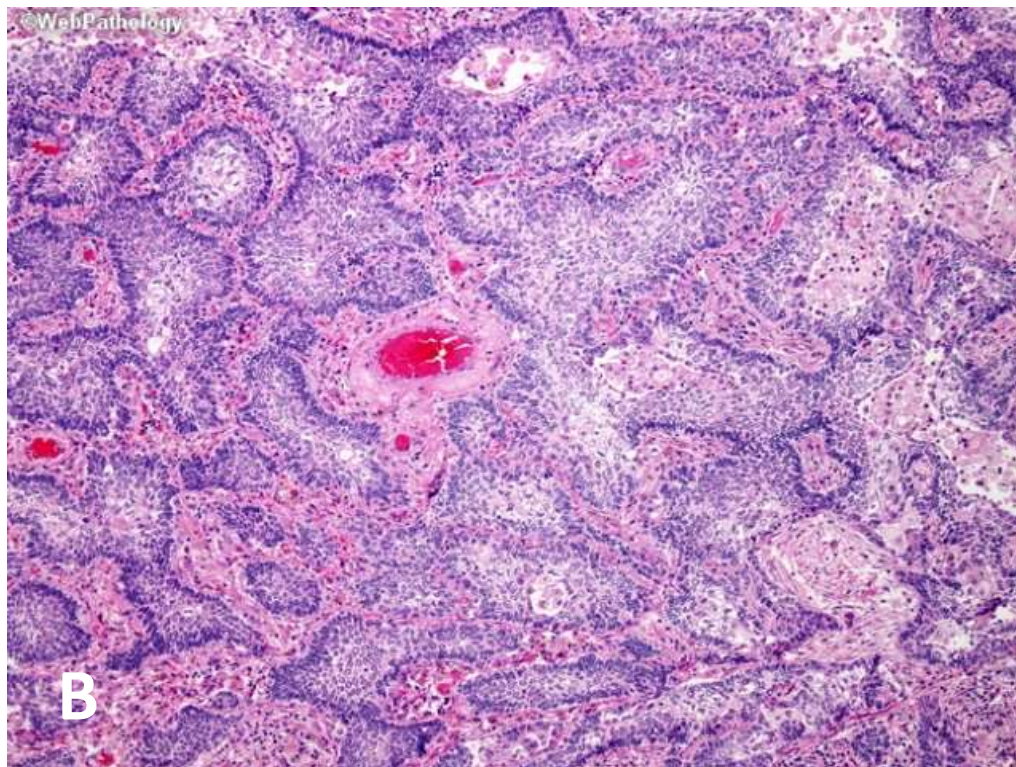
- Verrucous carcinoma
- Basaloid squamous cell carcinoma
- Spindle cell carcinoma
- Adenosquamous carcinoma
- Papillary squamous cell carcinoma

The most common type, found on the buccal mucosa, is verrucous carcinoma with heavily keratinized and well differentiated cells (Figure 7A-B). This variant therefore has a good prognosis.



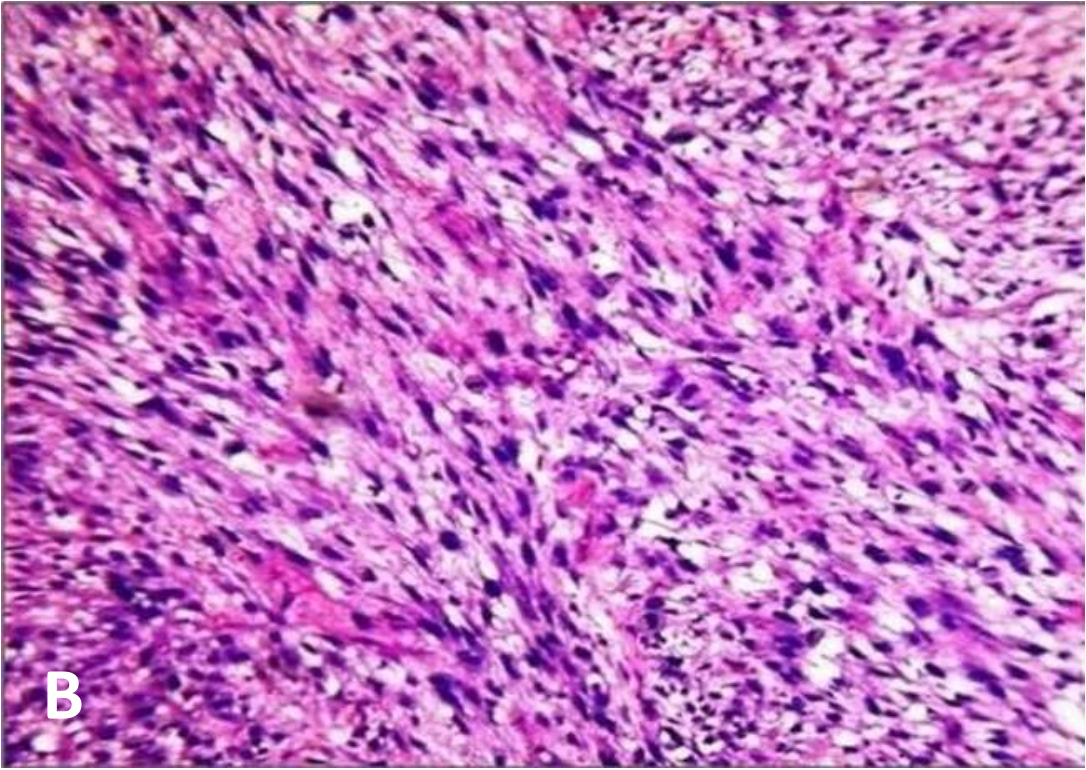
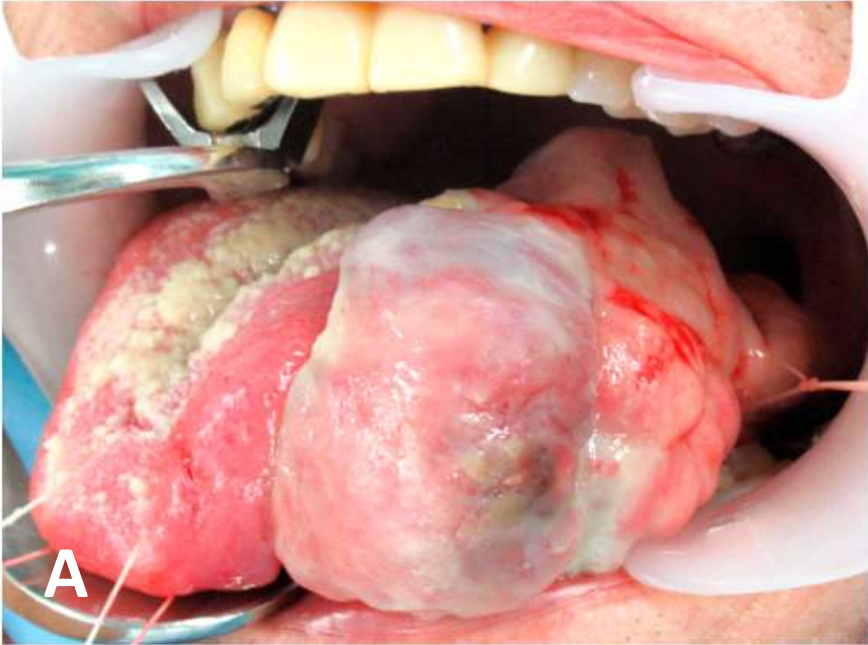
**Figure 7.** (A) Gross image of verrucous carcinoma of the buccal mucosa (21), (B) Histology of verrucous carcinoma (22)

Basaloid squamous cell carcinoma is most commonly found on the tongue and has a poor prognosis. It is composed of pleomorphic basaloid cells arranged in islands with peripheral palisading (Figure 8A-B).



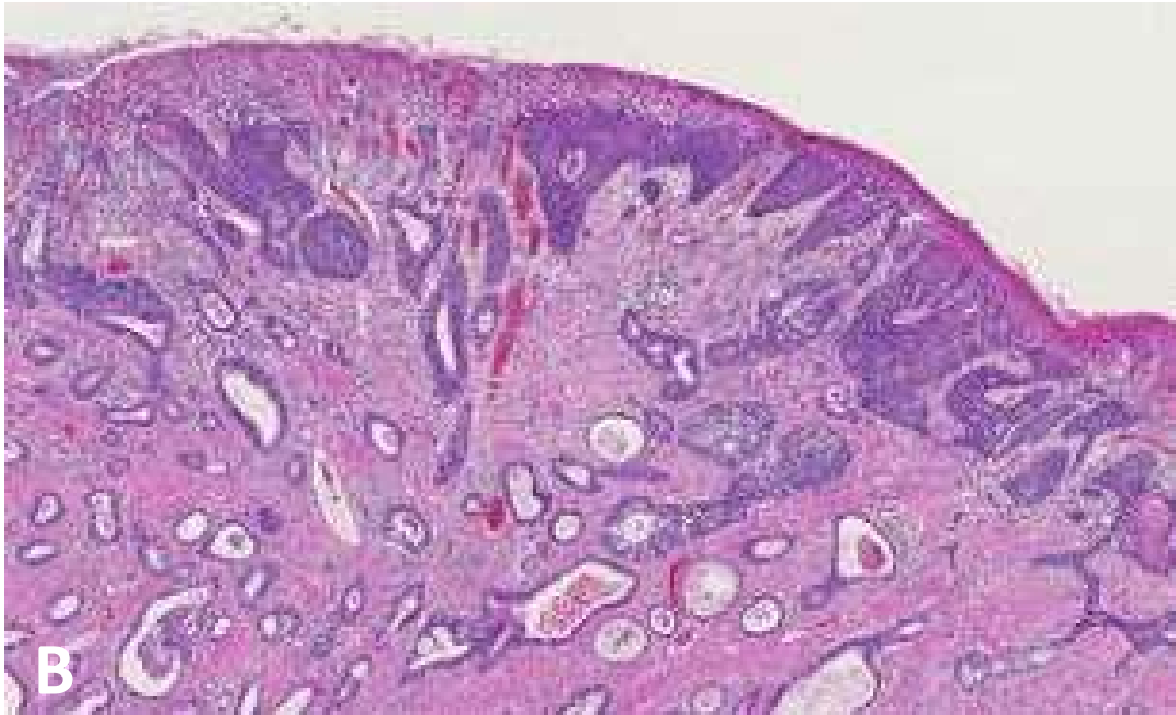
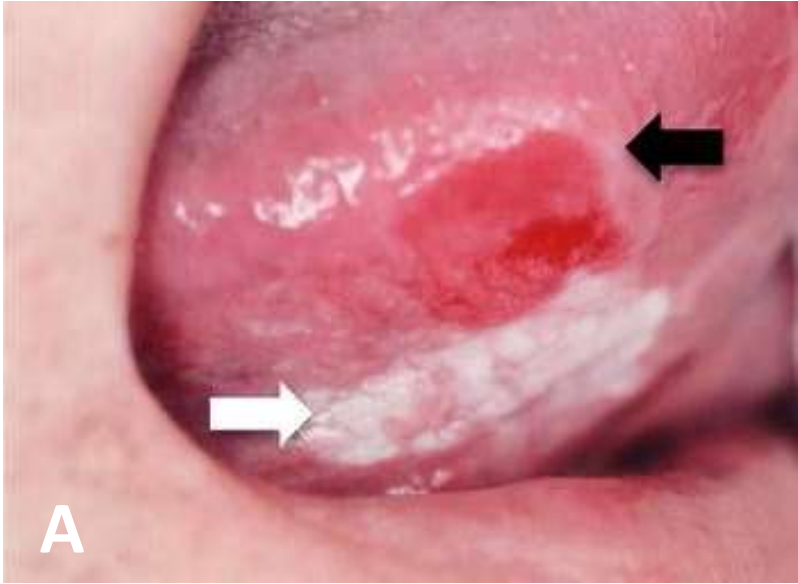
**Figure 8.** (A) Gross image of basaloid squamous cell carcinoma of the lateral tongue (23), (B) Histology image of basaloid squamous cell carcinoma (24)

Spindle cell carcinoma is also most commonly found on the tongue and associated with a poor prognosis (Figure 9A-B). It demonstrates sheets of malignant spindle cells which can look like sarcoma.



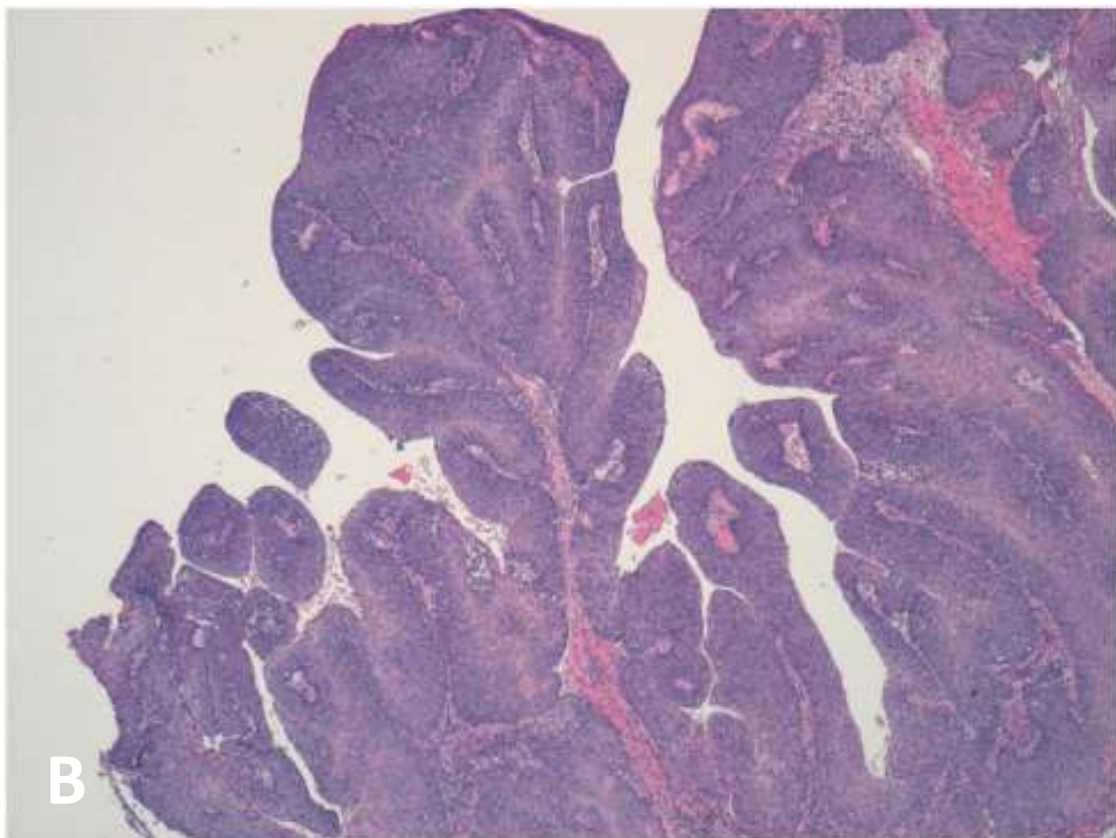
**Figure 9.** (A) Gross image of spindle cell carcinoma of the lateral tongue (25), (B) Histology image of spindle cell carcinoma (26)

Adenosquamous carcinoma histologically has features of squamous cell carcinoma and adenocarcinoma (Figure 10A-B). Similarly, to basaloid squamous cell carcinoma and spindle cell carcinoma it is mostly found on the tongue and has a poor prognosis.



**Figure 10.** (A) Gross image of adenosquamous carcinoma of the lateral tongue, (B) Histology image of adenosquamous carcinoma (27)

Papillary squamous cell carcinoma may look like a benign squamous papilloma, but with malignant features like stromal invasion (Figure 11A-B). It mostly arises in the gingivae or buccae and rarely at the tongue. Due to its exophytic and less invasive growth it has a better prognosis compared to conventional SCC (28).



**Figure 11.** (A) Gross image of papillary squamous carcinoma of the lateral tongue (29), (B) Histology image of papillary squamous carcinoma (30)



## 1.5. Clinical Signs and Symptoms of Oral Squamous Cell Carcinoma

Clinical signs and symptoms of oral cavity-OSCC include swelling, foreign body sensation, halitosis, bleeding, nonhealing and painless lesions (e.g., ulcers), painless lymphadenopathy in the head and neck region. While the tumor itself is not painful, infiltration or compression of nerves by the tumor may induce secondary pain, anesthesia, paresthesia and muscle paresis or palsy.

Involvement of the posterior structures (e.g., oropharynx, tongue base, tonsils) can cause sore throat, muffled voice, odynophagia, dysphagia and eustachian tube dysfunction with otalgia, otitis media and hypoacusis.

Regional lymph node swelling due to lymph node metastases is present during the initial presentation in about 50% of cases (31, 32).

Extranodal metastasis are most commonly found in the lungs, liver and bones (33). These respective organs may therefore cause additional organ specific signs and symptoms like dyspnoea, hemoptysis, obstructive pneumonia, pulmonary arterial embolism, intrahepatic icterus, metastatic bone pain and pathological bone fractures.

## 1.6. Diagnostic Approach of Oral Squamous Cell Carcinoma

Further diagnostic measures are needed after the patient presents with the above-mentioned clinical signs and symptoms.

Careful inspection, imaging, tissue sampling and histological work-up of lesions of the entire aerodigestive tract is mandatory since multiple sites may independently develop a cancerous lesion because of their common causative agents.

This is done via a triple approach:

- A panendoscopic approach involving laryngoscope, bronchoscope and esophagoscope, ideally coupled with sampling the suspected lesion (i.e., biopsy or fine needle aspiration).
- A radiological approach with ultrasonography, computerized-tomography (CT), positron-emission-tomography (PET) with 18F-fluorodeoxyglucose and magnetic-resonance-imaging (MRI), ultrasound- or CT-guided biopsy.
- A pathological approach with a thorough histopathological work up and final diagnosis.

Additional HPV testing can be performed. This holistic diagnostic approach demands a close interdisciplinary teamwork of clinicians, surgeons, radiologists and pathologists to finally make the diagnosis and plan the treatment.

### 1.7. Therapy of Oral Squamous Cell Carcinoma

Early surgical resection of operable tumors is recommended (34, 35). Adjuvant radiotherapy and / or chemotherapy depends on the staging of the cancer, while resection alone might be sufficient for early cancers, advanced cancers usually demand a more aggressive approach.

Inoperable tumors may be treated with radiotherapy and / or chemotherapy without prior surgery. A neoadjuvant approach with radiotherapy and / or chemotherapy to render the inoperable tumor into an operable tumor is also possible (36, 37).

In recent years, novel pharmaceutical approaches with targeted drugs like immune checkpoint inhibitors (e.g., pembrolizumab) and EGFR inhibitors (e.g., cetuximab), are also being recommended in certain conditions (38–40).

The surgical approach encompasses removal of the tumor, the affected lymph nodes and restoring the anatomy and physiology.

The removal of the tumor is usually done with a wide local excision of the tumor and the surrounding normal tissue. Maxillectomy, mandibulectomy, glossectomy or removal of any other structure in close proximity may be performed.

The affected lymph nodes are addressed with an additional ‘modified neck dissection’ surgery, removing lymph nodes in 5 regions ranging from the skull base to the thoracic inlet. Additional removal of the sternocleidomastoid muscle, accessory nerve and internal jugular vein describes a ‘radical neck dissection’. Removal of lymph nodes in less than 5 regions alone describes a ‘selective neck dissection’. The latter variant is the least aggressive with consequently the least operative morbidity and mortality.

Plastic surgery can be performed to restore the appearance and physiologic functions concerning airway, breathing, speech, ingestions of food and liquids. This is usually done with autografts and implants to cover, support or replace tissue defects and nonfunctional tissues.

## 1.8. Prognosis of Oral Squamous Cell Carcinoma

The five-year survival rate of OSCC is around 50% from the time of diagnosis (41, 42). This is mainly due to the delayed diagnosis, frequent local recurrence and metastases after surgical resection. Therefore, early diagnosis and treatment is the best way to improve the survival rate (43, 44). The delay in diagnosis is probably due to the lack or inconsistency of screening methods, occult symptoms and the inability of the patient to differentiate a malignant lesion from the more frequently occurring benign lesions of the oral cavity (e.g., aphthous ulcers).

Consequently, most patients with OSCC die from complications of local advancement of the cancer or distant metastases (45).

HPV positive OSCC seems to have a better response to radiotherapy and chemotherapy (46).

## 1.9. Podoplanin Protein

Podoplanin (PDPN) was given its name after it has been described on the surface of renal podocytes of rats (47). Various synonyms have since been used to describe PDPN, i.e.: gp36, gp38, canine gp40, T1 $\alpha$ ., PA2.26, Aggrus, OTS-8 or M2A oncofetal antigen (48–54). It is a mucin-like transmembrane sialoglycoprotein, consisting of 162 amino acids with a molecular weight of 38-40 KDa.

PDPN is specifically found in lymphatic endothelial cells, but devoid in blood endothelial cells (55).

It exhibits its physiologic and pathologic functions via protein-protein interactions. Some of its protein counterparts that it interacts with are C-type lectin-like receptor 2 (CLEC-2), ezrin and moesin members of the ERM (ezrin, radixin, moesin) protein family (56) and CD9 tetraspanin (57).

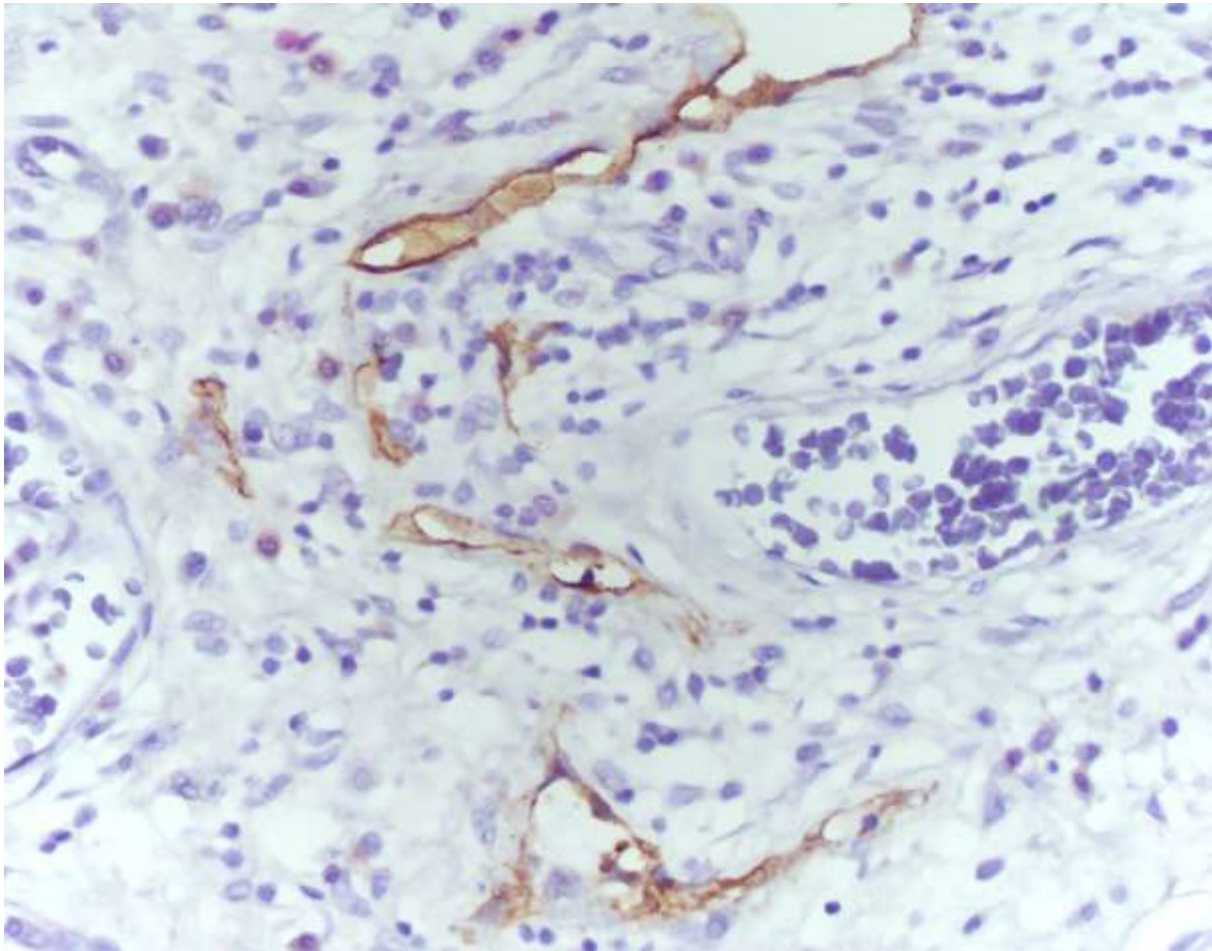
### 1.9.1. Physiological functions of Podoplanin

In humans, PDPN is physiologically expressed on lymphatic endothelial cells as demonstrated in Figure 12. Additionally it is expressed on mesothelial cells, glomerular podocytes, type 1 alveolar cells, some types of neurons, some fibroblasts and some immune cells (58, 59). However, it is not expressed in normal oral mucosa of humans (60).

PDPN contributes to the formation and maintenance of the lymphatic vascular system (61–63). As such, a defect with PDPN will lead to pathological lymphatic-blood vessel separation,

causing blood–lymphatic misconnections, lymphedema and blood-filled lymphatic vessels (51, 64).

Other physiological roles of PDPN include patterning and maintenance of intracranial blood vessels (65), formation of thrombocytes by contributing to megakaryocyte proliferation (66), regulating the response of innate and adaptive immune system during an inflammation (57, 58, 67–70), wound healing (71) and the development of the heart and lungs (72–74).



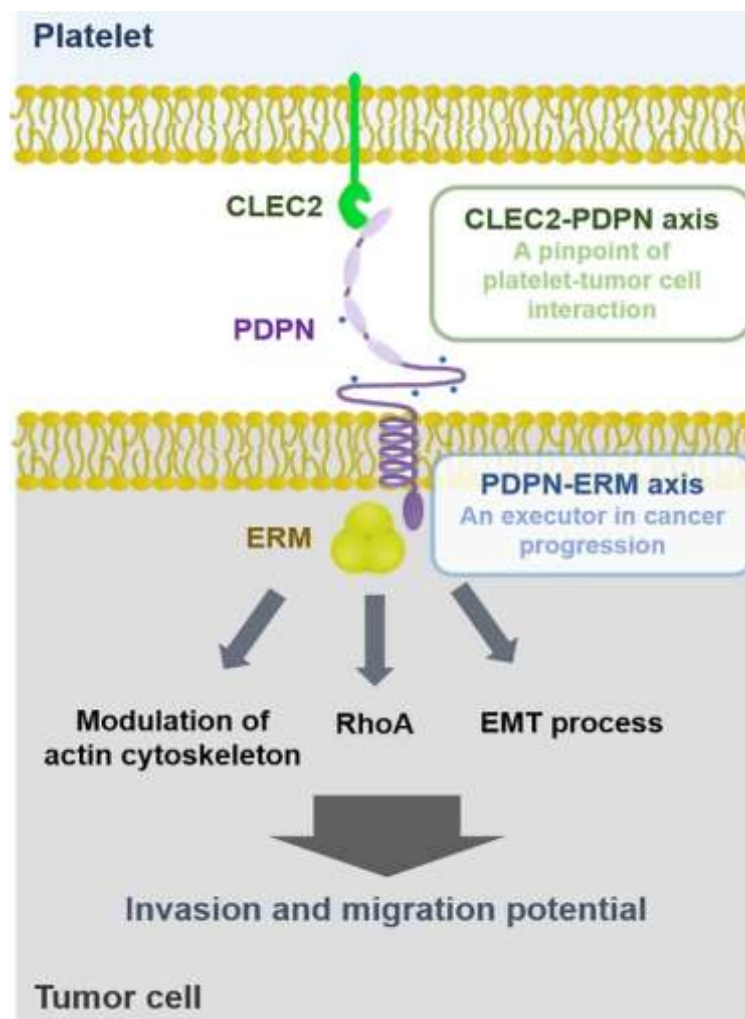
**Figure 12.** Lymphatic vessels in the subepithelial stroma are stained positive for PDPN (brown staining). Note that the blood vessel on the right with red blood cells in the lumen is PDPN negative. Magnification 400X, Olympus Image analyzer Cell D1 (Mentor’s personal archive).

### 1.9.2. Pathophysiology of Podoplanin

Cancer cells overexpressing PDPN (‘PDPN-positive’) were shown to have stem cell-like behavior with the ability to form heterogeneous cancer cells (75). This correlation has been demonstrated in SCC of the skin, larynx, esophagus and uterine cervix. Other non-squamous cell carcinomas associated with PDPN overexpression include mesothelioma, testicular seminoma, colorectal adenocarcinomas and brain tumors (50, 52, 76–79).

PDPN is known for causing tumor invasion and metastasis formation via various mechanisms (80, 81). These mechanisms include cell adhesion to the extracellular matrix (82), functional invadopodia formation, tumor lymphangiogenesis and Epithelial-To-Mesenchymal-Transition (EMT). The cells undergo EMT-mediated tissue invasion by losing their cell-cell tight junctions and changing their cell morphology (52, 83, 84).

However, PDPN is able to induce tissue invasion even without EMT (Figure 12). PDPN binding to Ezrin and Moesin (part of the ERM protein family) activates small Rho-GTPases, hereby allowing PDPN to anchor to the intracellular actin cytoskeleton and make them form protrusions on the cell surface (e.g., filopodia and invadopodia) allowing the affected cells to invade and migrate (56, 84–86). PDPN can thereby be found to be overexpressed in the invasive front of tumors. These protrusions also allow the aforementioned physiological immune responses and physiological embryogenesis (73, 84, 87).



**Figure 13.** Tumor cell interacting with platelets via the CLEC2-PDPN axis. PDPN is associated with ERM proteins that promote cancer cell migration and invasion through modulating actin cytoskeleton, RhoA, and EMT process, by B. Hwang et al (88)

Not only do PDPN-positive cancer cells spread locally, but also via hematogenous metastasis by PDPN-CLEC-2 interaction. CLEC-2 is a transmembrane receptor found on thrombocytes. The PDPN-CLEC-2 binding therefore leads to thrombocyte activation and aggregation around the cancer cells, forming a protective coating against the immune system and shear stress. In this regard, it was found that PDPN binding with the transmembrane protein CD9 tetraspanin prohibits PDPN-CLEC-2 interaction, which then disables its ability to hematogenously spread and develop a protective thrombocyte coating (89–91).

Further characteristics of PDPN-positive cancer cells are the predilection for lymph node metastasis and being less responsive to neoadjuvant radiotherapy and chemotherapy (92, 93).

### 1.9.3. Podoplanin in Oral Squamous Cell Carcinoma

PDPN overexpression is among the most frequently upregulated genes and it is particularly present in the early phases of malignant transformation of OSCC (55, 81, 89). PDPN detection can therefore help confirm if a dysplastic lesion has already undergone malignant transformation (9).

Additionally, the relative amount of circulating PDPN-positive cancer cells in the peripheral blood correlates with a poor clinical outcome (94–96).

### 1.10. HPV Infection in Oral Squamous Cell Carcinoma

As previously mentioned, oropharyngeal-OSCC is associated with HPV infections. The most common location of HPV in the oropharynx was found to be in the tonsils (97), followed by the base of the tongue and the Waldeyer's lymphatic ring (7).

HPV is a nonenveloped, double-stranded, circular DNA virus causing infections of the skin and mucous membranes with the risk of forming benign, premalignant and malignant lesions after several years. Over 120 HPV types can be classified, based on their virulence, as being low-risk or high-risk to form malignant lesions.

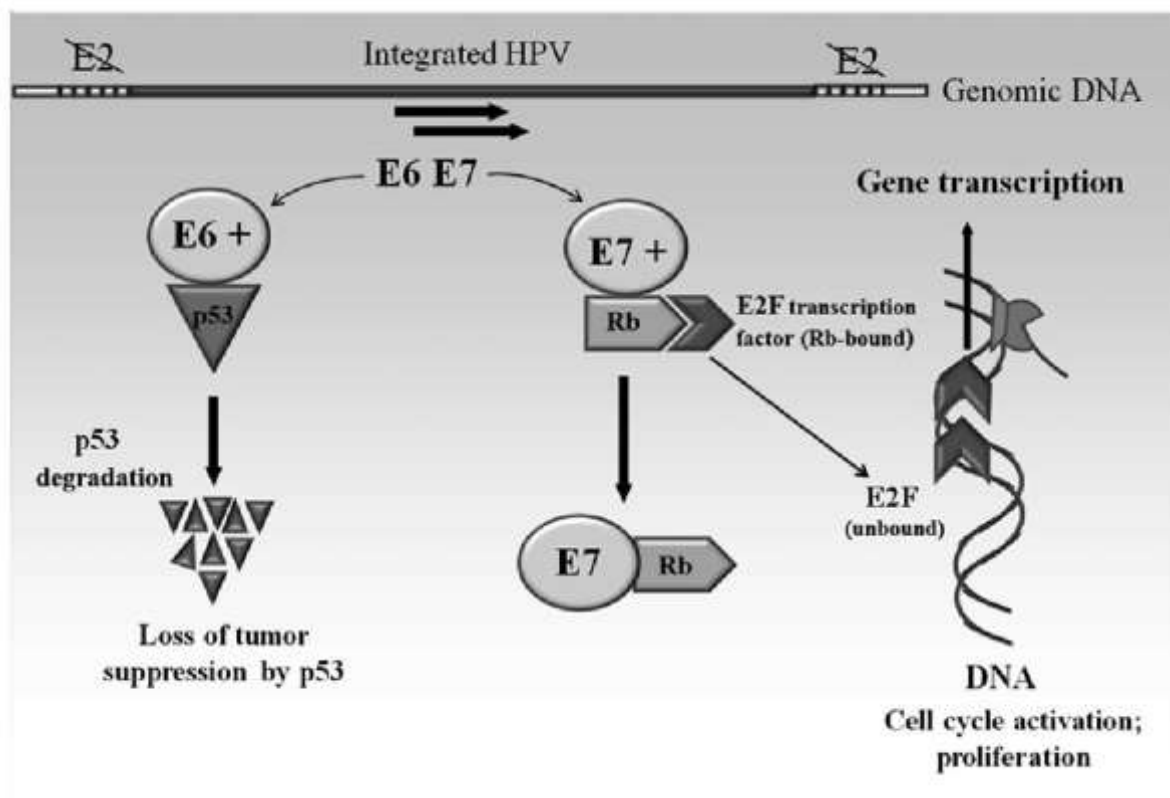
Low-risk HPV (e.g., type 6 and 11) cause anogenital tumors (e.g., condyloma acuminata and anogenital dysplasia) as well as non-anogenital tumors (e.g., oral warts, laryngeal papilloma, respiratory papillomatosis).

High-risk HPV (e.g., type 16, 18, 31 and 33) are responsible for anogenital cancers (e.g., cervical cancers) and non-anogenital cancers (e.g., oropharyngeal cancer, laryngeal cancer, pulmonary SCC).

The most common type involved in oropharyngeal-OSCC is type 16.

The typical route of transmission for the above-mentioned types is via sexual contact. Risk factors include damaged skin or mucosa, immunodeficiency, unprotected sex and uncircumcised males.

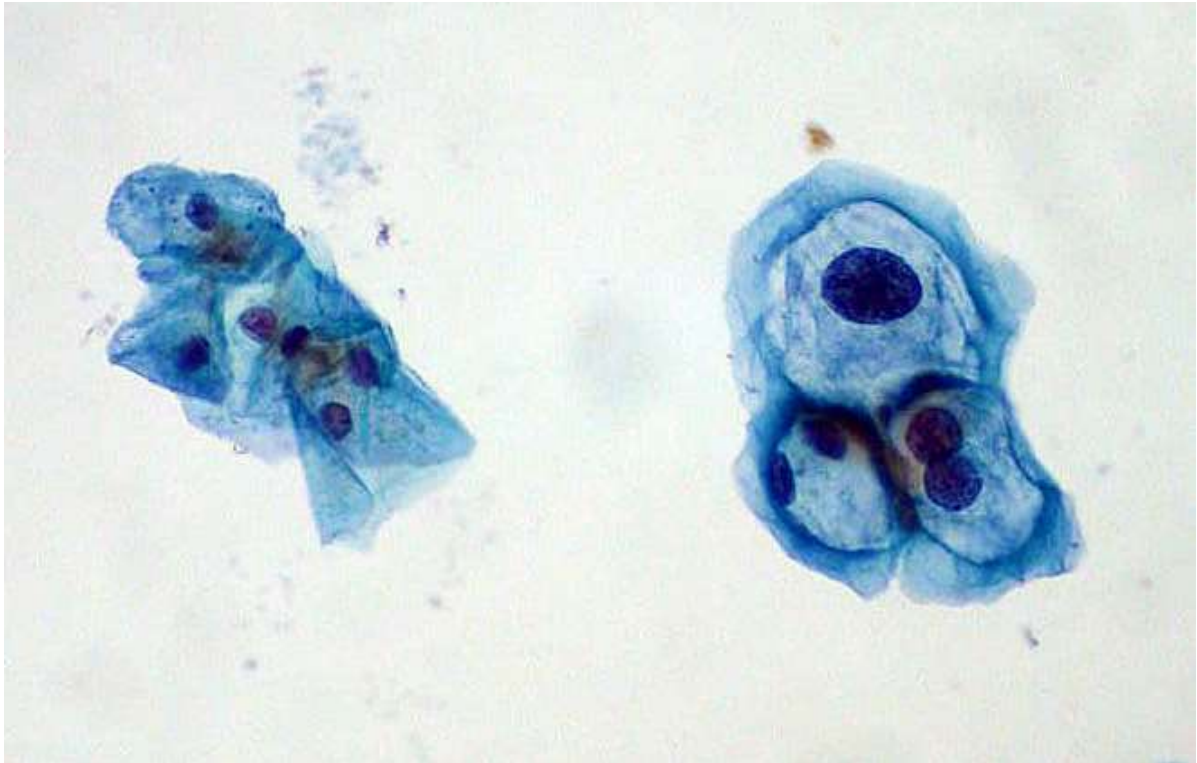
After the infiltration of HPV into human cells it induces the expression of E6 and E7 oncoproteins, which in turn cause oncogenesis by altering the host's genes (Figure 13). E6 promotes degradation of the host's p53-protein and E7 down-regulates the host's pRB-protein, both of them being tumor suppressors. The lack of p53- and pRB protein will lead to an uncontrolled cell cycle progression and cell proliferation.



**Figure 14.** HPV-induced oncogenesis via E6 and E7 oncoproteins, by B. Ruttkay-Nedecky et al. (98)

Additionally, HPV renders the infected cells susceptible for cancer formation by interchromosomal rearrangements, DNA methylation and p16-INK up-regulation (99, 100).

Histological changes include epidermal hyperplasia and hyperkeratosis. The infected squamous cells are referred to as Koilocytes, which are well-defined with clear cytoplasm, perinuclear halo and hyperchromasia (Figure 14).



**Figure 15.** Microscopic view on a liquid-based Pap-Smear. Normal squamous cells on left; HPV-infected cells ('Koilocytes') with mild dysplasia on right (by Ed Uthman, MD., July 2006, Link: <https://flickr.com/photos/euthman/194024495>, accessed in 5th July 2023)

Education and HPV vaccination are the best ways of preventing an HPV infection. The vaccine is either a 9-valent, 4-valent or 2-valent vaccine against the types 6, 11, 16, 18, 31, 33, 45, 52 and 58 (101).

#### 1.11.P16-INK Protein

P16-INK has multiple other names, among them are: p16, p16-INK4, p16-INK4A and many more. 'P16-INK4' as an acronym reveals a lot of information of this 'P-'rotein, namely its molecular weight of '16' kDa and its function as an 'IN-'hibitor of the cyclin-dependent 'K-'inase '4'.

It acts as a tumor suppressor by slowing down the progression of transition from the G1 phase to the S phase of mitosis. Its lack consequently can lead to uncontrolled cell proliferation and oncogenesis.



Some malignancies will down regulate p16-INK, while others do the exact opposite. For example, HPV-positive oropharyngeal-OSCC can up regulate the tumor suppressor p16-INK, explaining its more favorable outcome.

P16-INK can be detected with immunohistochemistry to aid in therapy planning and estimating the prognosis. Immunohistochemical staining for p16-INK is a well procedure for many squamous cell cancers, e.g., in oropharynx, cervix and urinary bladder. Since HPV can up regulate p16-INK, immunohistochemical staining of p16-INK can be used with a high sensitivity and moderate specificity as an alternative and indirect way to diagnose HPV infections (102).

## **2. AIMS AND HYPOTHESIS**

The goal of our research was to determine the immunohistochemical expression of PDPN and p16-INK in oral cavity-OSCC and oropharyngeal-OSCC. Furthermore, we wanted to investigate the correlation between the expression of the two immunohistochemical factors and the histological characteristics of the tumor. Additional parameters analysed were the pathological stage, histological grade, age, gender, involvement of lymph node and lymphovascular- and perineural tissue.

We hypothesize that immunohistochemical expression of PDPN will be present in OSCC and that it will correlate with worse histological grade and pathological state of the disease.

### **3. MATERIALS AND METHODS**

### 3.1. Samples

This research is a retrospective study conducted at University Hospital of Split, Clinical Institute for Pathology, Forensic Medicine and Cytology. There are no other cooperating institutions.

We included patients who were diagnosed with OSCC in the period from January 1st 2015 until December 31st 2017 at our Clinical Institute for Pathology, Forensic Medicine and Cytology. Patients who were diagnosed with OSCC in the specified period but did not have all the necessary clinical data and / or with insufficient materials for performing immunohistochemical staining for PDPN and p16-INK were excluded. We have finally included 20 samples of surgical materials and / or biopsy samples from patients diagnosed with OSCC.

The data were collected from the database of our Institution of Clinical Institute for Pathology, Forensic Medicine and Cytology, from which histopathological findings were extracted. Also, the respective paraffin blocks of the tumors were prepared for further pathological analysis as described below.

We recorded the following variables: age, gender, tumor localization, tumor size, histological tumor grade, presence of lymphovascular- and perineural invasion of the tumor, extension to regional lymph nodes and pathological tumor stage (pT) of the disease. Patients' age and gender and the localization of the tumor were extracted from the clinical files.

In case of a biopsy or subtotal resection, the complete size of the tumor was based upon the measurements given by the radiological reports (most commonly a computerized tomography of the head and neck region with iodine contrast enhancement). However, if a gross total resection was carried out, the pathologist performed a manual measurement of the complete size of the specimen and classified the tumor according to the 'Protocol for the Examination of Specimens from Patients with Cancers of the Oral Cavity' released by the College of American Pathologists (Version: 4.1.1.0, Date: Nov/2021) as shown in Figure 16.

<b>Protocol for the Examination of Specimens from Patients with Cancers of the Oral Cavity</b>	
<b>pT</b>	<ul style="list-style-type: none"> <li>not assigned (cannot be determined based on available pathological information)</li> </ul>
<b>pTis</b>	<ul style="list-style-type: none"> <li>Carcinoma *in situ*</li> </ul>
<b>pT1</b>	<ul style="list-style-type: none"> <li>Tumor less than or equal to 2 cm with depth of invasion (DOI) less than or equal to 5 mm</li> </ul>
<b>pT2</b>	<ul style="list-style-type: none"> <li>Tumor less than or equal to 2 cm with DOI greater than 5 mm</li> <li>or tumor greater than 2 cm and less than or equal to 4 cm with DOI less than or equal to 10 mm</li> </ul>
<b>pT3</b>	<ul style="list-style-type: none"> <li>Tumor greater than 2 cm and less than or equal to 4 cm with DOI greater than 10 mm</li> <li>or tumor greater than 4 cm with DOI less than or equal to 10 mm</li> </ul>
<p>pT4: Moderately advanced or very advanced local disease Superficial erosion alone of bone / tooth socket by gingival primary is not sufficient to classify a tumor as pT4</p>	
<b>pT4a</b>	<ul style="list-style-type: none"> <li>Moderately advanced local disease. Tumor greater than 4 cm with DOI greater than 10 mm or tumor invades adjacent structures only (e.g., through cortical bone of the mandible or maxilla or involves the maxillary sinus or skin of the face)</li> </ul>
<b>pT4b</b>	<ul style="list-style-type: none"> <li>Very advanced local disease. Tumor invades masticator space, pterygoid plates or skull base, and / or encases internal carotid artery</li> </ul>

**Figure 16.** Protocol for the Examination of Specimens from Patients with Cancers of the Oral Cavity released by the College of American Pathologists (Version: 4.1.1.0, Date: Nov/2021)  
DOI = Depth of invasion

We defined three histological grades (well differentiated, moderately differentiated and poorly differentiated) based upon the following cytological findings: mitotic figures, irregular nuclei, nuclear-cytoplasmic ratio, nuclear hyperchromasia, conspicuous nucleoli and necrotic cells.

The presence of lymphovascular invasion and perineural invasion of the tumor, the extension to regional lymph nodes and the pathological stage of the disease were extracted from the pathological reports.

The staging system most often used for OSCC is the American Joint Committee on Cancer (AJCC) TNM system, which is based upon (T) the size and extent of the Tumor, (N)

the spread to lymph nodes and (M) the metastasis to distant sites. We have performed the TNM staging according to the version released in 2022. Two patients who only received a biopsy of the tumor without assessment of the lymph node status were classified based upon (T) and (M) status.

### 3.2. Immunohistochemical analysis

For the purpose of immunohistochemistry, from each paraffin block of the tumor a 4  $\mu\text{m}$ -thick section was cut, mounted and dried at 37°C. Immunohistochemical analysis was fully automated and performed on the BenchMark ULTRA IHC/ISH Staining Module (Ventana, Tucson, Arizona, USA).

Following primary antibodies were obtained for the detection of PDPN and p16-INK: podoplanin (D2-40) and CINtec16 Histology. Both antibodies were ready to use antibodies (Ventana, Tucson, Arizona, USA). Ultra ViewDAB (Ventana, Tucson, Arizona, USA) and Ultra View RED (Ventana, Tucson, Arizona, USA) were used as a detection kit. Brown cytoplasm staining was considered positive for PDPN, and red cytoplasm staining was considered positive for p16-INK. Lymphatic vessels served as a positive control for PDPN and HPV related cervical carcinoma served as positive control for p16-INK.

The expression of PDPN in the malignant cells and peritumoral normal cells were determined and quantified by the HScore method with the following equation:

$$\text{HScore} = \sum P_i (i+1)$$

- 'i' = intensity of staining with a value of 1 (weak), 2 (moderate), or 3 (strong)
- 'P<sub>i</sub>' = percentage of stained cells of each intensity

10 representative fields of view were chosen for every sample and a HScore was calculated for each field of view. The HScore of the entire sample was considered the arithmetic mean of all HScores of the 10 individual fields of view.

The expression of PDPN in the surrounding surface squamous epithelium (SSE) was determined using a scoring system described by Kawaguchi et al. (9) as shown in Figure 17.

<b>0</b>	No PDPN expression observed in any part of the surface epithelium
<b>1</b>	PDPN expression restricted to the basal layer of the surface epithelium
<b>2</b>	PDPN expression observed in the basal and suprabasal layers at one area of the surface epithelium
<b>3</b>	PDPN expression observed in the suprabasal layer at two or three areas of the surface epithelium
<b>4</b>	PDPN expression observed in the suprabasal layer at more than three focal areas of the surface epithelium

**Figure 17.** Scoring expression of PDPN in the surrounding surface squamous epithelium according Kawaguchi et al (9)

### 3.3. Statistical analysis

All obtained data were entered into Excel tables, followed by statistical data processing with the MedCalc Statistical Software, Version 19.1.2 (MedCalc Software, Ostend, Belgium; medcalc.org; 2019, RRID:SCR\_015044), using the ‘Chi-Squared test’ and ‘Mann-Whitney U test’. The statistical significance was set as  $P < 0,05$ .

### 3.4. Ethics approval

The study was approved by the Hospital Ethics Committee of the University Hospital Centre in Split, Croatia under the reference number 2181-147-01/06/LJ.Z.-23-02. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.



## **4. RESULTS**

A total of 70% of the patients were male and the mean age at the time of diagnosis was 66±12 years.

The most common location of the tumor was the oropharyngeal mucosa noted in 9 (45%) cases, followed by the tongue in 7 (35%) cases and the tonsils in 4 (20%) cases. However, these differences were not statistically significant ( $P=0.386$ ).

The mean tumor size was 3.2±2 cm and the mean depth of invasion (DOI) 1±0.8 cm.

The general characteristics of the studied tumors are summarized in Table 1.

**Table 1.** General characteristics of the studied samples

Parameters	Results	$P^*$
Patients with oral squamous cell carcinoma	20	
Age (in years)	66±12	
Gender (N = 20)		
Male	14 (70%)	
Female	6 (30%)	
Tumor Localization (N = 20)		
Oropharyngeal Mucosa	9 (45%)	0.386
Tongue	7 (35%)	
Tonsils	4 (20%)	
Tumor size (in cm)	3.2±2	
Depth of tumor invasion (in cm)	1±0.8	

Results are presented as number (%) or mean ± standard deviation

\* Chi-square ( $\chi^2$ ) test

The tumors were most frequently moderately differentiated which was noted in 13 (65%) cases, 6 tumors (30%) were well differentiated and only one tumor (5%) was poorly differentiated, which was statistically significant ( $P=0.004$ ).

Lymphovascular invasion was noted in 7 cases (35%) and perineural invasion in 8 cases (40%), both findings weren't statistically significant ( $P=0.263$  and  $P=0.502$ ).

A total of 18 patients (90%) underwent gross total resection of the tumor with regional lymphadenectomy. However, two patients (10%) did not undergo surgical resection, but just received a biopsy, thereby leaving the lymph node status ambiguous.

At the time of diagnosis the most common pathological stage of the tumor was stage 3, indicating tumor greater than 2 cm and less than or equal to 4 cm with depth of invasion (DOI) greater than 10 mm or tumor greater than 4 cm with DOI less than or equal to 10 mm. Stage 3 was noted in 10 (50%) cases, followed by stage 1 in 6 (30%) cases, stage 2 in 3 (15%) cases and stage 4 in one (5%) case. The findings were statistically significant ( $P=0.027$ ).

The mean number of lymphatic vessels in the peritumoral connective tissue surrounding the invasive component of the tumor was  $13\pm 10$ .

The histological characteristics of the studied tumors are summarized in Table 2.

**Table 2.** Histological characteristics of the studied tumors

<b>Parameters</b>	<b>Results</b>	<b><i>P</i>*</b>
Histological grade (N = 20)		
Well differentiated	6 (30%)	0.004
Moderately differentiated	13 (65%)	
Poorly differentiated	1 (5%)	
Lymphovascular invasion (N = 20)		
Positive	7 (35%)	0.263
Negative	13 (65%)	
Perineural invasion (N = 20)		
Positive	8 (40%)	0.502
Negative	12(60%)	
Lymph node involvement (N = 20)		
Present	13 (65%)	
Absent	5 (25%)	
Data not available	2 (10%)	
Pathological tumor stage (pT) <sup>†</sup> (N = 20)		
Stage 1	6 (30%)	0.027
Stage 2	3 (15%)	
Stage 3	10 (50%)	
Stage 4	1 (5%)	
Number of lymphatic vessels in the peritumoral region <sup>‡</sup>		13±10

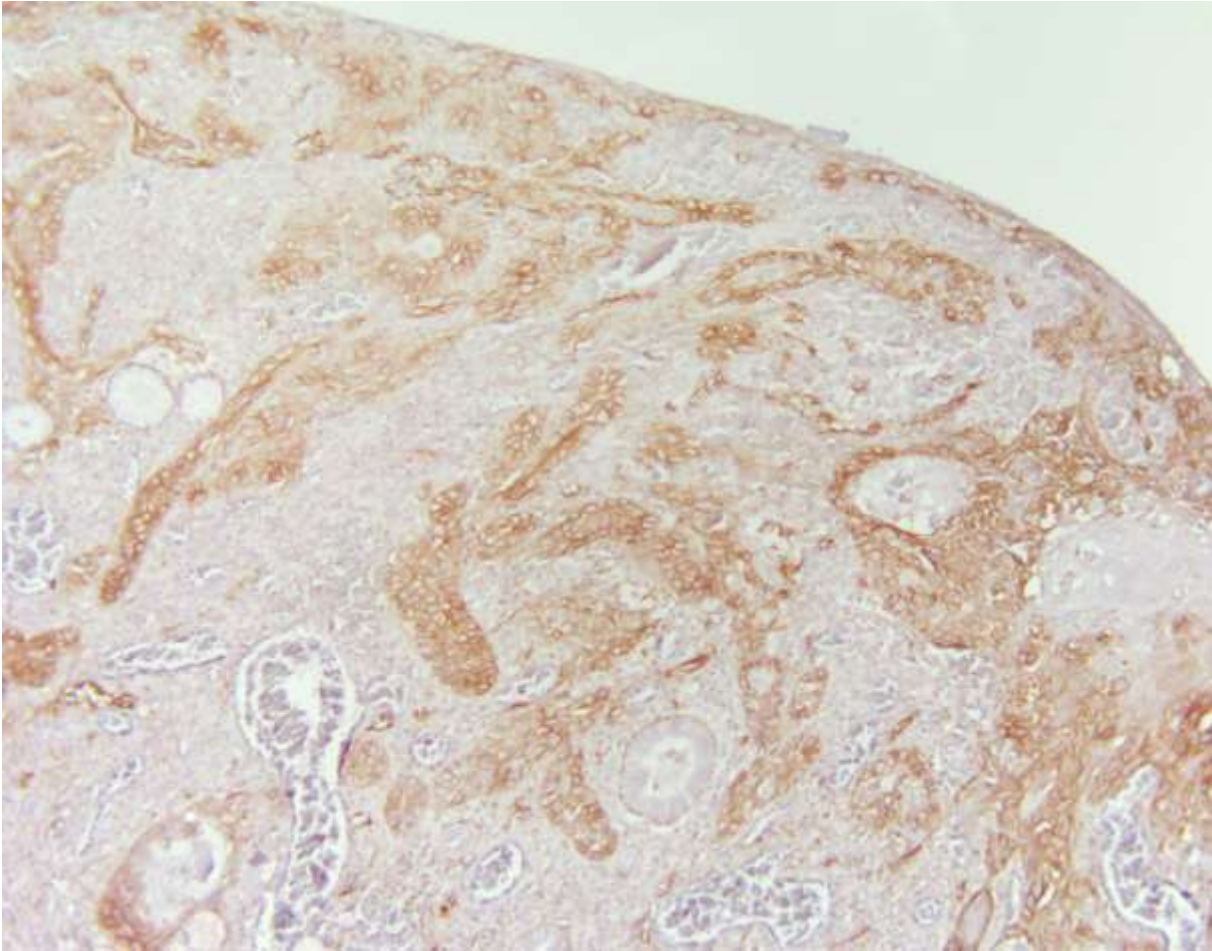
Results are presented as number (%)

\* Chi-square ( $\chi^2$ ) test

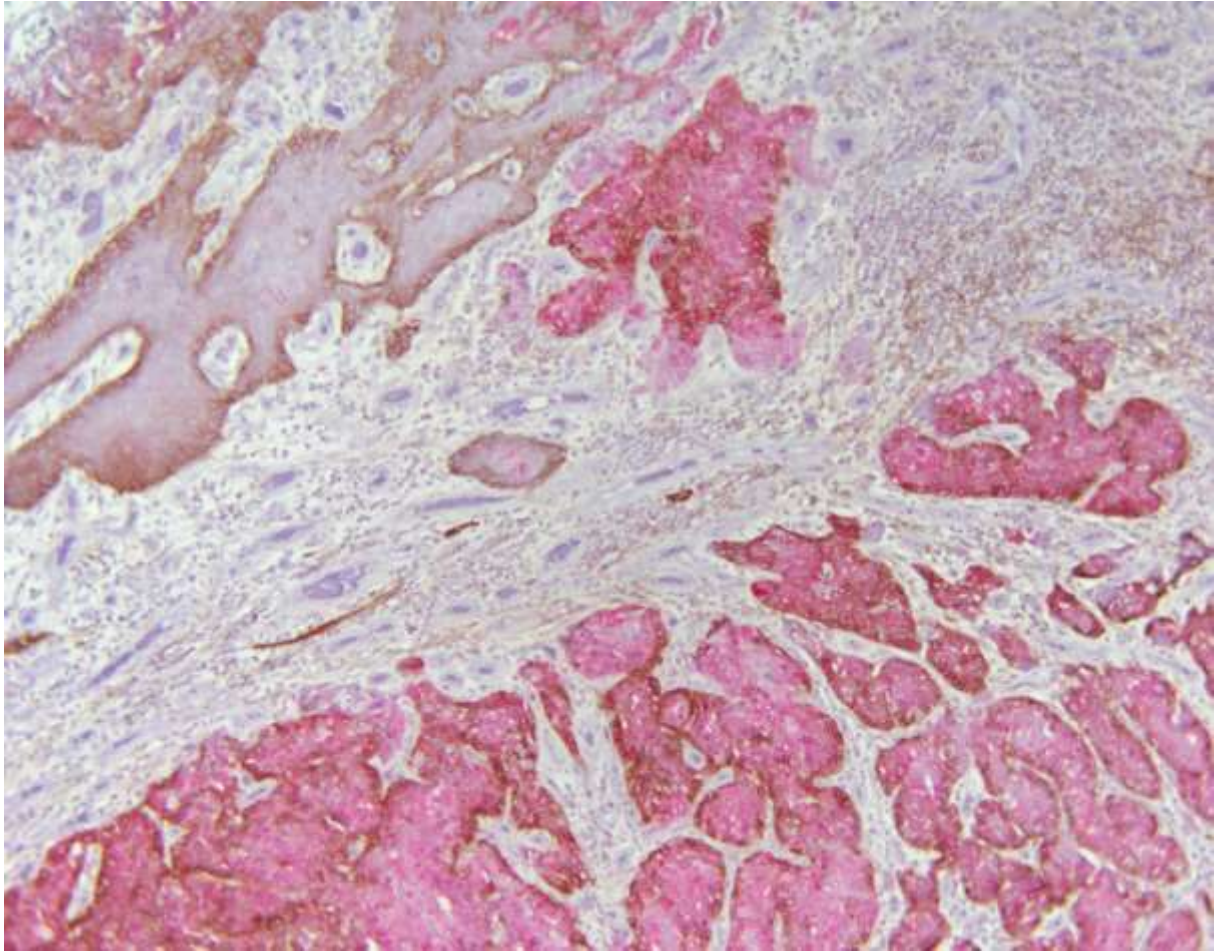
<sup>†</sup> Pathological tumor stage (pT) was assigned based on the guidelines provided by American Joint Cancer Committee (AJCC) in 2022

<sup>‡</sup> Connective tissue surrounding invasive component of the tumor

The double immunohistochemistry staining for PDPN and p16-INK in tumors is demonstrated in Figure 18 and Figure 19.



**Figure 18.** The image shows double immunohistochemistry staining for PDPN and p16-INK. Squamous cell carcinoma is positive for PDPN (brown staining of the membrane and cytoplasm), but negative for p16-INK. Magnification 100X, Olympus Image analyzer Cell D1 (Mentor's personal archive).



**Figure 19.** The image shows double immunohistochemistry staining for PDPN and p16-INK. Squamous cell carcinoma is positive for PDPN and p16 (brown staining of the membrane and cytoplasm indicative of PDPN, red staining of cytoplasm indicative of p16-INK). Note the PDPN positivity of the basal layer of squamous cell epithelium. Magnification 100X, Olympus Image analyzer Cell D1 (Mentor's personal archive).

Immunohistochemical expression of PDPN in tumors was assessed using the HScore method. The mean HScore was  $1.6 \pm 1.4$ . There was no statistically significant difference in expression of PDPN in tumors regarding the patient's gender; mean HScore for men was  $1.7 \pm 1.4$ , compared with  $1.3 \pm 1.4$  in female patients ( $P=0.497$ ).

PDPN HScore in tumors was higher in presence of lymphovascular invasion ( $1.83 \pm 1$ ) and lymph node involvement ( $1.7 \pm 1.3$ ) compared to tumors without lymphovascular invasion ( $1.5 \pm 1.6$ ) and without lymph node involvement ( $1.4 \pm 1.7$ ) the findings weren't statistically significant ( $P=0.603$  and  $P=0.767$  respectively).

On the contrary, the tumors with perineural invasion had lower PDPN HScore ( $1.3 \pm 1$ ) compared to tumors without perineural invasion ( $1.8 \pm 1.6$ ), the finding wasn't statistically significant ( $P=0.603$ ).

There was no statistically significant difference in PDPN HScore of the tumor regarding the histological grade of the tumor ( $P=0.217$ ).

The size of the tumor and PDPN HScore of the tumor are in positive but statistically insignificant correlation ( $P=0.597$ ).

PDPN expression was also noted in the surface squamous epithelium (SSE) surrounding the tumor and was assessed using PDPN score as noted in the literature (103).

The most common score for expression of PDPN in SSE was 0, noted in 9 (45%) cases, followed by score 1 and 2 noted in 4 (20%) cases each, while score 4 was noted in 2 (10%) cases and score 3 in one (5%) case. These findings were statistically significant ( $P=0.047$ ).

Majority of the tumors were p16-INK negative, precisely 85% of them which was statistically significant ( $P=0.004$ ). P16-INK positivity was also noted in the SSE surrounding the tumor in 8 (40%) cases, however this result was statistically insignificant ( $P=0.502$ ).

PDPN HScore in p16-ink positive tumors was lower ( $1.4\pm 1.3$ ) compared to p16-INK negative tumors ( $1.6\pm 1.4$ ), the finding wasn't statistically significant ( $P=0.829$ ).

The immunohistochemical results of PDPN and p16-INK are summarized in Table 3.

**Table 3.** Immunohistochemical expression of PDPN and p16-INK in the studied samples

Parameters	Results	<i>P</i> *
PDPN expression in the tumor (HScore)		
Mean	1.6±1.4	
in male	1.7±1.4	0.497
in female	1.3±1.4	
with lymphovascular invasion	1.83±1	0.603
without lymphovascular invasion	1.5±1.6	
with lymph node involvement	1.7±1.3	0.767
without lymph node involvement	1.4±1.7	
with perineural invasion	1.3±1	0.603
without perineural invasion	1.8±1.6	
in p16-INK positive tumors	1.4±1.3	0.829
in p16-INK negative tumors	1.6±1.4	
PDPN expression in the surface squamous epithelium <sup>†</sup> (N = 20)		
Score 0	9 (45%)	0.047
Score 1	4 (20%)	
Score 2	4 (20%)	
Score 3	1 (5%)	
Score 4	2(10%)	
p16-INK expression (N = 20)		
Positive expression in malignant cells	3 (15%)	0.004
Negative expression in malignant cells	17 (85%)	
Positive expression in SSE surrounding the tumor	8 (40%)	0.502
Negative expression in SSE surrounding the tumor	12 (60%)	

Results are presented as number (%) or mean ± standard deviation

\* Chi-square ( $\chi^2$ ) test

<sup>†</sup> PDPN expression in the epithelium was done using a scoring system described by Kawaguchi et al.(9).



## **5. DISCUSSION**

The huge advancements in diagnostic methodologies in the field of pathology together with the interest of initiating oncological therapy as early as possible, motivated pathologists to identify biomarkers to diagnose various cancers with high sensitivity and specificity. One of the common methods being used is immunohistochemical staining of target proteins.

The results of our study showed male and elderly predominance in patients diagnosed with OSCC. These findings are consistent with the literature, the average age at the time of OSCC diagnosis is 60 years and the male to female ratio is 5.8 vs 2.3 per 100,000 (104).

Majority of OSCC in our study were histologically moderately differentiated tumors with more advanced pathological disease states (i.e. pT3). These results are in accordance with the literature (2, 5, 27).

Fernanda Weber Mello et al. performed a systematic review with 22 cohort and 7 cross-sectional studies (105). They have found out that the analysed data, including a total of 2173 tumor samples, suggest a possible correlation between PDPN and lymph node involvement, worse histological grade and worse clinical stage of OSCC.

Only three (15%) tumor samples were p16-INK positive ( $P=0.004$ ), indicating that these three samples were likely HPV-positive. The prevalence of HPV-positive OSCC varies a lot depending on the population and the time of the study. For example, Antônio Carlos Oliveira et al. performed a systematic review and meta-analysis on the prevalence of HPV-positive OSCC in South America, they observed an overall prevalence of HPV in about 24% of patients diagnosed with oral cavity-OSCC and oropharyngeal-OSCC in South American patients (106). Other studies reported that the prevalence of HPV-positive OSCC has increased in the past decades from less than 20% to more than 70% in the United States and some European countries (107, 108)

Additionally, our study showed no statistically significant difference in PDPN expression and gender, perineural invasion, lymphovascular invasion and lymph node involvement. Also, PDPN does not seem to correlate with either p16-INK positive or negative tumors. However, the correlation between PDPN and tumor invasiveness and lymph node involvement was shown in multiple other studies (27, 60, 68, 82, 85, 87, 94). Our insignificant correlation might be due to our low sample count and different methodology. We performed a retrospective single center study including 20 tumor samples, thereby limiting the statistical significance. Furthermore, two patients underwent a biopsy instead of a total resection with lymph node resection, thus leaving an unclear lymph node status.

Further studies, ideally prospective and with larger sample counts, are needed to evaluate the sensitivity and specificity, as well as the practicability of immunohistochemical expression of PDPN and / or p16-INK as biomarkers to diagnose OSCC and other cancers as early as possible to improve morbidity and mortality. Additional investigations for the HPV-positive OSCC subgroup is also reasonable. Investigating PDPN expression in lymph node metastasis is yet another idea.

## **6. CONCLUSION**

The results of our study suggest that there are no statistically significant findings of PDPN in regards to histological characteristics of the tumor, local invasiveness or pathological stage of the disease, however we must keep in mind that our study had a limited sample size from only a single hospital center. Additionally only three tumors were p16-INK positive, thus limiting the interpretation of the data in regards to HPV-positive OSCC. Therefore, we can not conclude whether or not the expression of PDPN in OSCC or a HPV-positive status of the tumor has any influence on the disease. Further studies are necessary in order to illuminate the role of PDPN and p16-INK in regards to OSCC characteristics.

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## **8. SUMMARY**

**Objectives:** to determine the immunohistochemical expression of Podoplanin (PDPN) and p16-INK in oral cavity- and oropharyngeal squamous cell carcinoma (OSCC). Furthermore, we wanted to investigate the correlation between the expression of the two immunohistochemical factors and the histological characteristics of the tumor. Additional parameters analysed were the pathological stage, histological grade, age, gender, involvement of lymph node and lymphovascular- and perineural tissue.

**Materials and methods:** retrospective study conducted at University Hospital of Split, Clinical Institute for Pathology, Forensic Medicine and Cytology, including 20 patients with OSCC in the period from January 1st 2015 until December 31st 2017. From the pathohistological findings, the following data were recorded: age and sex of the patient, localization of the tumor, histological grade of the tumor, the presence of lymphovascular and perineural invasion, the presence of grafts in the regional lymph nodes and the pathological stage of the disease (pT). Immunohistochemical analysis was performed using the method of double staining for PDPN and p16-INK.

**Results:** A total of 70% of the patients were male and the mean age at the time of diagnosis was  $66 \pm 12$  years. There was no statistically significant difference in PDPN tumor expression in relation to patient gender ( $P=0.497$ ), tumor size ( $P=0.597$ ), tumor histological grade ( $P=0.217$ ), presence of perineural ( $P=0.603$ ) and lymphovascular invasion ( $P=0.603$ ) as well as tumor involvement of lymph nodes ( $P=0.767$ ). Stratified squamous epithelium (SSE) surrounding the tumor was PDPN negative (score 0) in 45% of cases, which was statistically significant ( $P=0.047$ ). Majority of the tumors were p16-INK negative ( $P=0.004$ ), as well as majority of SSE around the tumor ( $P=0.502$ ). Majority of the tumors (65%) were moderately differentiated, 30% were well differentiated, and only 5% were poorly differentiated ( $P=0.004$ ). Lymphovascular invasion was recorded in 35% of cases, and perineural in 40% of cases. The most common pathological stage of the disease was pT3.

**Conclusion:** There were no statistically significant findings of PDNP in regard to histological characteristics of the tumor, local invasiveness or pathology stage of the disease. Only three tumors were p16-INK positive. Further studies are necessary in order to illuminate the role of PDPN and p16-INK in regards to OSCC characteristics.

## **9. CROATIAN SUMMARY**

**Ciljevi:** odrediti imunohistokemijski izražaj Podoplanina (PDPN) i p16-INK u karcinomima pločastih stanica usne šupljine i orofarinksa (OSCC), te dobivene rezultate analizirati obzirom na histološke karakteristike tumora, dobe i spole pacijenata.

**Materijali i metode:** istraživanje je retrospektivno. Provedeno je na Odjelu patologije Kliničkog zavoda za patologiju, sudsku medicine i citologiju Sveučilišne bolnice u Splitu. U studiju je uključeno 20 pacijenata kojima je dijagnoza OSCC postavljena u razdoblju od 01.01.2015. do 31.12.2017. godine. including 20 patients with OSCC in the period from January 1st 2015 until December 31st 2017. Iz patohistoloških nalaza zabilježeni su sljedeći podaci: dob i spol pacijenta, lokalizacija tumora, histološki gradus tumora, prisutnost limfovaskularne i perineuralne invazije, postojanje presadnice u regionalnim limfnim čvorovima i patološki stadij bolesti (pT).

**Results:** Ukupno 70% pacijenata je bilo muškoga spola, a prosječna dob pri postavljanju dijagnoze je bila  $66 \pm 12$  godina. Nije bilo statistički značajne razlike u PDPN izražaju tumora u odnosu na spol pacijenata ( $P=0.497$ ), veličinu tumora ( $P=0.597$ ), histološki gradus tumora ( $P=0.217$ ), prisutnost perineuralne ( $P=0.603$ ) i limfovaskularne invazije ( $P=0.603$ ) kao ni zahvaćenost limfnih čvorova tumorom ( $P=0.767$ ). Višeslojni pločasti epitel (VPE) u okolini tumora je bio PDPN negativan (score 0) u 45% slučajeva, što je bilo statistički značajno ( $P=0.047$ ). Većina tumora (85%) tumora je bilo p16-INK negativno ( $P=0.004$ ), kao i VPE u okolini tumora ( $P=0.502$ ). Većina tumora (65%) je bilo umjereno diferencirano, 30% je bilo dobro diferencirano, a svega 5% slabo diferencirano ( $P=0.004$ ). Limfovaskularna invazija je zabilježena u 35% slučajeva, a perineuralna u 40% slučajeva. Najčešći patološki stadij bolesti je bio pT3.

**Conclusion:** Nije bilo statistički značajne razlike u PDPN izražaju obzirom na histološke karakteristike tumora, lokalnu invazivnost i patološki stadij bolesti. Samo u tri tumora bila je p16-INK pozitivna zbog čega je dobivene rezultate teško interpretirati. Daljnje studije su potrebne kako bi se rasvijetlila uloga PDPN u biološkom potencijalu OSCC.