"EVALUATION OF CD68 IN ORAL SQUAMOUS CELL CARCINOMA AND ITS CORRELATION WITH CLINICOPATHOLOGICAL PARAMETERS: A CROSS-SECTIONAL STUDY"

By

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LIST OF ABBREVIATIONS USED

OSCC	-	Oral Squamous cell carcinoma
TAM	-	Tumor Associated Macrophages
VEGF	-	Vascular endothelial growth factor
CAFs	-	Cancer Associated Fibroblasts
LOH	-	Loss of Heterozygosity
RT	-	Radiotherapy
EBRT	-	External beam radiation therapy
CRT	-	Chemoradiation therapy
IMRT	-	Intensity modulated radiation therapy
OL	-	Oral leukopenia
OE	-	Oral erythroplakia
SCC	-	Squamous cell carcinoma
IHC	-	Immunohistochemistry
BM	-	Buccal mucosa
RMT	-	Retromolar trigone
GBS	-	Gingivobuccal sulcus
WDSCC	-	Well differentiated squamous cell carcinoma
MDSCC	-	Moderately differentiated squamous cell carcinoma
PDSCC	-	Poorly differentiated squamous cell carcinoma
CDK	-	Cyclin- dependent kinase
EMT	-	Epithelial mesenchymal transition
TNM	-	Tumour, nodes and metastases
AJCC	-	American Joint Committee on Cancer
WHO	-	World Health Organisation

CDKN2A	-	Cyclin- dependent kinase inhibitor 2A
EGFR	-	Epidermal Growth factor receptor
H&E	-	Haematoxylin and eosin
DPX	-	Dibutylphthalate Polystyrene Xylene
GCO	-	Global Cancer Observatory

ABSTRACT

"EVALUATION OF CD68 IN ORAL SQUAMOUS CELL CARCINOMA AND ITS CORRELATION WITH CLINICOPATHOLOGICAL PARAMETERS: A CROSS-SECTIONAL STUDY"

Introduction: Oral squamous cell carcinoma (OSCC) is one of the top three cancers in India and ranks as the 8th most prevalent cancer worldwide. OSCC is complicated by the presence of tumor-associated macrophages (TAMs) in stromal cells, which play a major role in tumor progression and are involved in the proliferation and survival of tumor cells, angiogenesis, invasion of surrounding tissues, and metastasis.

Objectives- To detect TAMs through the expression of CD68 in OSCC and correlate it with clinicopathological parameters.

Materials and Methods: Two years prospective study of 56 histologically diagnosed OSCC cases. All cases were evaluated for histological grading, followed by assessment of CD 68 TAMs and their correlation with clinicopathological parameters. Statistical significance was set at $P < 0.05$.

Results: CD68 expression was observed in all OSCC cases. Females had more CD68 TAMs than males. There was a significant difference in CD68 TAMs the histological grade but no significant difference between individual IHC grades. A significantly higher number of CD68 TAMs was observed in tumors with lymph node metastasis and LVI.

Conclusion: The presence of TAMs in the stroma and around the tumor nest was confirmed. TAMs play an important role in tumor progression. This study highlights the used of CD 68 as a pan-macrophage marker that can serve as a prognostic indicator.

KEYWORDS: Oral squamous cell carcinoma, CD68, Tumors Associated Macrophages, Immunohistochemistry

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the top three cancers in India and ranks as the 8th most prevalent cancer worldwide[1]. OSCC is more common in males than females, with middle-aged and older men being the most at risk[2,56]. In 2020, 135,929 new cases and 75,290 deaths due to oral cancer were reported in India[3]. OSCC occurs mainly in patients aged 50-80 years of age[4]. Despite numerous advanced treatment options, the 5-year survival rate of patients with OSCC remains below 50%, primarily due to the aggressive nature of cancer and its tendency to metastasize to the cervical lymph nodes. The prognosis and survival of patients are influenced by nodal metastasis[5-7].

The incidence varies owing to region-specific epidemiological factors such as tobacco use, betel quid chewing, and alcohol consumption [8,85-86]. The development of oral cancer is also linked to infection with HPV 16[83-84]. OSCC is complicated by the presence of tumor-associated macrophages (TAMs) in stromal cells, which contribute to cancer progression and are associated with tumor cell growth and viability, formation of new blood vessels, infiltration of nearby tissues, and the spread of cancer to distant sites.[9-10]

In humans, the demonstration of TAMs is primarily based on the detection of antibodies against CD68[11]. In macrophages, CD68 role is to guide different subsets of macrophages to particular locations and permit them to roll over cells. More than 80% of studies used CD68 as pan-macrophage marker[62-63]. Many studies in thyroid, lung, esophagus, and hepatocellular carcinomas have shown that the existence of CD68 TAMs within the tumor microenvironment promotes tumor advancement and correlates with decreased 5-year survival rates. [6,62-64,76] However, some studies have

also shown no significant correlation between the proportion of CD68 TAMs and duration of survival in patients with OSCC.

Thus, the present study aimed to identify TAMs through CD68 expression in OSCC and to correlate it with clinicopathological parameters.

OBJECTIVE

1. To evaluate the expression of CD68 in Tumor Associated Macrophages (TAMs) in Oral Squamous Cell Carcinoma.
2. To correlate the expression of CD68 with clinicopathological parameters in Oral Squamous Cell Carcinoma

REVIEW OF LITERATURE

ANATOMY OF ORAL CAVITY:

The oral cavity, the initial section of the digestive system, serves various functions including digestion, phonation, sensory perception, protection, respiration, and social interaction.[12] Its digestive role involves chewing, saliva production, forming a bolus for swallowing, and the swallowing itself. The oral cavity opens at the oral fissure, bordered by the lips, and extends posteriorly to the entrance of the pharynx. Lateral walls were formed by the cheeks. The proper oral cavity is enclosed by U-shaped dental arches of the maxilla and mandible, forming anterior and lateral boundaries. Its roof is comprised of hard and soft palates, while the floor is formed by the mylohyoid muscles (known as the diaphragma oris) along with parts of the tongue.[12,18]

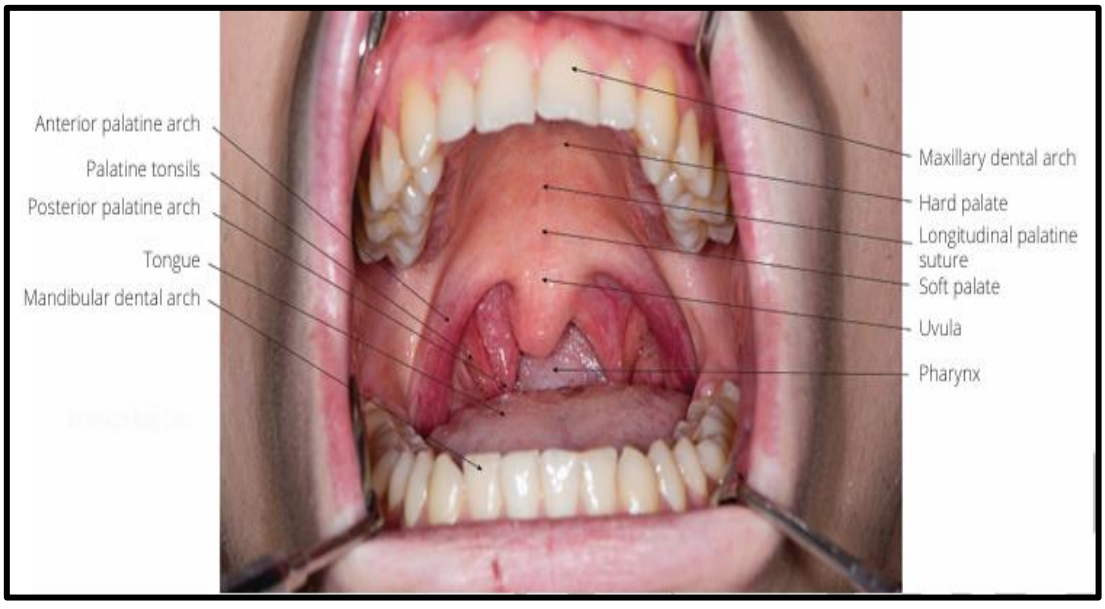


Figure 1: Anterior view of oral cavity

(Image retrieved from Vodanović M. Basic Anatomy of the Oral Cavity. In: Brkić H, Dumančić J, Vodanović M, editors. Biology and Morphology of Human Teeth. Jastrebarsko: Naklada Slap; 2021. p. 1-14.)

COMPONENTS:

It comprises of:

- Lip
- Buccal mucosa
- Lower alveolar ridge
- Upper alveolar ridge
- Retro molar trigone (retro molar gingiva)
- Hard palate
- Anterior 2/3 of tongue and
- Floor of the mouth.

LIPS:

The exterior of the lip is composed of the skin and orbicularis oris muscle, whereas the inner lip is composed of a mucous membrane. It is rich in blood vessels and nerves.[18]

TONGUE

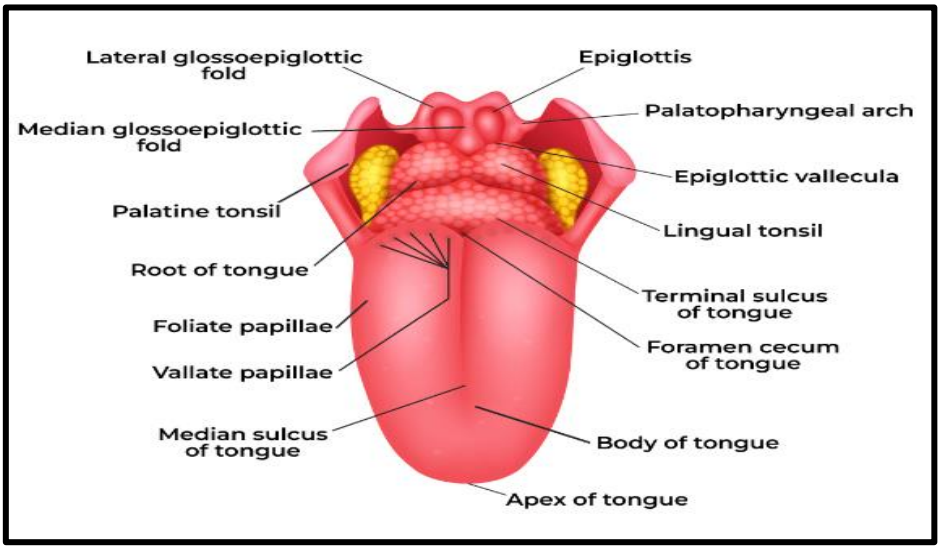


Figure 2: Structure of tongue

(Image retrieved on 25/02/2025 https://www.geeksforgeeks.org/tongue-structure/)

It is divided into 3 parts.

1. Oral (anterior 2/3rd)
2. Pharyngeal (posterior 1/3rd)
3. Root (base)

It is divided into the left and right halves by a median septum. There were two surfaces: dorsal and ventral. A V-shaped groove separates the dorsal surface into two parts: the anterior 2/3rd and posterior 1/3rd. Tongue upper surface is covered with numerous visible projections called lingual papillae. These structures serve various functions, including mechanical and tactile sensations, taste perception, and aiding in the mixing of food.[13-14]

FLOOR OF THE MOUTH:

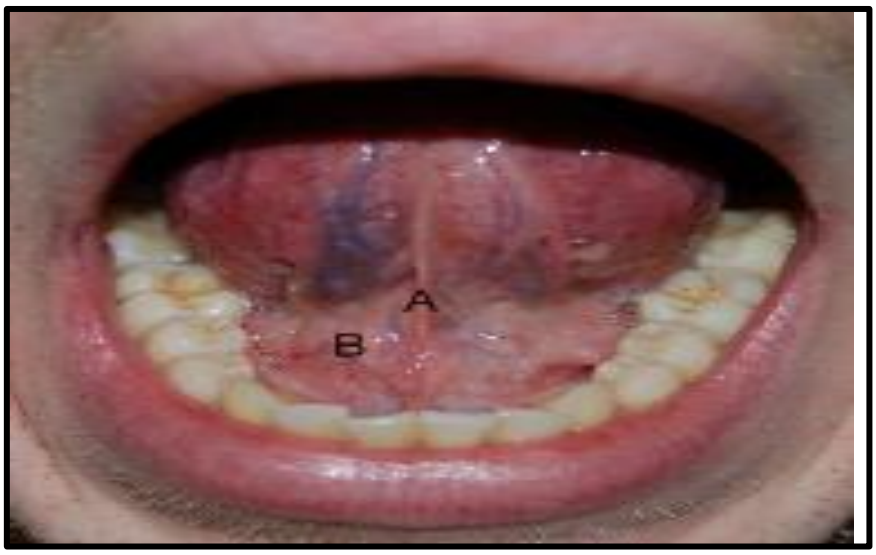


Figure 3: Floor of mouth A: lingual frenulum; B: sublingual papillae

(Images retrieved from Christopoulos A. Mouth Anatomy. In: Meyers AD, editor. Medscape Reference. Updated December 24, 2024.)

This mucosal region forms a horseshoe shape situated between the lower alveolar ridge gingiva on the outer edges and lateral border of the tongue in the centre. It stretches backward and terminates at the tonsillar areas on both the left and right sides. The FOM is connected to the tongue by the frenulum, which is a mucosal fold in the midline. The openings in both the sublingual and submandibular glands are located within this region.[15]

HARD PALATE:

It comprises of maxillary and palatine bones, and forms the roof of the oral cavity.

RETRO MOLAR TRIGONE:

Situated beyond the 3rd molar, this anatomical structure consists of a triangular mucosal area that covers the anterior surface of the mandible's ascending ramus.

BLOOD SUPPLY AND LYMPHATIC DRAINAGE

The oral vestibule and oral cavity receive blood supply from the branches of the external carotid artery, primarily the facial, maxillary, and lingual arteries. The facial artery supplies the lips, with venous drainage occurring via the facial vein, which joins the retromandibular vein to form the common facial vein and drains into the internal jugular vein. The upper cheek region receives blood supply from the angular artery, which is a terminal branch of the facial artery.[13-16]

Among the terminal divisions of the external carotid artery, the maxillary artery supplies the cheek mucosa, teeth, gingiva, and palates. It has three segments: the mandibular, pterygoid, and pterygopalatine. Venous drainage in this region is

managed by the deep facial vein, which connects to the facial vein and drains into the internal jugular vein.[13-16]

The lingual artery and its branches supply the tongue and floor of the mouth, whereas the lingual veins drain these regions. The lingual veins converge into a common trunk with the facial vein, and eventually drain into the internal jugular vein. [13-16]

LYMPHATIC DRAINAGE OF ORAL CAVITY

The primary groups of lymph nodes involved in drainage were as follows:

a. Jugulodigastric lymph nodes
b. Submental lymph nodes
c. Submandibular lymph nodes

The submandibular lymph nodes receive most of the lymphatic drainage from the gingiva, except in the area close to the lower incisors, which drain into the submental nodes.

The lymph nodes from the palate flow predominantly to the jugulodigastric nodes, with some vessels draining into the retropharyngeal nodes.

The lateral third of the dorsum of the tongue, along with its lateral edges and part of its ventral surface, drain into the ipsilateral submandibular nodes.

Primary lymph drainage from the tongue flows to the bilateral submandibular lymph nodes, with some vessels reaching the nodes of the juguloomohyoid.

Lymphatic drainage from the circumvallate papillae is directed to the jugulodigastric and jugulo-omohyoid-specific nodes, which are located on both sides. [13-16]

NERVE SUPPLY

The trigeminal nerve, also known as cranial nerve V, is responsible for sensory innervation of various oral and facial structures. Specifically, the maxillary (V2) and mandibular (V3) branches supply sensations to the lips, cheeks, gums, teeth, hard palate, and oral floor.[16]

HISTOLOGY OF ORAL CAVITY

The oral mucosa is lined with a stratified squamous epithelium composed of keratinocytes**.** Basal cells are responsible for maintaining the epithelial thickness through continuous replication. These cells consist of stem cells, which divide infrequently, and transit-amplifying cells, which undergo division more frequently over short periods. The turnover time of the oral squamous epithelium is slower than that of the gastrointestinal mucosa, with the buccal epithelium taking approximately 25 days and the gingival epithelium taking approximately 50 days.[17-18]

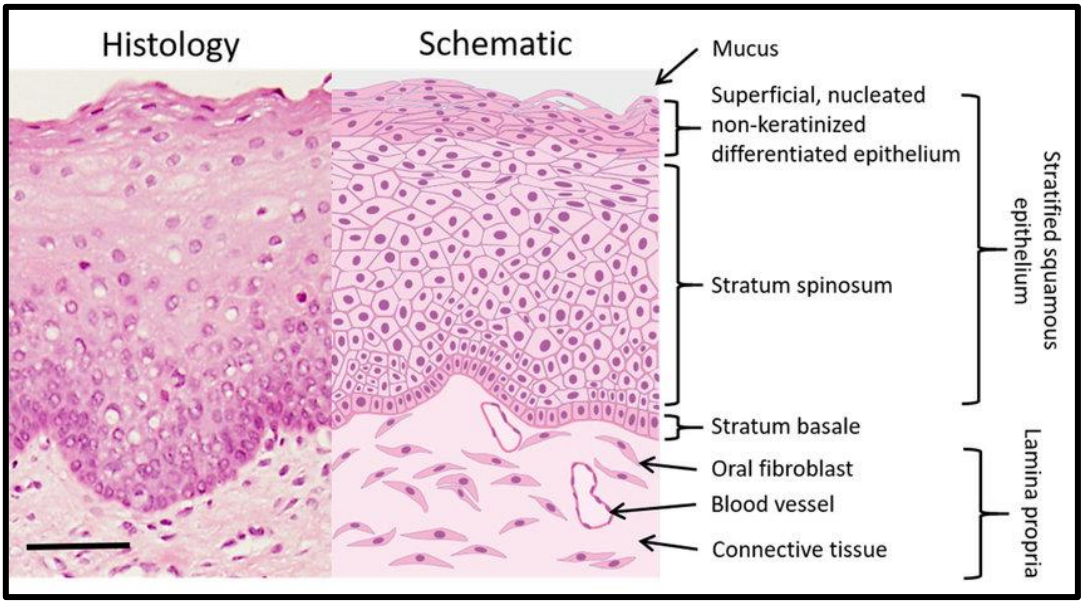


Figure 4: Histological (left) and schematic (right) image of the buccal oral mucosa

(Image courtesy: Prof. Keith Hunter, Unit of Oral Pathology, University of Sheffield).

Table 1: VARIATION IN EPITHELIAL LINING ACROSS ORAL CAVITY

The oral cavity and specific mechanical needs of the region influence the thickness and degree of keratinization.

Epithelium type	Layer	Site/Location
Orthokeratinised	Thick	Hard palate, gingiva
Parakeratinised	Thick	Gingival, dorsal Tongue, alveolar mucosa
Non keratinised	Thick	Buccal and labial mucosa
Non keratinised	Thin	Ventrolateral tongue, FOM, soft palate and gingival sulcus

PATHOGENESIS:

It is a multifactorial process driven by a combination of environmental influences, genetic factors, and molecular mechanisms that ultimately result in the conversion of healthy cells into malignant cells.[7]

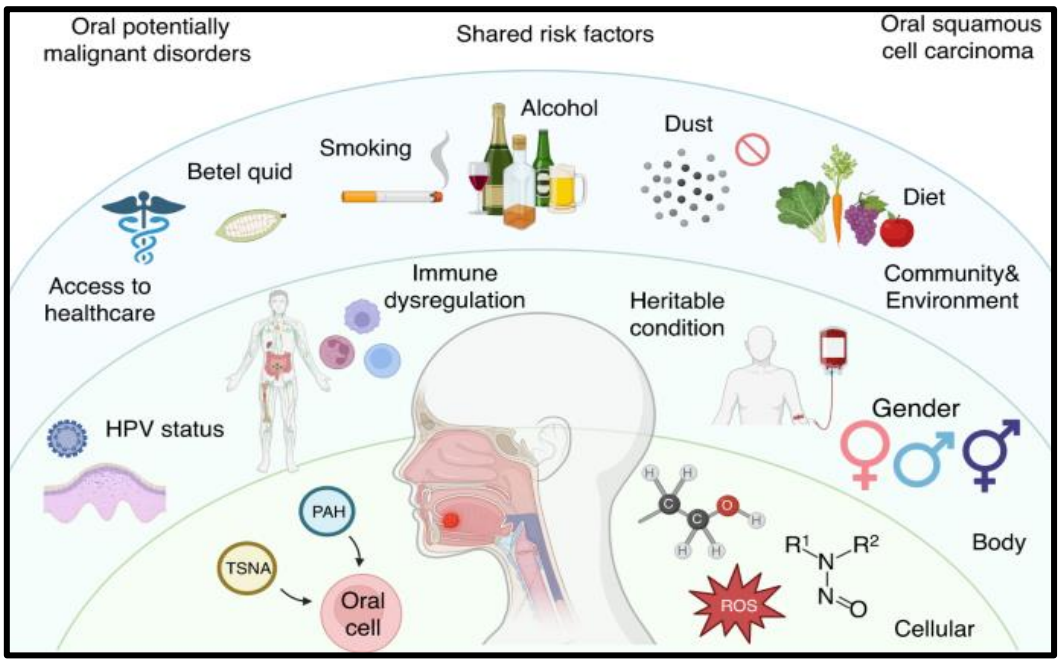


Figure 5: Risk factors of OSCC

(Image retrieved from Tan Y, Wang Z, Huang C. Oral squamous cell carcinomas: state of the field and emerging directions. *Int J Oral Sci*. 2023 Sep 22;15(1):45.)

1. ETIOLOGY

- **Cigarette Smoking**

Raises oral cancer risk 2–4 times and 6–15 times when combined with alcoholism.[7]

The risk increased 8.5-fold for low/medium-tar and 16.4-fold for high-tar cigarette smokers.

- **Betel Chewing**

Betel quid with areca nuts, lime, catechu, and tobacco is a major risk factor.[19-20]

It is strongly associated with buccal and labial cancers.

Betel quid was classified as a significant risk factor by IARC in 1986.[7]

- **Alcohol Consumption**

Chronic alcoholism combined with smoking is a dominant risk factor, present in 75% of oral cavity cancer cases.[5,7,21]

- **Family History**

A family history of head and neck cancer significantly increased the risk of developing oral cancer.[22-23]

- **HPV Infection**

High-risk types, especially HPV-16, inactivate tumor suppressor proteins (p53 and pRb) through E6 and E7 oncoproteins.[5,7,24-25]

- **Diet and Nutrition**

Deficiencies in antioxidants (e.g., vitamins A, C, and E) and micronutrients, such as zinc, impair the immune response and promote carcinogenesis.[5,7,21,24]

- **Chronic Inflammation**

Irritants, such as poor hygiene or betel nut chewing, cause inflammation and release cytokines and growth factors that foster tumor growth.[21.25]

- **Other oral conditions**

Oral submucosal fibrosis, oral lichen planus, lupus erythematosus, dyskeratosis congenita, and Fanconi anemia, are linked to increased oral cancer risk. [21,81]

2.TUMOR MICROENVIRONMENT AND ANGIOGENESIS

- **Angiogenesis**

Tumors use VEGF to form new blood vessels, thereby ensuring a steady supply of oxygen and nutrients.[8,26]

- **Cancer-Associated Fibroblasts (CAFs)**

Secretes matrix components and growth factors that support tumor growth.[10]

3.TUMOR PROGRESSION AND METASTASIS

- **EMT**

Epithelial cells lose polarity and adhesion and gain invasive mesenchymal properties (e.g., E-cadherin loss and vimentin upregulation).

- **Spread**

OSCC metastasizes to lymph nodes and less often to distant organs, such as the lungs and liver, with lymphatic involvement affecting prognosis.[25,82]

4. GENETIC MUTATIONS AND MOLECULAR ALTERATIONS

- **Oncogene Activation**

Mutations in genes, such as HRAS, KRAS, and EGFR, drive proliferation and tumorigenesis.[7,10,75]

- **Tumor Suppressor Inactivation**

Mutations in **p53**, **CDKN2A (p16)**, and **RB1** disrupt apoptosis and cell cycle control,[7,10]

FIELD CANCERISATION MODEL:

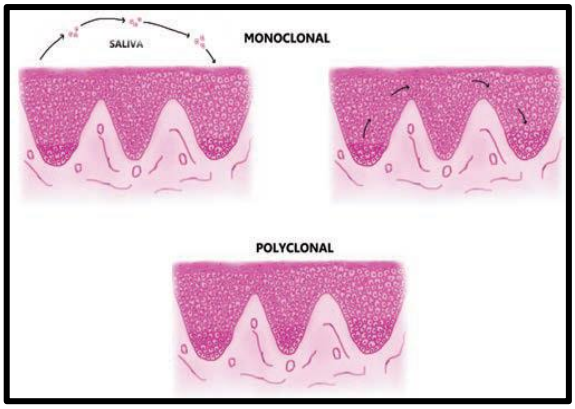


Figure 6(a). FIELD CANCERISATION MODEL

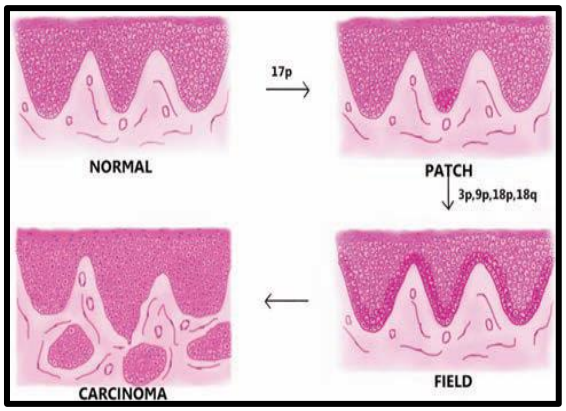


Figure 6(b). PATCH FIELD CANCERISATION MODEL

(Images retrieved from Mohan M and Jagannathan N. Oral field cancerization: An update on the current concepts. Oncology Reviews. 2014 Jun 30;8(1).)

The theory of field cancerization states that the entire oral epithelium is susceptible to cancer due to repeated exposure to carcinogens and the accumulation of genetic mutations in key genes, including oncogenes and tumor suppressor genes.[27]

Recent studies have refined the concept that various oral carcinomas originate from distinct cellular clones in the **patch field carcinoma model**[27], indicating that genetic mutations in stem cells within the basal layer of the oral epithelium are passed down to their daughter cells. Over time, this mutated group of cells expands and may remain undetectable, although it can sometimes manifest as conditions such as leukoplakia or erythroplakia.[7,81]

CATEGORISATION OF POTENTIALLY MALIGNANT CONDITIONS IN ORAL CAVITY [5,28,32]

- Precancerous Lesions
  - Leukoplakia
  - Erythroplakia
  - Erythroleukoplakia
- Precancerous conditions
  - Oral submucous fibrosis.
  - Oral lichen planus.
  - Chronic hyperplastic candidiasis (candida leukoplakia).
  - Syphilis.
  - Actinic keratosis.
  - Sideropenic dysphagia.
  - Leukokeratosis nicotina-palatinae
  - Discoid lupus erythematosus.

PREMALIGNANT LESIONS OF THE ORAL SQUAMOUS CELL CARCINOMA

LEUKOPLAKIA [7]

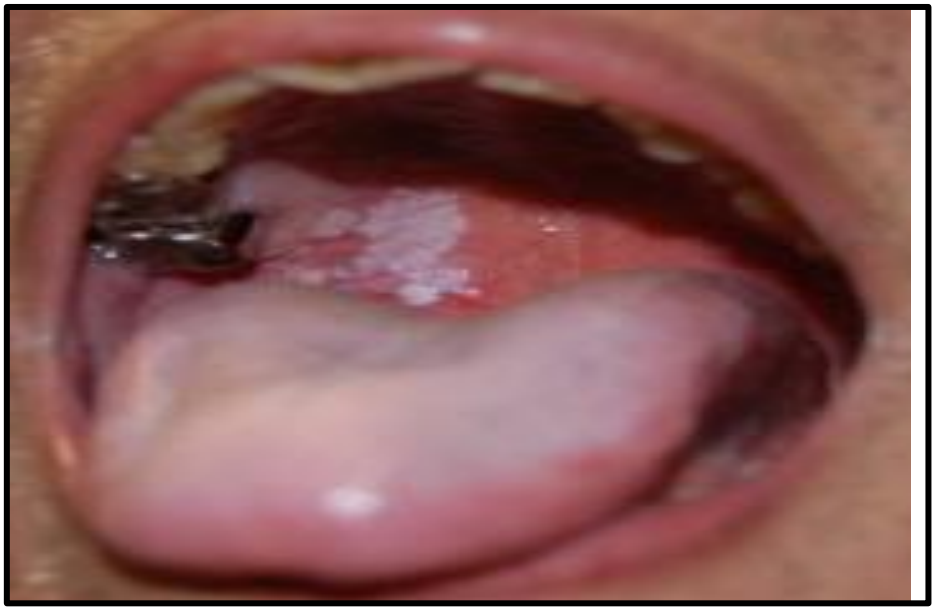


Figure 7: Leukoplakia in soft palate

(Image retrieved from Vodanović M. Basic Anatomy of the Oral Cavity. In: Brkić H, Dumančić J, Vodanović M, editors. Biology and Morphology of Human Teeth. Jastrebarsko: Naklada Slap; 2021. p. 1-14.)

The WHO Collaborating Centre defined oral leukoplakia (OL) as a persistent, white, non-scrapable lesion characterized as "a predominantly white plaque of uncertain risk, having ruled out other known diseases or disorders that do not pose an increased cancer risk." [28-31]

The prospective risk of malignancy in patients with OL ranges from 1 to 30%.[32]

ERYTHROPLAKIA

Oral erythroplakia (OE) has a significant risk of malignant transformation, with about 50% of patients facing the possibility of developing dysplasia, carcinoma in situ, or invasive cancer.[33]

It is defined as a "primarily bright red patch that cannot be clearly diagnosed as any specific disease, either clinically or pathologically."[34] Research suggests that between 85% and 90% of early stage OSCC cases initially manifest as OE.[35-36]

SQUAMOUS CELL CARCINOMA (SCC):

According to the World Health Organization, SCC is classified as an epithelial tumor that displays varying degrees of differentiation and exhibits a significant tendency to spread to the lymph nodes.

CONVENTIONAL SCC

Conventional SCC can be categorized into two types: keratinizing and non-keratinizing. Among these, the keratinizing variant was the most common.[42-43,81]

Based on the formation of keratin pearls and the level of differentiation, it can be further divided into the following categories:

1. **Well-differentiated SCC (WDSCC)**: Characterized by the keratinization of individual cells, the presence of keratin pearls, nuclei that are slightly to moderately hyperchromatic and pleomorphic, and have limited mitotic activity.

2. **Moderately Differentiated SCC (MDSCC)**: Marked by reduced keratinization, significant nuclear abnormalities, and heightened cell division activity.

3. **Poorly Differentiated SCC (PDSCC) is** characterized by significant atypical with undifferentiated cells, high mitotic rate, and few keratin production.

VARIANTS OF SCC:

- **VERRUCOUS CARCINOMA:** [37-38]

A well-differentiated type of SCC occurs in the oral cavity and larynx.

Macroscopically, it presents as a slow-growing, locally aggressive tumor with papillomatous protruding lesions and expansile margins.

Microscopically, the tumor had papillary projections resembling clubs, characterized by mature squamous cell proliferation, whereas the epithelial surface exhibited church-spire keratosis.

- **BASALOID SCC:** [39]

This uncommon but extremely virulent form most frequently occurs in the pyriform fossa. It typically appears as an ulcerated growth with underlying induration in the submucosa.

Histologically, it comprises of basaloid as well as squamous cellular elements and frequently features cystic cavities and comedo-type necrosis. Basaloid SCC is characterized by early recurrence and a high propensity for local metastasis.

- **ACANTHOLYTIC SCC**:

This rare variant is commonly found in sun-exposed areas. The most frequent sites were the supraglottic, larynx, and hypopharynx. Histologically, it is characterized by the presence of acantholytic squamous cells that form pseudolumina.

- **PAPILLARY SCC:**

It is most commonly observed in the laryngeal and pharyngeal cavities.

Macroscopically, it appeared as a broad, fragile growth, with a polyp-like appearance.

Microscopically, it is characterized by tumor cells arranged in a polypoidal configuration supported by a fibrovascular core. Tumor cells showed marked pleomorphism and stromal invasion. Precursor lesions include papilloma and mucosal hyperplasia.[40]

- **SPINDLE CELLS SCC:**

The most common site of this tumor is the larynx. It consists of conventional SCC components and spindle cell elements that infiltrate underlined connective tissue. Despite its epithelial origin, it resembles a tumor of mesenchymal origin.[41]

Macroscopically, it appears as a polyp-like structure with a narrow stalk, which in some instances may spontaneously detach and be expelled through the expectoration of sputum.

Microscopically, it is distinguished by the presence of two distinct populations of malignant squamous and spindle cells.

- **ADENOSQUAMOUS VARIANT OF SCC:**

This highly malignant neoplasm is predominantly observed in laryngeal and hypopharyngeal regions. It originates from pluripotent cells situated in the basal layer and is typically associated with unfavourable prognosis.[42-43]

Microscopically, the tumor exhibited both adenocarcinoma and squamous cell carcinoma elements, with the adenocarcinoma component displaying mucin-positivity.

- **LYMPHOEPITHELIAL CARCINOMA:**

A poorly differentiated carcinoma was marked by significant lymphocytic infiltration. It is most commonly found in the larynx and hypopharynx and is associated with a poor prognosis.[42-43]

- **GIANT CELL CARCINOMA**

Poorly differentiated carcinomas are characterized by numerous multinucleated giant cells. These cells are characterized by their cytoplasm, which contains neutrophils and cellular debris. It is most commonly found in the larynx and hypopharynx and is associated with a poor prognosis.[32]

PROGNOSTIC FACTORS

1. Patient Factors:

- Age and sex
- Ethinicity type
- Lifestyle and socioeconomic status

2. Tumor Factors:

- **Size**:

T1/T2 considered "low-risk" and T3/T4 "high-risk."

Larger tumors are associated with cervical involvement, higher recurrence, and poor prognosis.[45-47]

- **Site**:

Common sites are the tongue, FOM, and buccal mucosa, which are more common in Asian populations.[5]

Anterior oral cavity tumors generally have a better prognosis than those in posterior regions, with 5-year survival rates decreasing for more posteriorly located tumors.[24,45]

- **Locoregional Metastasis**:

Bilateral nodal involvement has the worst prognosis, followed by contralateral and ipsilateral.[48-49]

- **Histology and Degree of Differentiation** [5]

- **Surgical Margins**:

The presence of clear margins after surgery is crucial for the prognosis.[98]

- **Invasive Front**:

The pattern of tumor invasion (e.g., cohesive vs. non-cohesive) reflects biological behavior and prognosis. Endophytic growth correlated with higher recurrence.[5,52.73]

- **Perineural and endoneural invasion**:

Tumor size, grade, invasive front, and nodal involvement. This indicates a potential for regional and distant metastases.[5,52,73]

- **Depth of invasion:**

The staging system incorporates this crucial prognostic indicator.[52]

- **Tumor Volume/Thickness**:

Tumor thickness is a better predictor of prognosis than tumor diameter.[5,48-49,77]

- **Growth Speed**:

Faster growth may indicate more aggressive biological behavior.[5,96]

- **Tissue eosinophilia:**

Eosinophilic infiltration is associated with a favourable prognosis.[50,51]

THERAPEUTIC PROCEDURES FOR OSCC:

LIPS:

- Surgery and radiotherapy (RT) are equally effective for early stage cancers.
- Elective treatment may involve a combination of approaches, especially in patients with neck involvement.[53]

FLOOR OF THE MOUTH :

Surgical Options:

- **Wide Local Excision**: For lesions up to 5 mm, it includes removal of the submandibular gland if involved.
- **Marginal Mandibulectomy:** Removal of the rim of the mandible and the primary lesion, often followed by postoperative RT.
- **Segmental Mandibulectomy**: Removal of the affected bone and free flap reconstruction.[53]

Radiotherapy Options:[53]

- **Brachytherapy/Intraoral Cone RT**: Used for superficial T1 tumors.
- **External Beam Radiation Therapy (EBRT)** targets the anterior floor and the neck nodes.
- **Interstitial Irradiation**: For T1 and T2 tumors extend minimally to the tongue.
- **Intraoral Cone Irradiation**: Used to treat well-defined superficial lesions.[97-100]

ORAL TONGUE :

Surgical Options:[53-54]

- **Partial Glossectomy and Primary Closure**: For early-stage lesions; deeper lesions may require extensive resection and free flap repair.
- **Skin Grafting and Flap Restoration**: Standard for moderate-stage progression.
- **Near-Total/Total Glossectomy and Laryngectomy**: For advanced (T4) cases.

Radiotherapy Options:[53-54]

- **Intraoral cone RT or interstitial RT** was administered as part of the treatment plan.
- **Postoperative RT/CRT**: Often combined with surgery for comprehensive management.

BUCCAL MUCOSA :

- **Small Benign Lesions (<1 cm)** were treated with primary closure after the excision.
- **Lesions (2-3 cm)**: Surgical excision is the standard treatment.
- **Small Lip Commissure Lesions**: Treated with radiotherapy (RT).
- **Advanced lesions**: Bone resection may occur if the mandible or maxilla is affected, followed by reconstruction using free flaps for soft tissue or bone

repairs. Bilobed free flaps are preferred for full-thickness cheek reconstruction in order to achieve better cosmetic outcomes.

- **Radiotherapy Options**: Include electron beam irradiation, intraoral cone irradiation, and interstitial irradiation to protect the normal tissues on the opposite side of the body.[53-54]

RETROMOLAR TRIGONE, HARD PALATE AND GINGIVA :[53-54]

- **Lower Alveolar Ridge**: Surgery alone or combined with postoperative radiotherapy (RT) or chemoradiotherapy (CRT) is preferred. Advanced cases may require segmental mandibular excision and free-flap reconstruction.
- **Retromolar trigone**: Early stage, small lesions are treated surgically. Advanced carcinomas are managed by surgery plus postoperative RT or CRT. Superficial and extensive lesions were treated with RT alone.
- **Hard Palate and Upper Alveolar Ridge**: Resection, with or without CRT or RT, is the preferred approach.
- **Radiotherapy Options**: Intraoral cone irradiation for small lesions, mixed beam, or intensity-modulated radiation therapy (IMRT) is used for well-lateralized lesions.[97-100]

CD68 IMMUNOHISTOCHEMISTRY

Cluster of differentiation 68 (CD68) is a glycoprotein that binds to low-density lipoproteins and is expressed in monocytes/macrophages.[8,11] It is a useful marker for various cells of the macrophage lineage, that is, monocytes, histiocytes, Kupffer cells, giant cells, and osteoclasts.

CD68 antibodies recognise a 110kd glycoprotein on tissue macrophages and blood monocytes.[8] In humans the demonstration of TAMs is primarily based on detection of antibodies to CD68.[55] In macrophages, CD68 role is to guide different subsets of macrophages to particular locations and permit them to roll over the cells.

CD68 AND TUMOR-ASSOCIATED MACROPHAGES IN OSCC

Tumor-associated macrophages (TAMs) play major roles in tumor progression and contribute to tumor cell proliferation, survival, angiogenesis, tissue invasion, and metastasis [26,55]

In OSCC cases examined by Ahlam T et al.[8] TAMs are prominently found along the invasion front, emphasizing their role in identifying and eliminating tumor cells. Moreover, these macrophages were also dispersed throughout the tumor parenchyma, a distribution pattern similar to that observed by ElRouby[6] and Liu et al.[58] indicating their recruitment to the tumor site and their capacity to impact the neoplastic process.

Macrophages originating from circulating monocytes are essential contributors to both the innate and adaptive immunity[59-60]. Macrophages can be classified into two main types: the M1 phenotype, which is typically found in inflamed tissues, and the M2 phenotype, which is associated with cancer-related inflammation.[55-56] M1

macrophages are integral to the immune system, contributing to defense mechanisms through phagocytosis and the generation of inducible Nitric Oxide Synthase (iNOS) and Reactive Oxygen Species (ROS) to combat harmful pathogens.[56] In contrast, M2 macrophages are induced by chemokines and polarizing cytokines released by tumor cells, allowing them to evade the immune system, avoid destruction, and subsequently promote their proliferation.[55,75,80]

CD68 is widely recognized as a pan-macrophage marker and has been used in over 80% of studies[8]. Medrek C et al.[61] reported that CD68+ macrophage infiltration in the stroma of breast cancer is strongly related with size of tumor and serves as an independent prognostic indicator.[79] However, some studies, including Ahlam T,[8] found no significant association between CD68+ TAMs and patient survival outcomes in cases of OSCC.

He et al.[9] reported a significant association between CD68 expression and lymph node metastasis. Explaining these findings is challenging because macrophages comprise M1, M2, and undifferentiated monocytes/macrophages. The functional heterogeneity of these mixed cell populations likely contributes to OSCC's initiation and advancement.[80,94-95]

Lu et al.[11] observed a relative increased in CD68+ TAMs in large, recurring tumor, with lymphatic metastasis cases having progressive clinical stages. However, Kazumasa et al.[57] found no significant association between increased macrophage number, stage, and pathological grade of OSCC.

ROLE OF CD68 [8,26,57,101]

- **Tumor Growth:** TAMs secrete growth factors (e.g., EGF, VEGF, and FGF) that promote cell proliferation and survival.
- **Angiogenesis:** CD68+ TAMs produce VEGF, which supports the formation of blood vessels.
- **Immune Evasion:** M2 TAMs release IL-10, inhibit cytotoxic immune cells, and recruit Tregs, aiding tumor evasion.
- **Metastasis:** TAMs secrete MMPs that degrade the ECM, thereby facilitating invasion and metastasis.

PROGNOSTIC VALUE OF CD68 [62-64]

- **Aggressiveness:** Higher CD68 levels are linked to more aggressive tumors and poorer outcomes, often observed at the invasive tumor front.
- **Prognostic Marker:** CD68 analysis helps to assess TAM density and polarization, indicating patient prognosis.

ROLE OF CD68 IN OTHER DISEASES [65-67]

- **Atherosclerosis:** CD68 marks activated macrophages in the plaques, contributing to inflammation and plaque rupture.
- **Cancer:** High CD68 expression in tumors correlates with poor prognosis and tumor progression.
- **Neurodegenerative Diseases:** In Alzheimer's disease, CD68-expressing microglia release pro-inflammatory and neurotoxic molecules, exacerbating damage.
- **Autoimmune Disorders:** In conditions such as rheumatoid arthritis, CD68+ macrophages promote chronic inflammation and tissue damage

MATERIALS AND METHODS

STUDY DESIGN: Cross sectional study

SOURCE OF DATA: All the total surgical excision specimen of oral carcinoma received at Department of Pathology, Jawaharlal Nehru Medical College, KLE'S Dr. Prabhakar Kore Hospital and Medical Research Centre, Belagavi, Karnataka

STUDY PERIOD: January 2023 to December 2024.

SAMPLE SIZE: A total of 56 cases of oral squamous cell carcinoma.

SELECTION CRITERIA:

INCLUSION CRITERIA: All the total surgical excision specimen that were histopathologically diagnosed as oral squamous cell carcinoma.

EXCLUSION CRITERIA:

1. Biopsies specimens.
2. Cases diagnosed as with premalignant lesions or dysplasia
3. All poorly preserved and inadequate specimens.

ETHICAL CLEARANCE: The ethical clearance was acquired from

Institutional Ethics Committee, JNMC, Belagavi prior to commencement of the study.

STUDY METHODS:

Patients detailed history, age, gender, tumor location and size were retrieved from histopathology records.

- Total surgical excision specimen of OSCC received in the histopathology laboratory were numbered and fixed overnight in 10% formalin.
- The following day, the specimens were processed for grossing, and representative tissue sections were placed in different capsules.
- Tissue Processing:
    - Dehydration: Tissues undergo dehydration in upgraded alcohol solutions.
    - Clearing: Tissues were cleared in xylene.
    - Impregnation: Tissues were impregnated with paraffin wax in a tissue processor.
- Tissues were removed from the capsules and embedded in molten wax to prepare blocks.
- Two sections of 3-4 microns thickness were obtained.
- Slides Preparation:
    - One section was stained with hematoxylin and eosin (H&E) for histological grading and histopathological evaluation.
    - Other sections for immunohistochemistry (IHC) were precoated with poly-l-lysine and immunostained with a primary antibody against tumor-associated macrophages (CD68).
    - The stained slides were dipped in xylene and mounted with a coverslip using Dibutylphthalate Polystyrene Xylene (DPX).

CD68 IMMUNOHISTOCHEMISTRY STAINING

TRIS - EDTA buffer (Cat#PS009)- required amount of buffer is prepared. **(ANNEXURE V)**

PRINCIPLE OF PROCEDURE:

CD68 (Clone:KP1) Mouse Monoclonal Antibody was used to detect antigens in formalin-fixed, paraffin-embedded (FFPE) tissue samples. The antigen-antibody complex was visualized using an enzyme-linked (HRP/AP) secondary antibody that specifically binds to the primary antibody. This interaction is then revealed by enzymatic activation of the chromogen, producing a visible reaction at the antigen location. Each phase of this process requires a specific duration and optimal temperature.

POSITIVE CONTROL FOR CD68 MARKER: Tonsil

INTERPRETATION: Brown staining in the cytoplasm was taken as immunopositive.

- Both H & E and IHC staining procedures are explained in ANNEXURE (IV and V)

EVALUATION OF IMMUNOSTAINING [8,68]

- CD68 highly expressed in TAMs exhibiting brown cytoplasmic staining was considered positive.
- CD68 IHC slides were assessed using an Olympus BX41 microscope.
- The sections were scanned at low power to determine the areas with the most intense staining for CD68 (hotspots) in the stroma and around the tumor nest.

- Selected high-power field pictures from the five hot spots at x400 magnification were taken using a JENOPTIK SUBRA digital camera using GRYPHAX software.
- Positively stained cells were counted in these 5 Hot spots at x400 magnification using **QuPath 0.5.1 version software**.
- The mean values were calculated, and the results were presented as the average percentage of CD68 positive cells per high-power field and were graded accordingly.
- To eliminate observer bias, the count was performed by three observers with good interobserver agreement.

Table 2: CRITERIA FOR CD68(+ve) CELLS GRADING [8]

GRADE	CRITERIA
0	Less than 10% cells showing positive immunostaining
1	11-50% cells showing positive immunostaining
2	Greater than 50 % cells showing positive immunostaining

HISTOPATHOLOGICAL GRADING FOR OSCC

Acoording to **MODIFIED BRODER GRADING SYSTEM** [87]

1. Well differentiated squamous cell carcinoma (WDSCC) (Grade 1)
2. Moderately differentiated squamous cell carcinoma (MDSCC) (Grade 2)
3. Poorly differentiated squamous cell carcinoma (PDSCC) (Grade 3)

Based on the evaluation of keratin formation, pleomorphism and mitotic activity.

PARAMETERS STUDIED:

The following parameters were evaluated.

1. Age
2. Sex
3. Site
4. Histology grade of tumor
5. Depth of invasion (DOI)
6. Lymph node metastasis
7. Lymphovascular invasion (LVI)
8. Perineural invasion (PNI)
9. Bony invasion
10. Overall CD68 (+) expression.

STATISTICAL ANALYSIS:

- The data obtained were analyzed using the statistical software R version 4.4.2. and Microsoft Excel.

- Statistical tests used in the study were Chi square test, Shapiro Wilk test and QQ plot, Mann Whitney U test, Kruskal Wallis test, Dunn's test and Spearman's rank correlation test.

- Percentage of CD 68 positivity TAM and their association with clinicopathological parameters were studied and expressed as percentages and proportions.

- A p-value less than or equal to 0.05 was considered statistically significant.

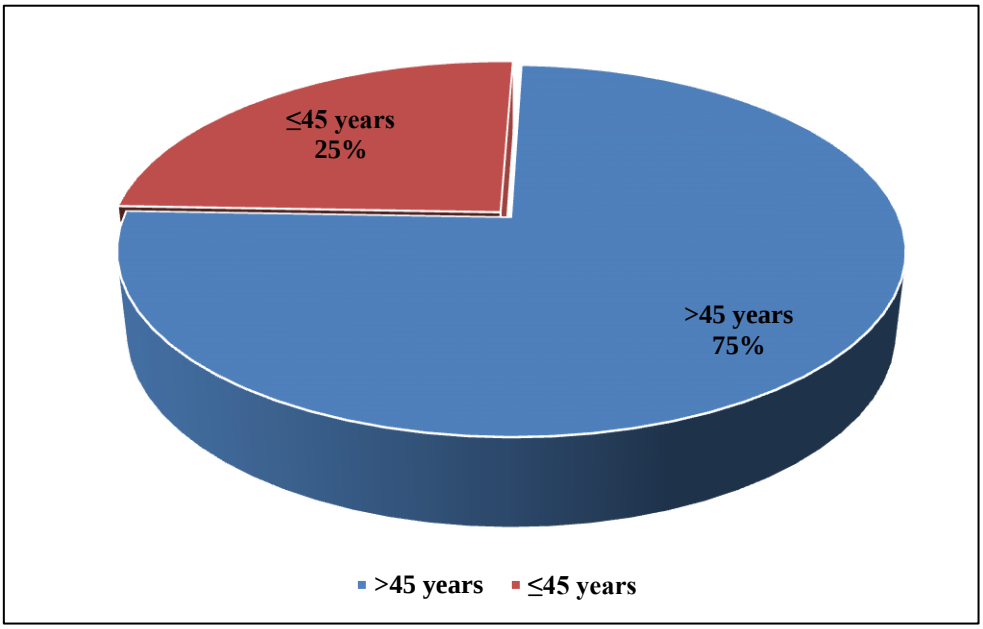
RESULTS

In this study, 56 histologically diagnosed OSCC cases were examined. All cases were evaluated by H&E staining for histological grading, followed by the assessment of CD 68 positive TAMs and their correlation with clinicopathological parameters.

Table 3: Distribution of OSCC according to age and sex

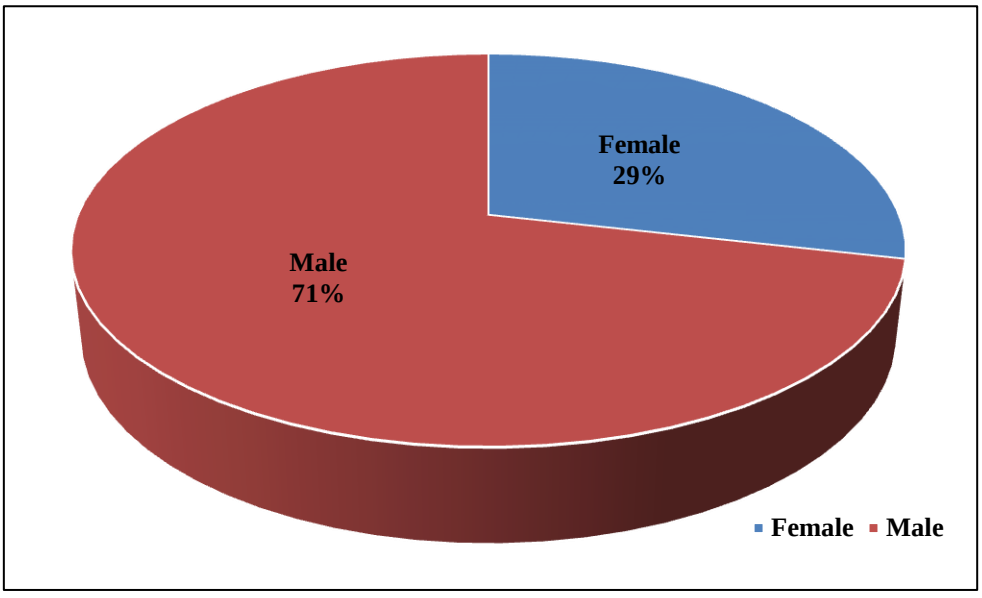
Variables	Sub Category	Number of cases (%)
Age (years)	>45 years	42 (75%)
	≤45 years	14 (25%)
	Mean ± SD	50 ± 10
Sex	Female	16 (29%)
	Male	40 (71%)

Figure 8: Distribution by Age



Out of 56 cases, the majority (75%) were older than 45 years, and the remaining 25% were aged 45 years or younger. The mean age was 49.95 ± 9.84 years.

Figure 9: Distribution according to sex

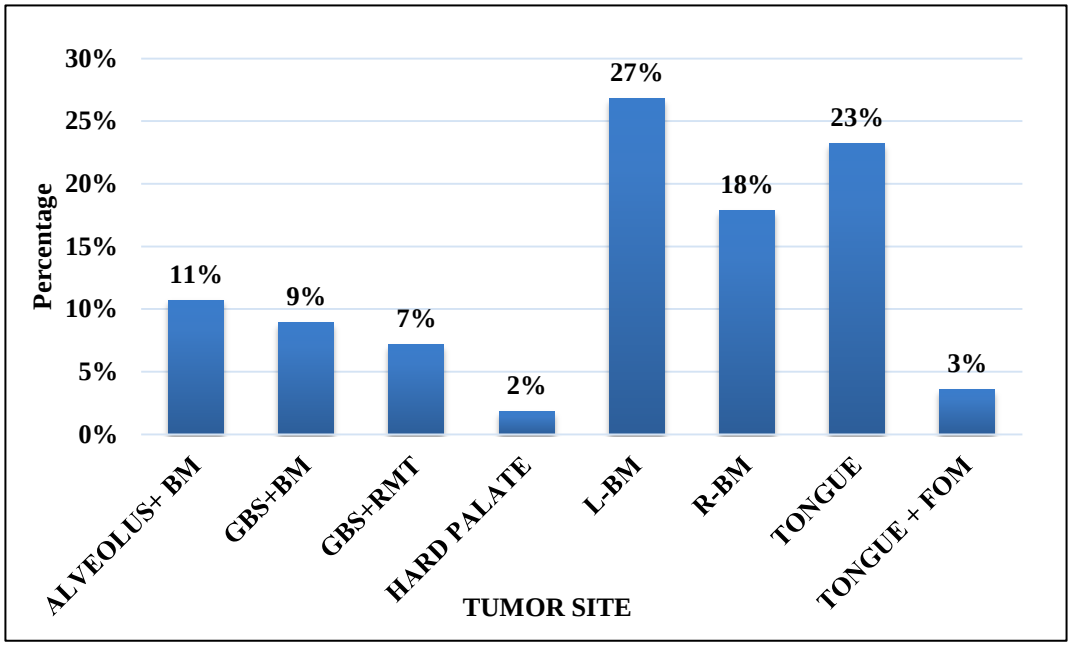


Among the cases, there were 40 males (71%) and 16 females (29%), giving a male-to-female ratio of 5:2.

Table 4. Distribution of OSCC according to tumor site

Tumor Site	Number of cases	%
Alveolus + BM	6	11%
GBS+BM	5	9%
GBS+RMT	4	7%
Hard palate	1	2%
L-BM	15	27%
R-BM	10	18%
Tongue	13	23%
Tongue + FOM	2	3%

Figure 10: Distribution according to tumor site



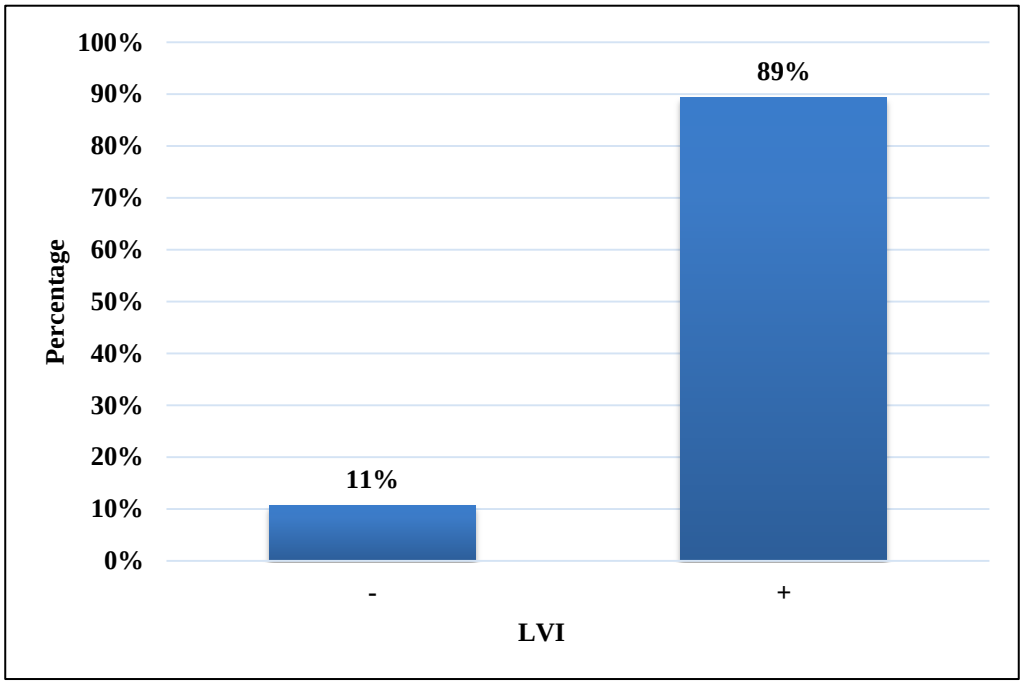
In the study population, 25 cases (45%) involved the buccal mucosa, with L-BM (27%) and R-BM (18%), followed by the tongue in 13 cases (23%). Other sites were Alveolus + BM (11%), GBS + BM (9%), and GBS + RMT (7%). Less frequently, tumors were found in the hard palate (2%).

Table 5: Distribution according to other clinicopathological parameters

Variables	Sub Category	Number of subjects	%
LVI	-	6	11%
	+	50	89%
PNI	-	1	2%
	+	55	98%
Bone invasion	-	42	75%
	MAXI+	2	4%
	MND+	12	21%
Lymph Node Metastasis	-	18	32%
	+	38	68%
Histology Grade	WDSCC	4	7%
	MDSCC	43	77%
	PDSCC	9	16%
IHC Grade	0	16	28%
	1	34	61%
	2	6	11%

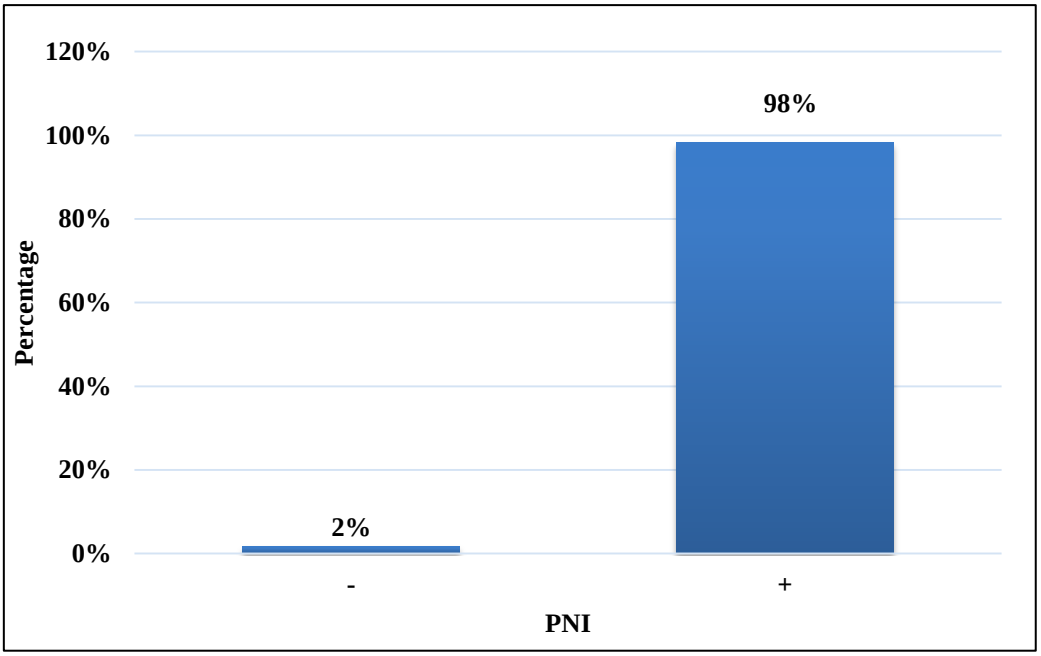
Of the 56 cases,14 (25%) had bone invasion, with 12 (21%) showing invasion in the mandible (MND+) and 2 (4%) showing invasion in the maxilla (MAXI+).

Figure 11 Distribution according to LVI



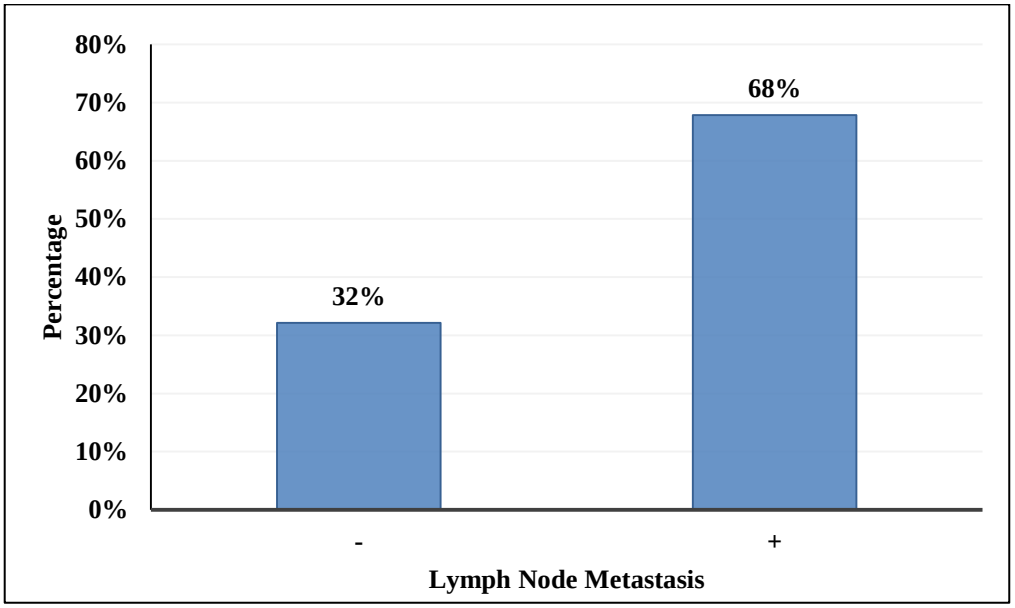
Among the study population, 50 cases (89%) out of 56 showed LVI

Figure 12: Distribution according to PNI



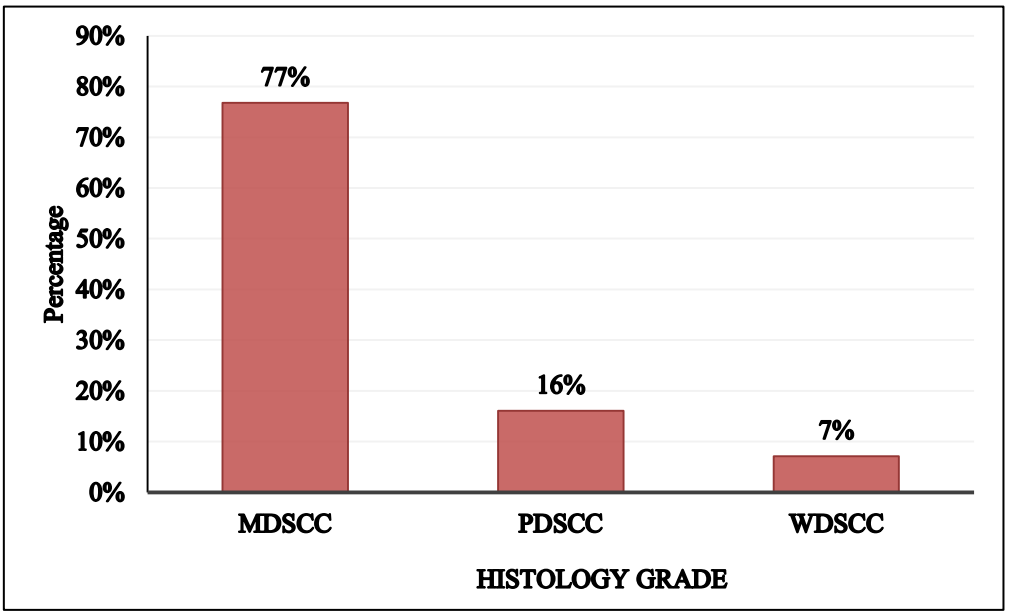
The PNI was observed in the majority of cases 55 patients (98%).

Figure 13: Distribution according to Lymph Node Metastasis



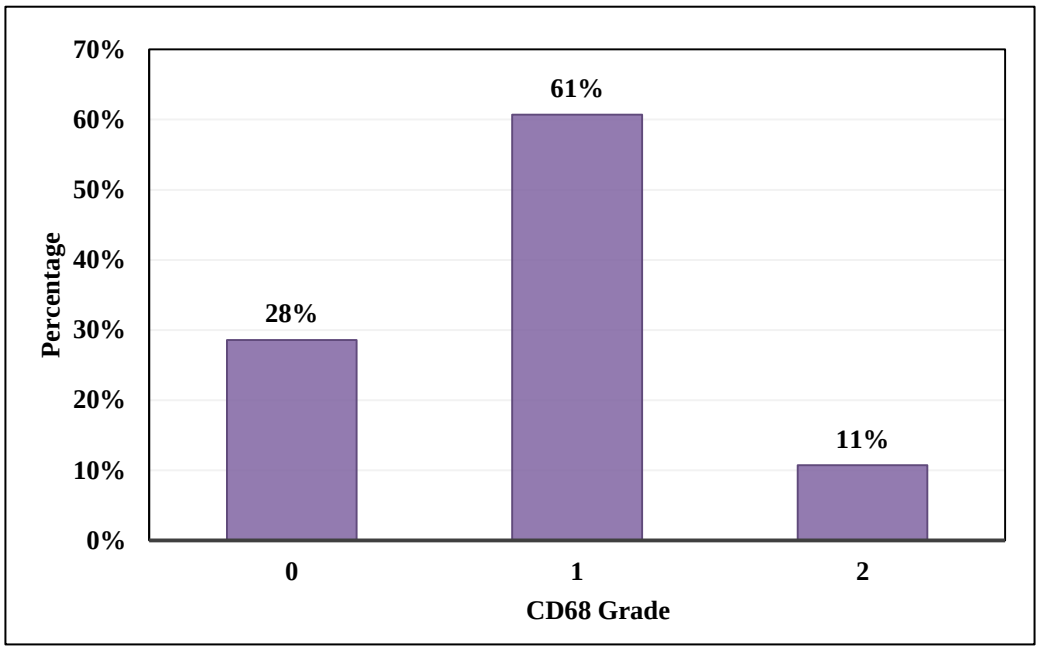
Lymph Node Metastasis was observed in 38 cases (68%) and 18 cases has no nodal metastasis.

Figure 14: Distribution according to the histological grade of OSCC



Among the 56 cases 43 (77%) were MDSCC, the most frequently observed histological grade followed by PDSCC 09 cases (16%) and 04 cases (7%) of WDSCC

Figure 15: Distribution according to CD68 IHC grade



Among the 56 OSCC cases, 34 (61%) were graded as Grade 1,16 (28%) were grade 0, and 6 (11%) were grade 2.

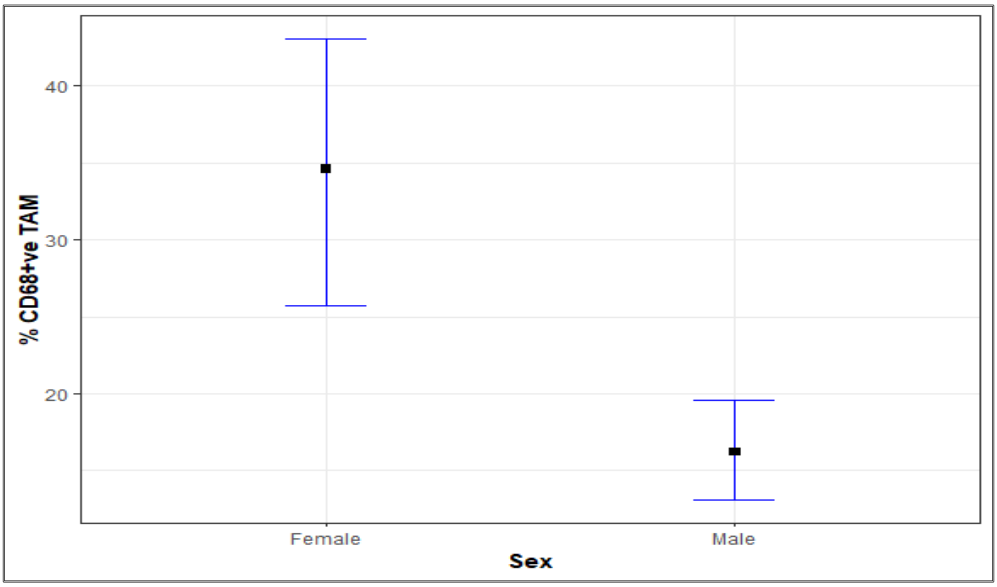
Table 6: Correlations of CD68+TAM percentage with age and sex

Variables	Sub Category	% CD 68+veTAM		p-value
		Mean ± SD	Median (Min,Max)	
Age (years)	>45 years	21 ± 16	19.5 (1, 59)	0.7261^{MW}
	≤45 years	23 ± 16	20.5 (6, 58)	
Sex	Female	35 ± 18	27 (1, 59)	$\mathbf{< 0.001^{MW}*}$
	Male	16 ± 11	17 (1, 45)	

*Abbreviation: MW – Mann Whitney U test, * indicates statistical significance.*

There was no significant difference in % CD 68+veTAM between individuals older than 45 years and those 45 years or younger (p-value = 0.7261).

Figure 16: Mean plot of %CD68+ve TAM over Sex



In our study, there was a significant difference in % CD 68+veTAM with females, showing a higher mean %CD68+ve TAM compared to males ($p < 0.001$). This suggests that female patients have a higher number of TAMs than male patients do.

Table 7: Correlation of % CD 68+veTAM with DOI

Variables	Correlation coefficient	p-value
% CD 68+veTAM with DOI	0.0108	0.9370^{SP}

Abbreviation: SP – Spearman's rank correlation test.

In our study, the DOI ranged from 0.3cm to 2cm, and Spearman's rank correlation test showed that there was no significant correlation between % CD 68+veTAM and DOI (p = 0.9370).

Table 8: Correlation of % CD 68+veTAM at the tumor site

Tumor Site	Yes	No	p-value
BM	23 ± 16 22.5 (1, 59)	18 ± 14 16.5 (3, 58)	0.2023^{MW}
Tongue	19 ± 14 17 (3, 58)	22 ± 16 22 (1, 59)	0.3995^{MW}
GBS	23 ± 19 17 (5, 56)	21 ± 15 21 (1, 59)	0.9200^{MW}
Alveolus	27 ± 11 27.5 (15, 45)	21 ± 16 18.5 (1, 59)	0.1485^{MW}
RMT	11 ± 5 10 (5, 17)	22 ± 16 21.5 (1, 59)	0.1150^{MW}
FOM	27 ± 16 27 (16, 38)	21 ± 16 20 (1, 59)	0.5959^{MW}
Hard palate	42	21 ± 15 19 (1, 59)	0.2043^{MW}

The distribution of % CD68+ve TAM across different tumor sites in our study did not show any significant differences ($p > 0.05$, for all comparisons).

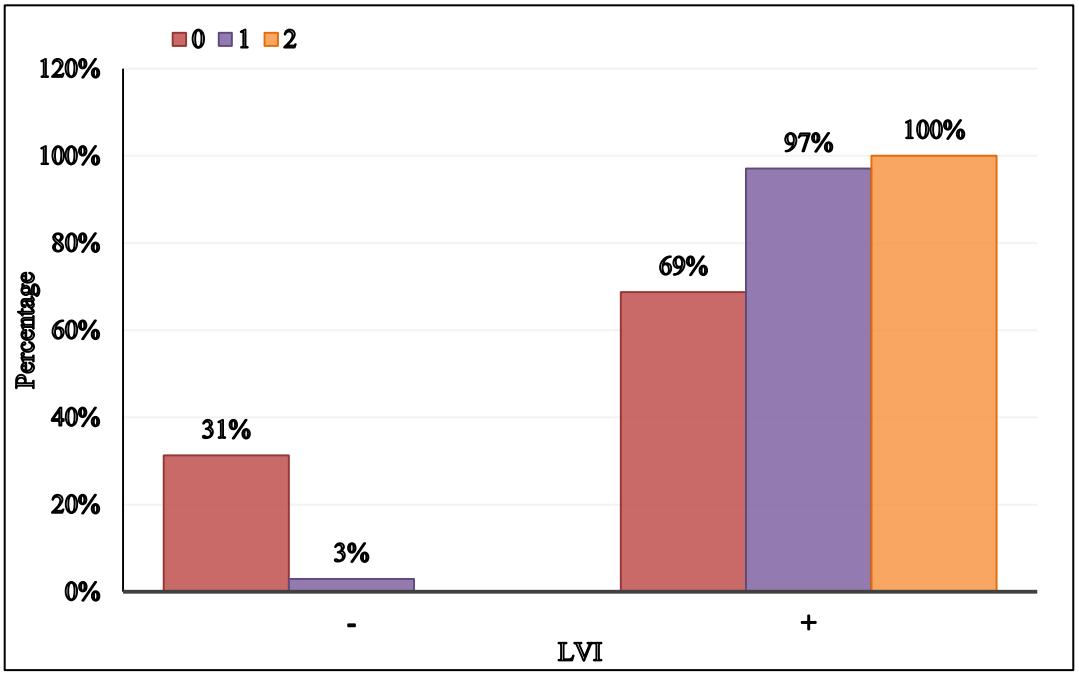
These results suggested that tumor location may not be a key factor influencing CD68+ve TAM expression in OSCC.

Table 9: Correlation of LVI with CD68 IHC grade

Variables	Sub Category	CD68 IHC grade			p-value
		0	1	2	
LVI	-	5 (31%)	1 (3%)	0	**0.0240^{MC}***
	+	11 (69%)	33 (97%)	6(100%)	

MC – Chi square test with Monte Carlo simulation.

Figure 17: Correlation between LVI and CD68 IHC grade

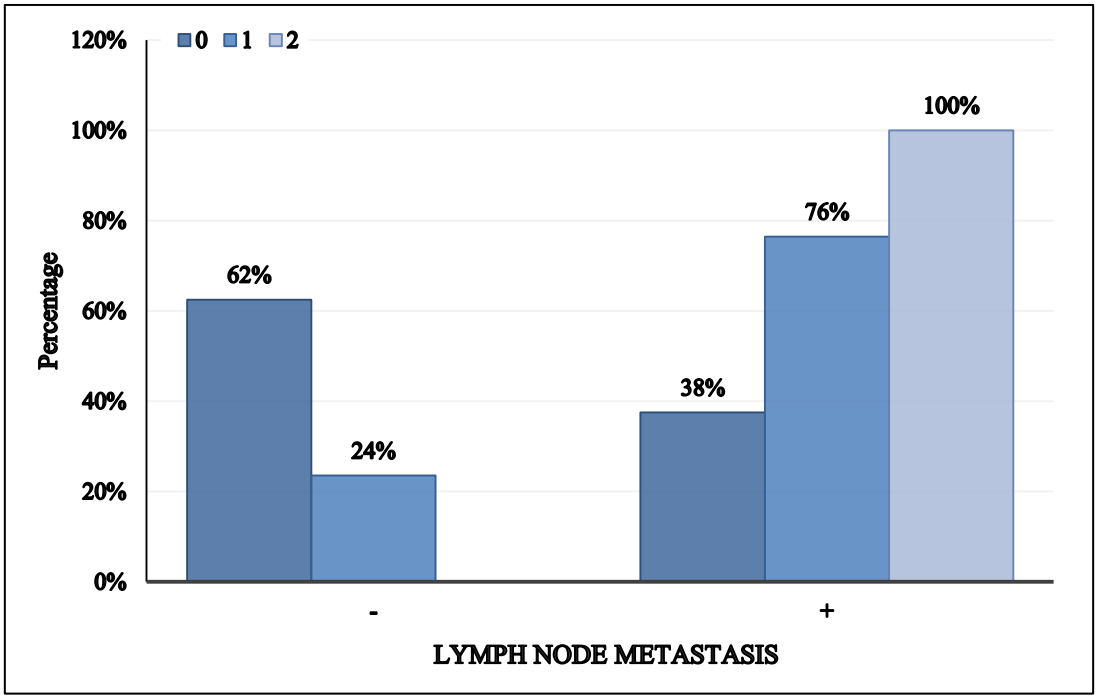


A significant association was observed in our study between LVI and CD68+TAMs grade (p = 0.0240), with a higher prevalence of LVI in the higher grades (100% in grade 2).

Table 10: Correlation of Lymph Node Metastasis with CD68 IHC grade

Variables	Sub Category	CD68 IHC grade			p-value
		0	1	2	
Lymph Node Metastasis	-	10 (62%)	8 (24%)	0	**0.0050^{MC}***
	+	6 (38%)	26 (76%)	6 (100%)	

Figure 18: Correlation of Lymph Node Metastasis to CD68 IHC grade



In our study, lymph node metastasis showed a significant correlation with CD68 IHC grade (p = 0.0050), increasing from 38% in grade 0 to 76% in grade 1, and 100% in grade 2.

Table 11: Correlation of others clinicopathological parameters with CD68 IHC grade

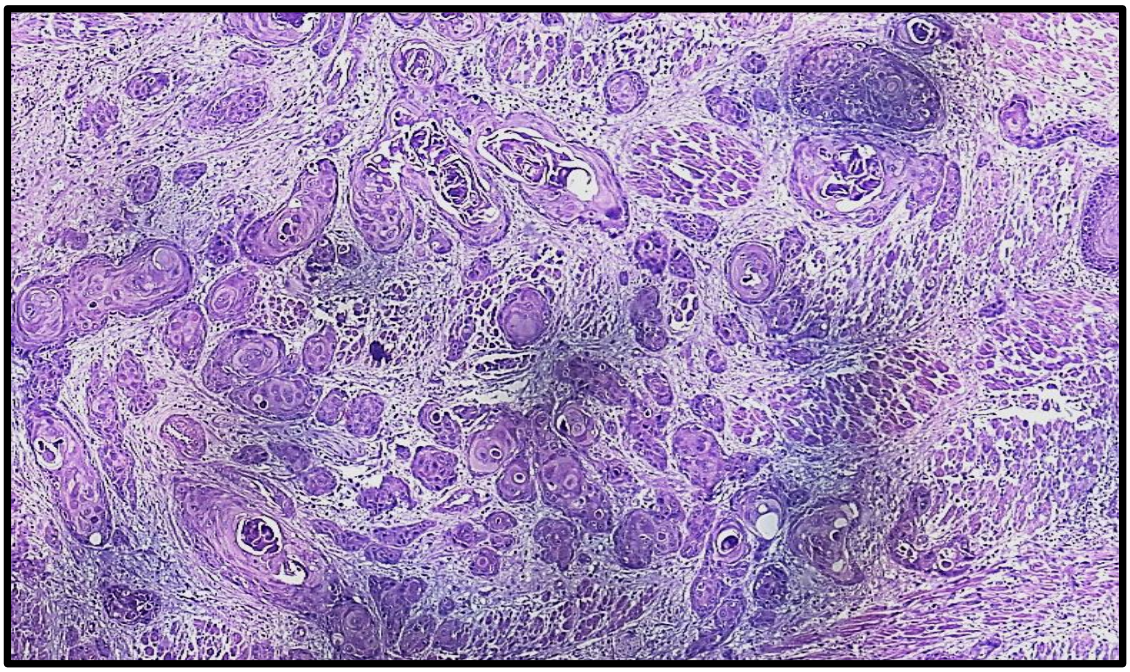
Variables	Sub Category	CD68 IHC grade			p-value
		0	1	2	
PNI	-	1 (6%)	0	0	0.3983[MC]
	+	15 (94%)	34 (100%)	6 (100%)	
Bone Invasion	-	14 (87%)	24 (71%)	4 (67%)	0.5447[MC]
	+	2 (13%)	10 (29%)	2 (33%)	
Histology Grade	WDSCC	2 (13%)	2 (6%)	0	0.1864[MC]
	MDSCC	9 (56%)	28 (82%)	6 (100%)	
	PDSCC	5 (31%)	4 (12%)	0	

In the present study, the PNI was present in almost all cases across all IHC grades, although no statistically significant association was found (p = 0.3983).

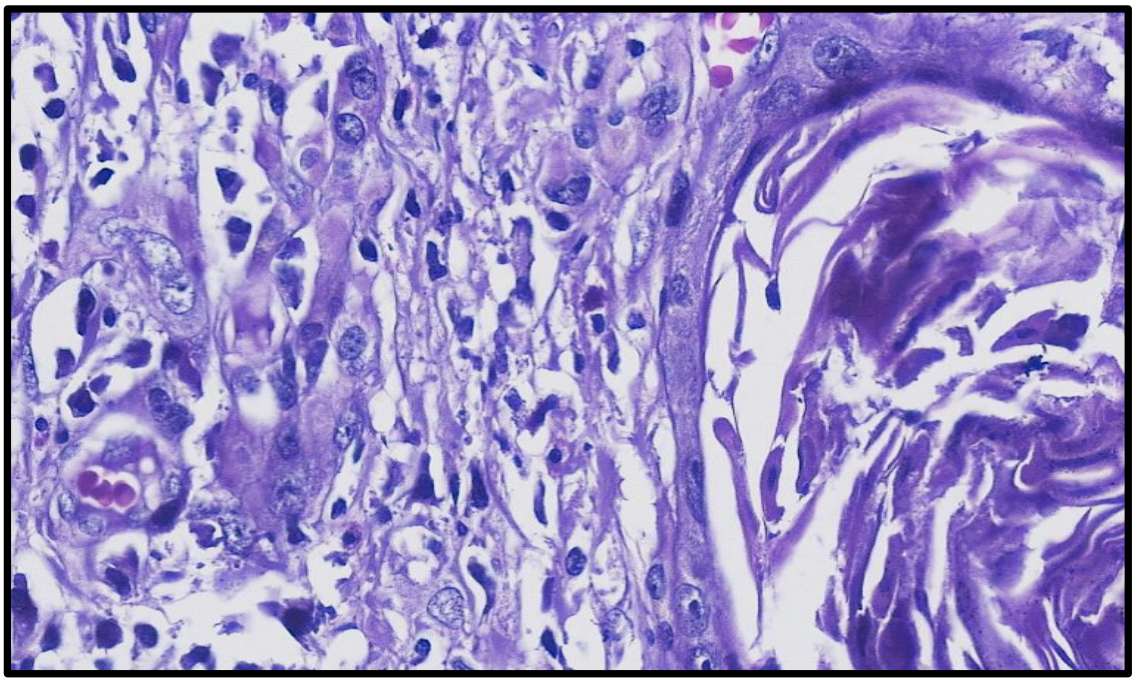
Even in the case of bone invasion, no significant correlation was found in our study (p = 0.5447).

In our study, histological grade was not significantly associated with IHC grade (p = 0.1864), although MDSCC was the predominant histological type in all IHC grades.

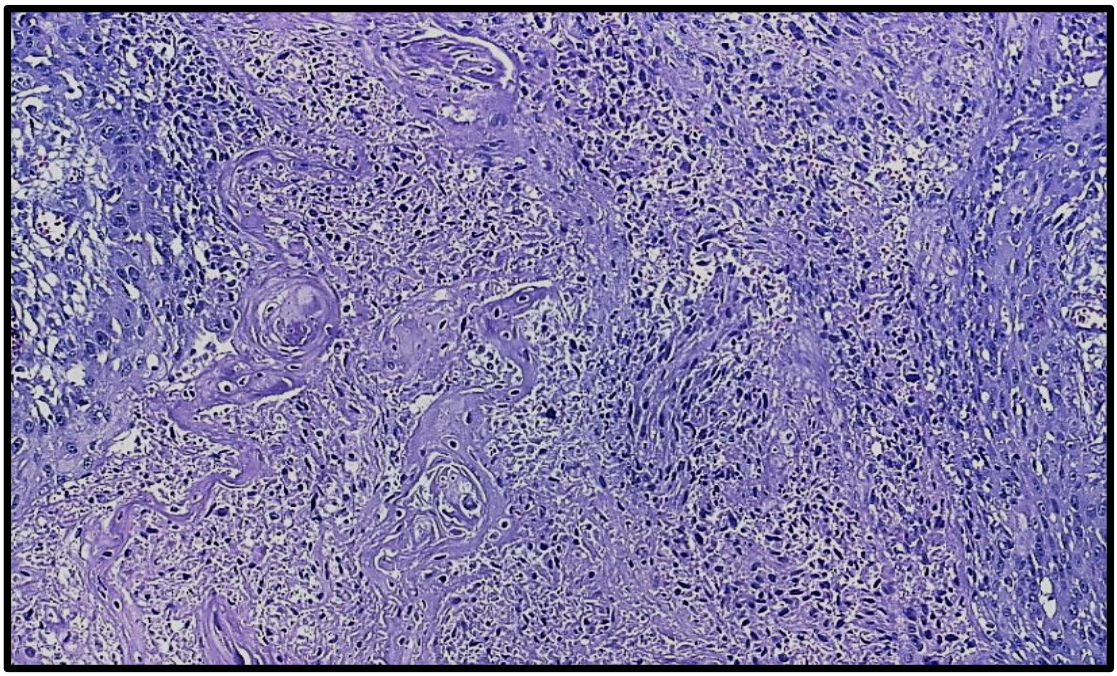
PHOTOMICROGRAPHS



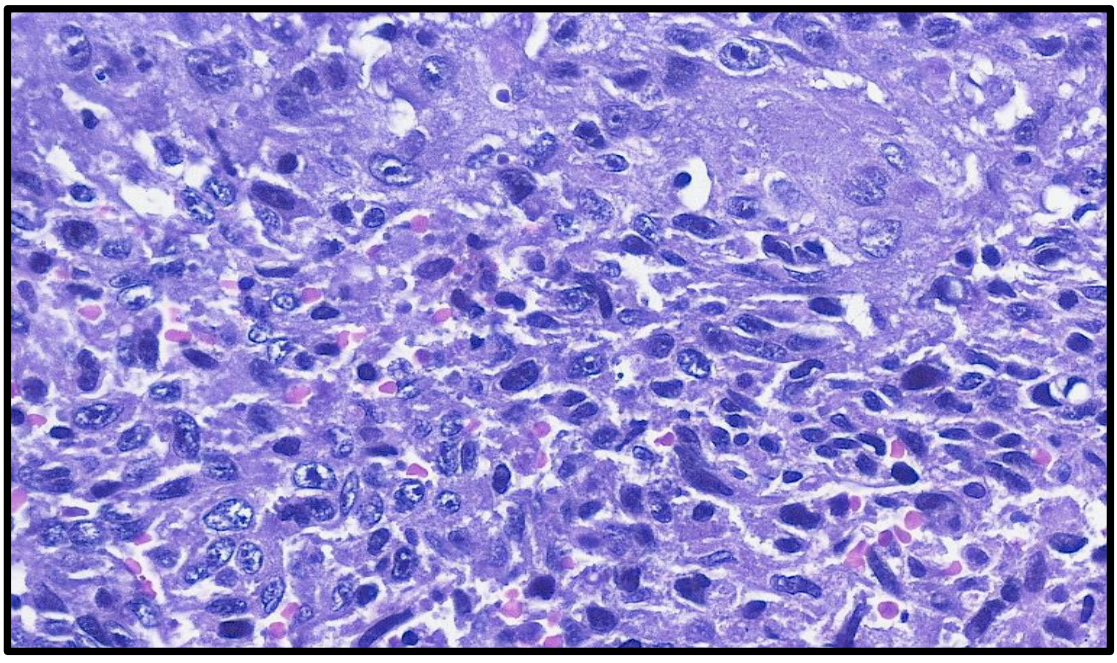
Photomicrograph 1. H&E, 100X- Well differentiated OSCC



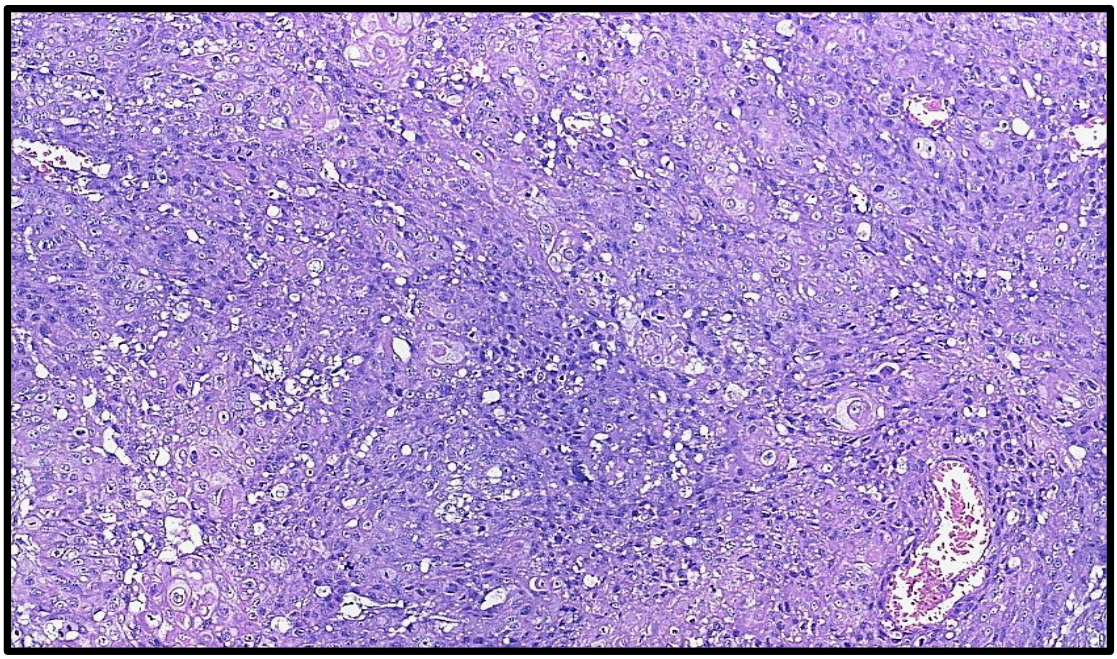
Photomicrograph 2. H&E, 400X- Well differentiated OSCC



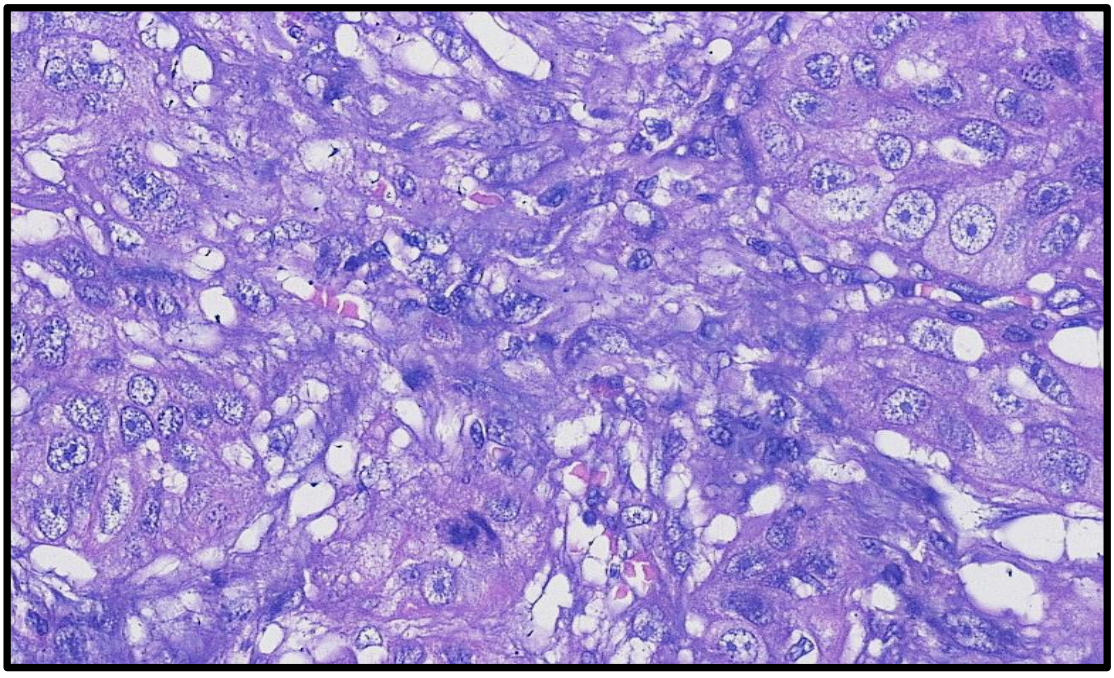
Photomicrograph 3. H&E, 100X- Moderately differentiated OSCC



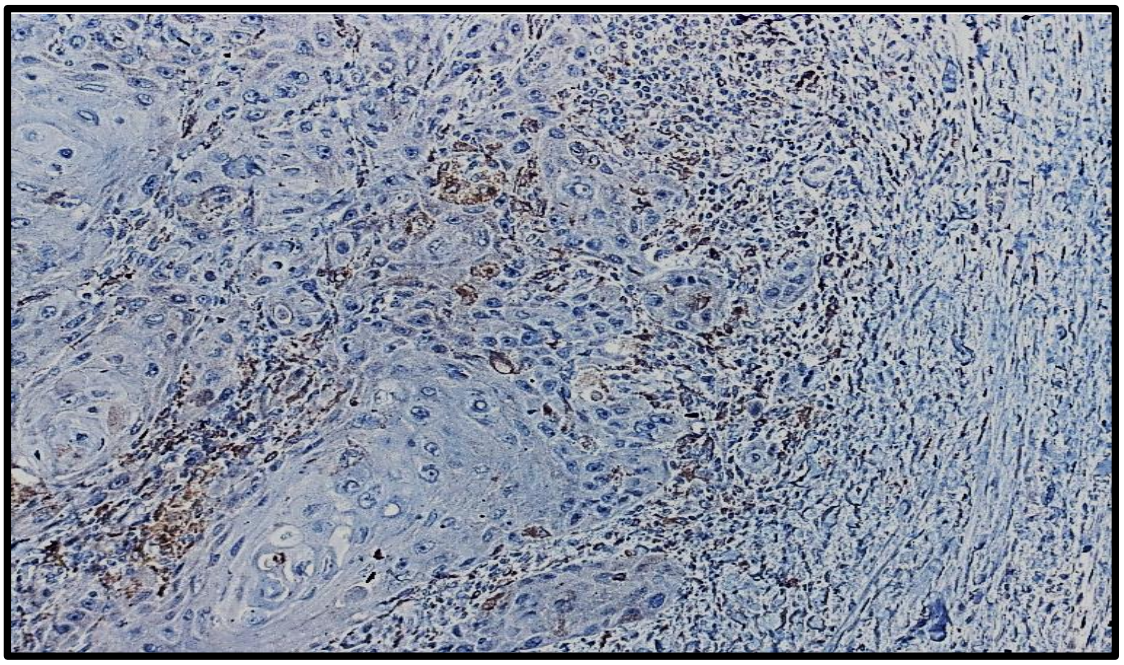
Photomicrograph 4. H&E, 400X- Moderately differentiated OSCC



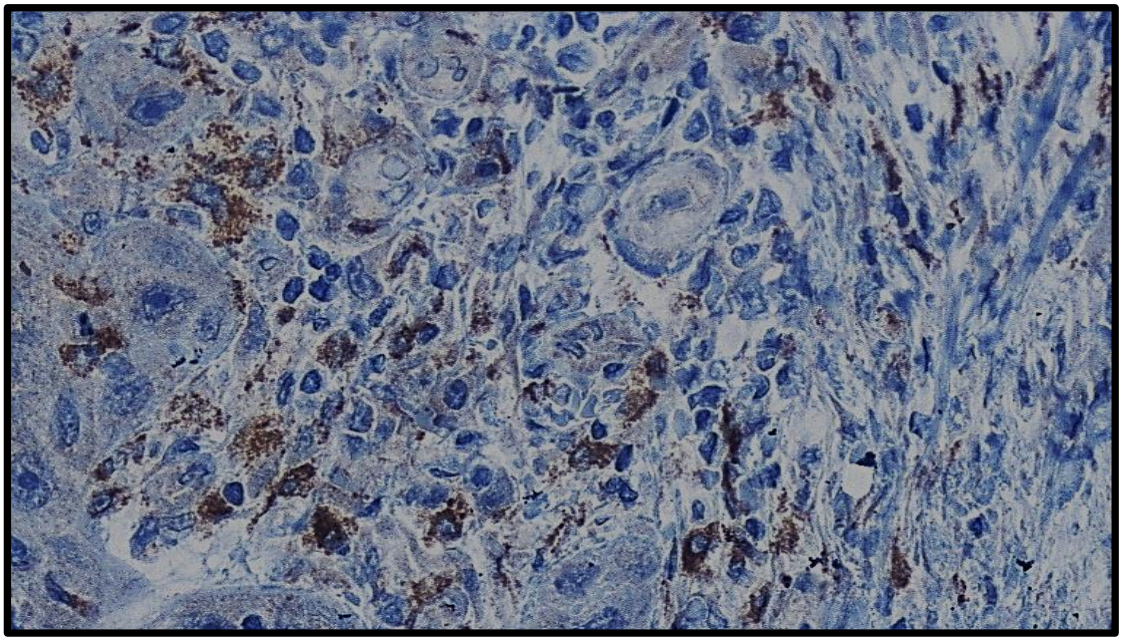
Photomicrograph 5. H&E, 100X- Poorly differentiated OSCC



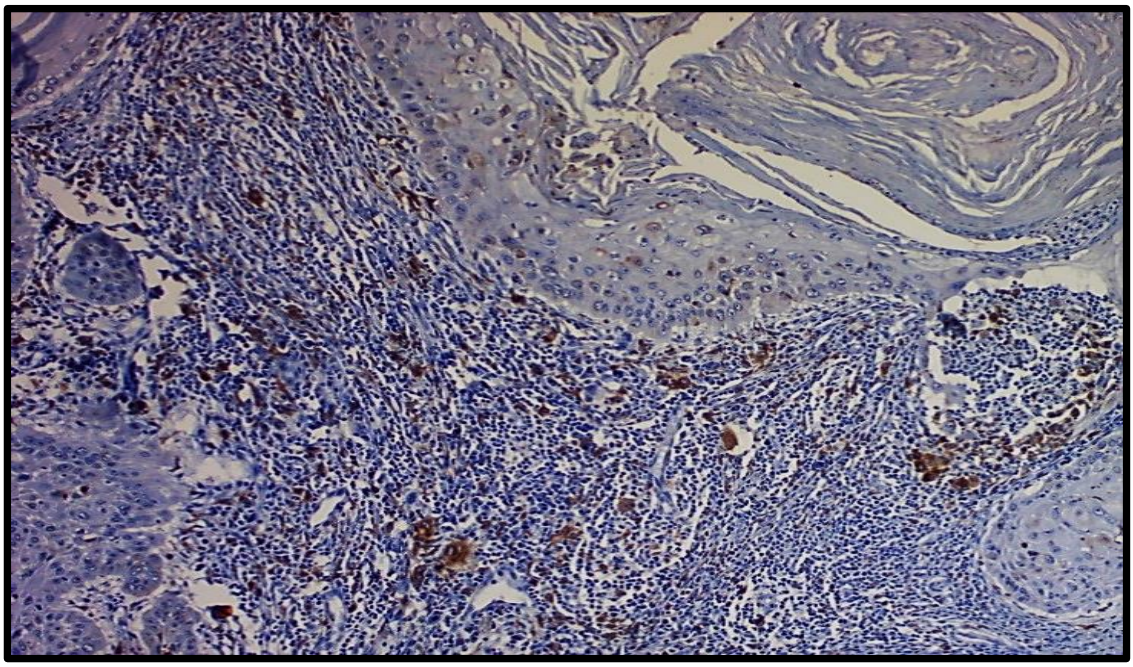
Photomicrograph 6. H&E, 400X- Poorly differentiated OSCC



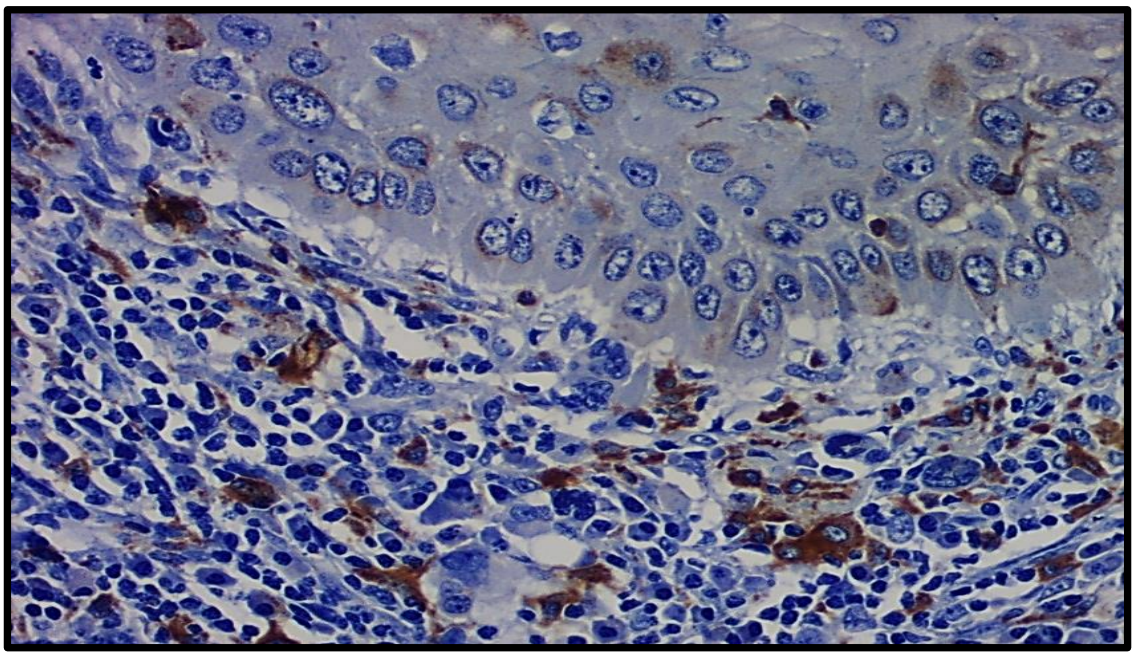
Photomicrograph 7. IHC CD68- GRADE 0 (Less than 10% of tumor cells) 100X



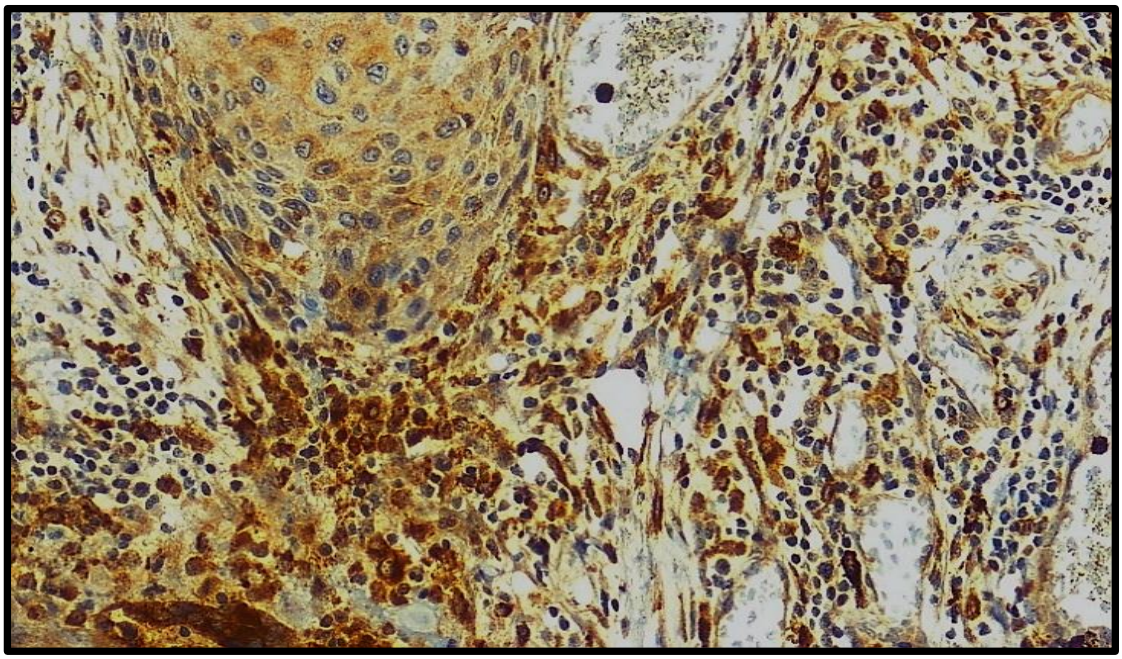
Photomicrograph 8. IHC CD68- GRADE 0 (Less than 10% of tumor cells) 400X



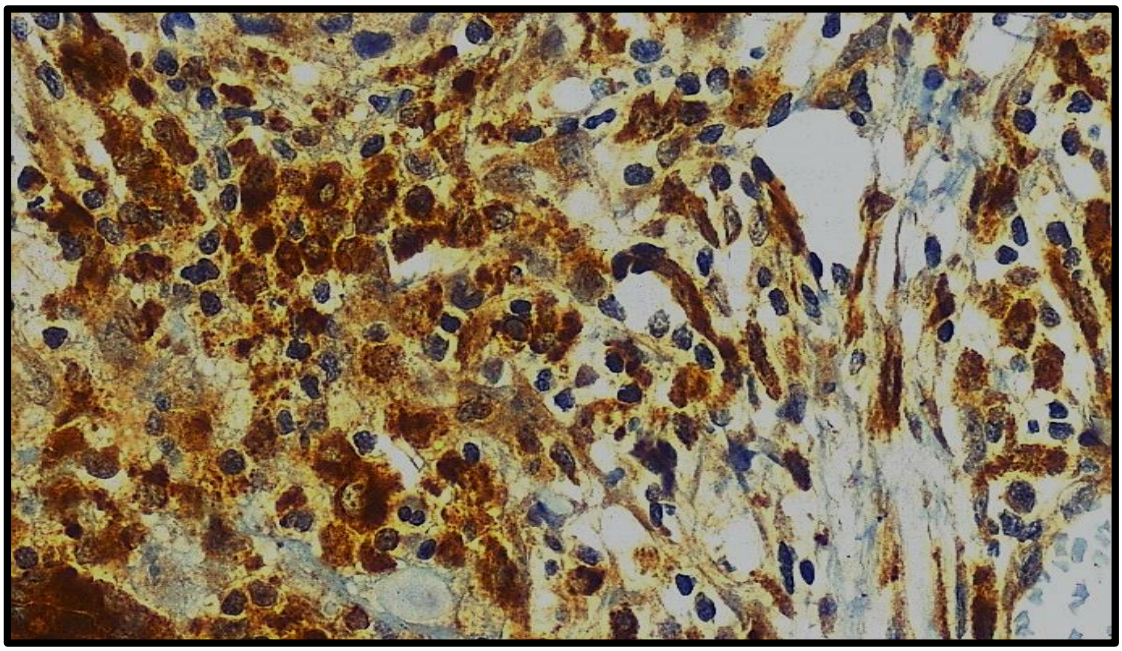
Photomicrograph 9. IHC CD68- GRADE 1 (11-50% of tumor cells) 100X



Photomicrograph 10. IHC CD68- GRADE 1 (11-50% of tumor cells) 400X



Photomicrograph 11. IHC CD68- GRADE 2 (Greater than 50 % of tumor cells) 100X



Photomicrograph 12. IHC CD68- GRADE 2 (Greater than 50 % of tumor cells) 400X

DISCUSSION

According to the Global Cancer Observatory (GCO), 377,713 cases of OSCC were reported globally in 2020, with the highest prevalence observed in Asian countries.[80] According to the GCO, the incidence and mortality of OSCC are predicted to increase by up to 40% by 2040 [69.] Despite advancements in treatment, the five-year survival rate for OSCC remains below 50%, ranking as the 7th leading cause of death among males and the 10th leading cause of death among females in Europe.[70-72] This has encouraged numerous researchers to explore the molecular mechanisms and roles of various cells and molecules in the development and progression of cancer with the aim of utilizing them for therapeutic applications.

TAMs are classified into two categories: classically activated M1 and alternatively activated M2 phenotypes.[80,88] Their activation state is influenced by signals from the microenvironment, allowing them to shift along a spectrum between M1 and M2 phenotypes.[56,68,80]

Solid tumors are known to exhibit abnormal growth and often become hypoxic owing to inadequate blood and oxygen supply.[56,89] TAMs are attracted to these hypoxic regions and serve as key components of the tumor stroma. TAMs are considered major contributors to tumor progression, influencing tumor cell proliferation and survival, angiogenesis, tissue invasion, and metastasis.[(26)(80)]

In the current study, clinicopathological and CD68 IHC evaluations were performed in 56 OSCC cases. To comprehend the molecular processes involved in development, advancement, and spread of cancer, new therapeutic treatments can be developed for OSCC. An effort was made to investigate the role of CD68 expression

and its association with prognosis with the goal of facilitating targeted therapies to improve outcomes.

Table 12: COMPARISON OF MEAN AGE WITH MEAN CD68(+) TAMs

Study	Mean age (years)	Mean CD68 (+) TAMs		p-value
		≤ 45 years	≥45 years	
Ahlam T et al	56 ± 15	40 ± 7	36 ± 5	0.098
Saghafi et al	61 ± 18	16 ± 12		0.145
Present study	50 ± 10	23 ± 16	21 ± 16	0.726

The present study showed that the incidence of OSCC ranges from 29 to 71 years. Most of the patients were older than 45 years of age. This could be due to widespread tobacco use as well as the tobacco belt area in the study group [92]. The p values showed an insignificant correlation between age and mean CD68(+) TAMs in these studies.

However, in a study in Iran, Saghafi et al.[73] found that with increasing age, the staining of the CD68 marker slightly decreased, which is similar to the findings of Ahlam et al.[8] and the present study.

Table 13: COMPARISON OF SEX WITH MEAN CD68(+) TAMs

Study	M:F	Mean CD68(+) TAMs		p-value
		Male	Female	
Ahlam T et al	1.7:1	35 ± 6	40 ± 6	0.023
Present study	5:2	16 ± 16	35 ± 18	<0.001

The current study showed that females have higher CD68+ve TAM than males ($p < 0.001$), which is similar to the findings of Ahlam et al.[8]

Table 14: COMPARISON OF SITES OF OSCC

Study	Site of Lesion
Saghafi et al (Iran)	Tongue > >Buccal vestibule
Omar E.A et al (Saudi)	Tongue and FOM
Mulla F.I et al	BM> Tongue>GBS>Alveolar ridge> RMT
A. Sudhakaran et al.	BM> Lip> Alveolus
Present study	BM> Tongue>Alveolus>GBS>Hard palate

In our study, the buccal mucosa was the most frequently affected site (35 %), and the least frequently affected site was the hard palate (2%). Sudhakaran et al.[74] and C. Scully et al [22.] However, Saghafi et al. and Omar et al.[5] reported that the tongue was the most common site of involvement. This could be a result of the different habits of individuals across various geographical regions.[73,92]

Table 15: COMPARISON OF MEAN CD68 SCORE WITH HISTOLOGY GRADE OF OSCC

Study	Histology grades of OSCC			p-value
Ahlam T et al	WDSCC	MDSCC	PDSCC	0.168
Mean CD68 score	36 ± 5	37 ± 7	43 ± 6	
Bagul N et al				
Mean CD68 score/ rank	27	24	26	**0.00 (χ2-28.824)**
Present study				
Mean CD68 score	13 ±12	25 ± 16	10 ± 11	**0.0228**

In our study, there was a significant difference in CD 68 (+ve) TAMs across histological grades (p =0.0228) and between PDSCC and MDSCC (p = 0.0344); although no significant found between WDSCC and MDSCC (p =0.3343) or WDSCC and PDSCC (p = 0.7336). In the present study, the most common histological grade observed was MDSCC (77%) with a maximum CD68 expression and PDSCC (16%) with a minimum CD68 expression, which was similar to the studies conducted by Lo Muzio L et al.[76] and Wei et al.[75,80] which revealed a pattern connecting inflammatory infiltrates with tumor differentiation; dense inflammatory infiltrates were observed in well and moderately differentiated tumors, whereas poorly differentiated cancers appeared to have fewer inflammatory infiltrates [56,93-94]

In contrast, Ahlam et al.[8] and Kazumasa et al.[57] showed no significant difference in TAMs distribution across histological grades and stages of OSCC, also CD 68 (+ve) TAMs were higher in PDSCC, which is completely opposite to our findings.

SITE OF CD68 DISTRIBUTION:

In our study CD68 positive TAMs were mainly observed in the tumor stroma and around tumor nests. A similar distribution was observed by Devendra et al.[68] Bagul et al.[56] and El-Rouby et al.[6] This indicates the recruitment of TAMs into the tumor microenvironment and their potential to influence tumor behaviour.[88,94] This also implies that TAMs play a role in tumor cytotoxicity and in clearing tumor debris.[6,56,80,88]

LYMPHOVASCULAR INVASION AND LYMPH NODE METASTASIS:

The present study found significant differences between lymph node metastasis ($p = 0.0050$) and LVI ($p = 0.0240$) with the IHC grading of CD68; however, no correlation was found between LVI and % CD68 TAMs. The present study observed a higher number of CD68 (+ve) TAMs in tumors with lymph node metastasis, which is similar to the findings of Lu et al.[11] and He et al.[9] This shows that TAMs contribute to tumor progression by triggering the release of the proangiogenic cytokine VEGF-A, which helps to form new blood vessels within the tumor microenvironment, supplies nutrients and oxygen to rapidly growing tumor cells, and facilitates the escape of tumor cells through these newly formed vessels, which promotes metastasis.[95,101-103]

SUMMARY AND CONCLUSIONS

- This cross-sectional study was done at Department of Pathology, JNMC, KLE'S Dr. Prabhakar Kore Hospital and Medical Research Centre, Belagavi, Karnataka by collecting data and blocks of Oral squamous cell carcinoma cases from January 2023 to December 2024
- Our study aimed to evaluate the CD 68 marker in OSCC and its relationship with various clinicopathological parameters.
- All 56 OSCC slides were stained with the CD68 antibody, examined for the number of CD 68+TAMs, and graded accordingly.
- The patients ages ranged from 29 to 71 years old. The number of CD68+TAMs decreases with age.
- With a sex ratio of M:F- 2:5, females showed more CD68+TAMs than males.
- The most frequent site of OSCC is the buccal mucosa, followed by the tongue and alveolus; there was no correlation between the tumor site and CD68 expression.
- No association was found between bone invasion and the depth of invasion of tumors with CD68 expression.
- In our study, PNI was present in almost all cases across all CD68 grades, although no statistically significant association was found.
- There was a significant difference in CD68 TAMs with histological grade ($p=0.0228$) in our study, which showed an increased number of TAMs in MDSCC and WDSCC compared to PDSCC, revealing the tumor's immunogenic profile or a sign of nodal metastasis, which could require supplementary treatment after surgery. However, no significant difference was found between the tumor grades and individual IHC grades of CD68, implying

that individual IHC grades had no significant role in indicating tumor progression.

- In our study, CD68+TAMs were mainly distributed in the stroma and around the tumor, suggesting their recruitment to the tumor microenvironment, which may contribute to antitumor responses or tumor progression.
- Tumors exhibiting lymph node metastasis and lymphovascular invasion (LVI) showed a higher presence of CD68+TAMs, which contributed to tumor metastasis.
- Thus, CD 68 is a pan-macrophage marker that can serve as a prognostic indicator.

LIMITATIONS

1. The sample size was limited (56 cases), and the patient could not be followed up due to time and financial constraints.
2. Another possible limitation is that only one marker is not sufficient for allocating macrophages towards the M2 phenotype.
3. Not many studies were available in the literature which were focus on correlation with clinicopathological parameters.

FUTURE PERSPECTIVE

- CD68 can serve as a prognostic marker to assess tumor progression, aggressiveness, and the likelihood of relapse.
- Advanced OSCC remains difficult to treat, leading to poor 5-year survival and increased mortality rates. Many studies have suggested that the tumoricidal activity of macrophages could aid in clinical applications, prognosis, and immunotherapy.

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ANNEXURES

ANNEXURE - I INFORMED CONSENT FORM

"EVALUATION OF CD68 IN ORAL SQUAMOUS CELL CARCINOMA AND ITS CORRELATION WITH CLINICOPATHOLOGICAL PARAMETERS"

Principal Investigator:

Guide/Co Investigators:

Introduction: In India there are increasing death due to oral cancer and the study aims at detecting CD68 which will help in better prognosis of patient with oral squamous cell carcinoma.

Procedure: During this study, you will be asked questions regarding history and background and you are supposed to answer to the best of your knowledge. If you agree to enroll yourself in this study, you will be interviewed regarding your present, past and family history and your clinical manifestations.

Withdrawal from participation in the study: Participation in this study in voluntary. You will be free to decide whether to participate in this study or continue participation once enrolled. In case you decide to withdraw your participation, you are free to do so. However, please convey the decision to the principal investigator.

Possible benefits from participating in the study: You will/will not get any benefits by participating in this study. The data gathered will help population at large.

Possible risks from participating in the study: There are no risks involved in participating in this study

Privacy and confidentiality: The information collected from you will be coded, to prevent any person to identify you. Your identity will never be revealed. The data collected from you will be kept confidential and only processed or aggregated data will be used for publication.

Financial incentives: You will not receive any payment for participating in this study.

Authorization for publication of aggregated data: Results obtained after processing of the aggregated data will be published for scientific purpose and or presented to scientific groups. However, your identity will never be revealed.

Questions: If you have any question or complaints with regard to your right as study participant you may contact Dr Harsha Hegde, Chairperson, Ethical committee of JNMC, 0831-2473777 Extension 4052.

Legal rights: By signing this consent form, we are not waving any of your legal rights

CONSENT STATEMENT

I voluntarily agree to take part in this study by signing below. I may withdraw at any time. I am not giving up any legal rights by signing this form. My signature below indicates that I have read or it has been read to me this entire consent form and have had all my questions answered.

Name of the participant: (Signature/thumbprint)

Name of the witness: (Signature/thumbprint)

Name of the investigator: (Signature)

Date:

ANNEXURE II – PROFORMA

PROFORMA FOR ORAL SQUAMOUS CELL CARCINOMA

PATIENT HISTORY

Name: Age: Sex: IP no. and HPR no.:

History:

Clinical diagnosis with stage:

Type of sample: Biopsy/Complete excision/Any other procedure

Gross:

Size:

Tumor site:

Depth of invasion:

Bony invasion:

Perineural invasion:

Lymphovascular invasion:

No. of Lymph nodes involved:

Groups of lymph node:

Microscopy:

1. Histopathological diagnosis and grade:

2. IHC staining:

- Percentage of tumour cells stained by CD68:
- Grade based on staining intensity:

ANNEXURE III- TNM STAGING OSCC

The latest TNM staging for oral squamous cell carcinoma (OSCC) follows the guidelines set by the 8th edition of the AJCC updated in 2018. The staging system classifies tumors based on:

- **T (Tumor)**: Size and extent of the primary tumor.
- **N (Node)**: Regional lymph node involvement.
- **M (Metastasis)**: Presence of distant metastasis

PRIMARY TUMOR (T)

T Category	**T CRITERIA**
Tx	Primary tumor cannot be assessed.
Tis	Carcinoma in situ.
T1	Tumor ≤ 2 cm, depth of invasion (DOI) ≤ 5 mm.
T2	Tumor ≤ 2 cm, DOI > 5 mm and ≤ 10 mm, or Tumor > 2 cm ≤ 4 cm, DOI ≤ 10 mm.
T3	Tumor > 4 cm or any tumor with DOI > 10 mm.
T4a	Moderately advanced disease; Lips and Oral cavity tumor invades bone, nerves, or skin of the face.
T4b	Very advanced disease; invades masticator space, pterygoid plates, skull base, or encases the internal carotid artery.

NOTE:

- T4a and T4b have been added to the new TNM staging system.
- Superficial erosion of the bone/tooth socket (alone) by a gingival primary is not sufficient to classify the tumor as T4.

REGIONAL LYMPH NODE (N) :

N Category	Clinical N (cN) Criteria	Pathological N (pN) Criteria
Nx	Regional lymph nodes cannot be assessed.	Regional lymph nodes cannot be assessed.
N0	No regional lymph node metastasis.	No regional lymph node metastasis.
N1	Single ipsilateral node ≤ 3 cm, no extra nodal extension (ENE(–)).	Single ipsilateral node ≤ 3 cm, ENE(–).
N2	**N2a**: Single ipsilateral node > 3 cm but ≤ 6 cm, ENE(–).	Single ipsilateral node > 3 cm but ≤ 6 cm, ENE(–); or single node ≤ 3 cm with ENE(+).
	N2b: Multiple ipsilateral nodes, none > 6 cm, ENE(–).	Multiple ipsilateral nodes, none > 6 cm, ENE(–).
	N2c: Bilateral/contralateral nodes, none > 6 cm, ENE(–).	Bilateral/contralateral nodes, none > 6 cm, ENE(–).
N3	**N3a**: Node > 6 cm, ENE(–).	Node > 6 cm, ENE(–).
	N3b: Any node(s) with overt ENE(+).	Single ipsilateral node > 3 cm with ENE(+); or multiple nodes with ENE(+); or single contralateral node ≤ 3 cm with ENE(+).

- AJCC 8 TNM STAGING now includes separate classifications for clinical (cN) and pathological (pN) neck nodes across all subsites.
- Extra nodal extension (ENE) has been incorporated into the N category for most cancers, excluding viral-related types and mucosal melanoma.

DISTANT METASTASIS (M):

M0	No distant metastasis
M1	Distant metastasis present

- AJCC 8 TNM staging eliminates the Mx category, which refers to distant metastasis that cannot be assessed.

PROGNOSTIC STAGE GROUPING

Stage 0	Tis, N0, M0
Stage I	T1, N0, M0
Stage II	T2, N0, M0
Stage III	T3, N0, M0 or T1-3, N1, M0
Stage IVA	T4a, N0-1, M0 or T1-4a, N2, M0
Stage IVB	T4b, Any N, M0 or Any T, N3, M0
Stage IVC	Any T, Any N, M1

ANNEXURE- IV

HEMATOXYLIN AND EOSIN STAINING PROTOCOL

1. Deparaffinize in Xylene I and II and III changes. (III change use warmed xylene)

(5 minutes in each)

2. Rehydrate using

    a. Absolute ethanol 100% (5 minutes)

    b. Absolute Ethanol 100% (5 minutes)

3. Rinse in distilled water (5 minutes)

4. Rinse in running tap water (5 minutes)

5. Stain in Harris's haematoxylin by progressive method (2 minutes) Fresh and filtered

6. Rinse in running tap water (20 minutes)

7. Decolorize in 1% acid alcohol (1 second)

8. Rinse well in tap water (5 minutes)

9. Immerse in hot water bath, 55˚C for blueing (3 seconds)

10. Rinse in tap water (5 minutes)

11. Counterstain in Eosin (15 seconds)

12. Dehydrate with absolute alcohol 100% (2-4 dips)

13. Clear in xylene I and II (5 minutes)

14. Mount with DPX.

Stock solution – Eosin:

Stock – 1% aqueous Eosin – Y

Stock – 1% aqueous Phloxin B

Working Solution – Eosin:

100ml stock Eosin

10 ml stock Phloxin B

780 ml 95% Ethanol

4 ml glacial acetic acid

Working Solution – Hematoxylin

Harris Hematoxylin, 1 litre

Working solution – 0.25% Acid alcohol

95% Ethanol, 2578 ml

dH2O, 950 ml

HCl, 9ml

Result: Nuclei – blue, cytoplasm – pink, RBCs – red.

Reference: Bancroft D, Laylon C. The haematoxylin and eosin, In: Kim SS Ed, Bancroft's Theory and practice of histopathological techniques. &th Ed., China, Churchill Livingstone; 2013: p173-187.

ANNEXURE- V

CD68 IMMUNOHISTOCHEMISTRY STAINING PROTOCOL

PROCEDURE**:**

1. Tissue section is cut on a microtome with 3 microns thickness and collected on coated slides
2. The sections were then incubated overnight at 37°C. Before test bake it at 60°C for 1 hour
3. Deparaffinise steps-
    - Xylene I- 10 minutes
    - Xylene II- 10 minutes
    - Absolute alcohol I- 10 minutes
    - Absolute alcohol II- 10 minutes
    - Rinse in water- 5 minutes
    - Rinse in distilled water- 1 minute
4. Antigen retrieval (TRIS-EDTA buffer)- Buffer solution
5. Required amount of buffer is prepared and cook the slides in pressure cooker for 3 whistles
6. Allow it to cool to room temperature for 15 minutes
7. Wash with wash buffer 2 times with gap of 30 seconds each
8. Apply 3% hydrogen peroxide- 8 to 10 minutes
9. Primary monoclonal CD68 (Clone:KP1) Mouse Monoclonal antibody; PathnSitu, is incubated for 30 to 60 minutes in closed chamber at room temperature
10. Wash with wash buffer 3 times with gap of 30 seconds each

11. Apply polymer Horseradish peroxidase (HRP) for 25 to 30 minutes in closed chamber at room temperature
12. Wash with wash buffer 3 times with gap of 30 seconds each
13. Apply Diaminobenzidine (DAB) substrate for 10 minutes
14. Wash with water for 2 minutes
15. Wash with distilled water for 1 minute
16. Counter stain with Haematoxylin- 3minute
17. Blueing in warm water- 1minute
18. Clear in xylene and mounted with DPX.

PREPARATION OF REAGENTS

1. Antigen retrieval Buffer

TRIS EDTA Buffer (Cat#PS009) - pH: 8.5 to 9.0

Preparation:

TRIS Base- 1.21 gram

EDTA (atomic number: 372)- 0.37 gram

Dissolve in 1000ml of water

2. Wash buffer

TRIS BUFFERED SALINE (TBS)- pH: 7.2 to 7.6

Preparation:

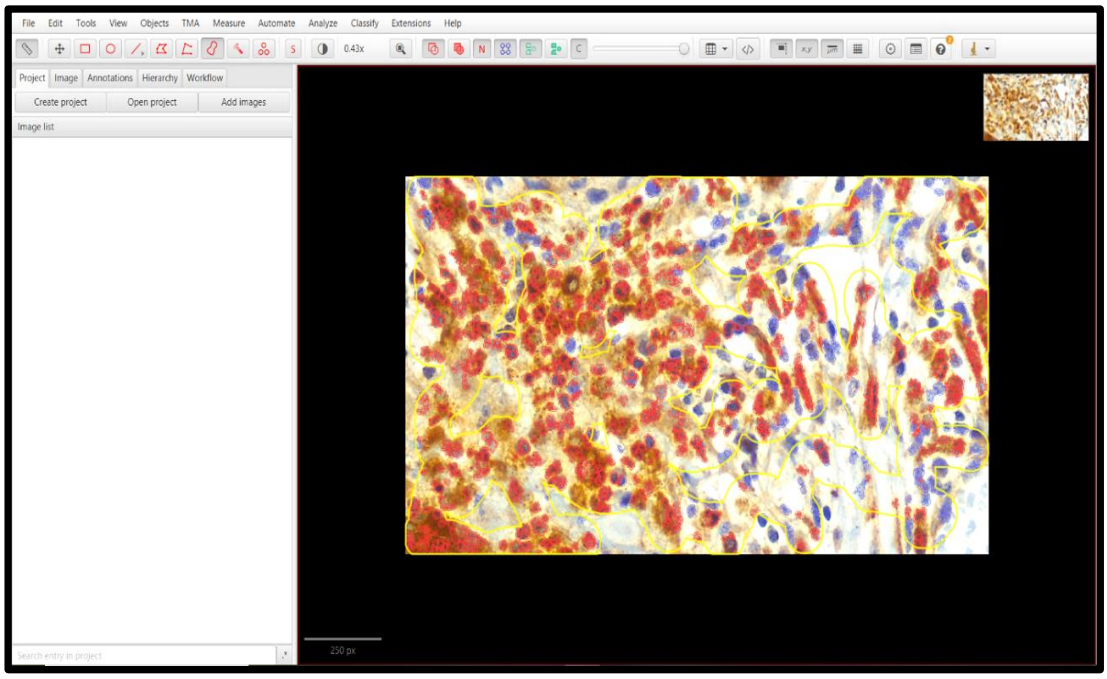
TRIS Base- 8.6 gram

NaCl- 9.6 gram

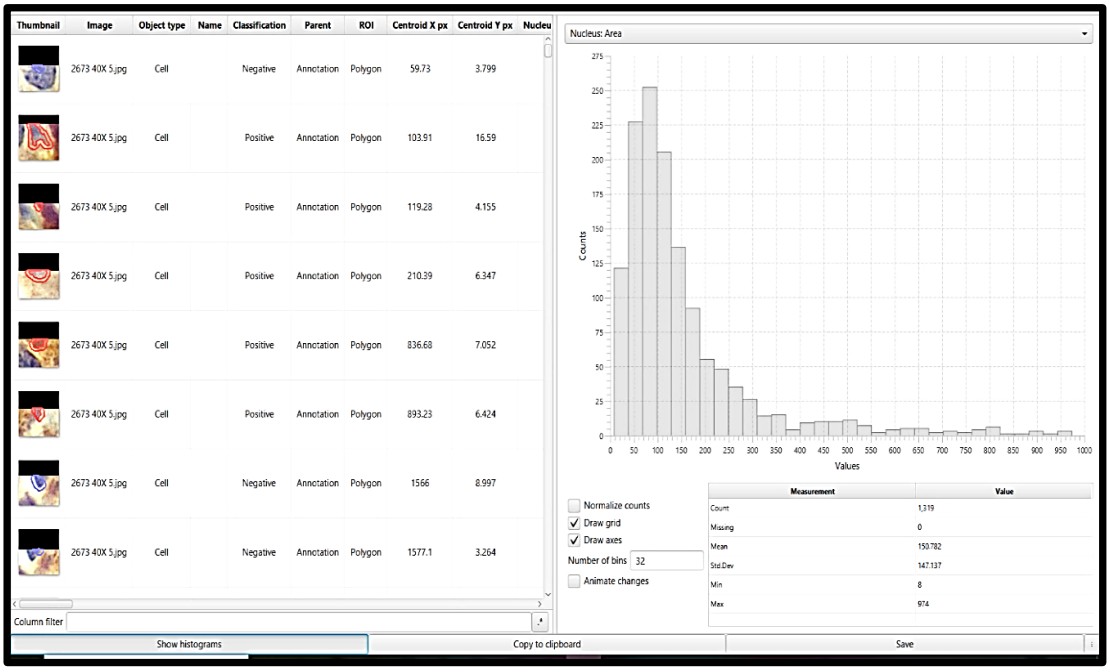
Dissolve in 1000ml of water.

Adjust pH by using concentrated HCl

ANNEXURE- VI QuPath CELL DETECTION IMAGES



PHOTOMICROGRAPH 13: CD68 (+) TAMs ARE HIGHLIGHTED IN RED



PHOTOMICROGRAPH 14: COUNTING THE CD68 (+) CELLS

ANNEXURE – VII KEY TO MASTER CHART

M - Male

F - Female

GBS - Gingivobuccal mucosa

RMT – Retromolar trigone

FOM- Floor of mouth

BM - Buccal Mucosa

(R) - Right

(L) - Left

FOM - Floor of mouth

LVI - Lymphovascular invasion

DOI - Depth of invasion

PNI - Perineural invasion

MAXI - Maxilla

MND - Mandible

WLE - Wide local excision

HM – Hemi mandibulectomy

PMML - Pectoralis major myocutaneous flap

MRND - Modified radical neck dissection

RND - Radical neck dissection

ND - Neck dissection

PDSCC - Poorly differentiated Squamous cell carcinoma

MDSCC - Moderately differentiated Squamous cell carcinoma

WDSCC - Well differentiated Squamous cell carcinoma

% - Percentage

ANNEXURE – VIII MASTER CHART

SL NO	HPR NO.	AGE/SEX	SURGERY PERFROMED	TUMOR SITE	TUMOR SIZE	DOI	LVI	PNI	BONE INVASION	LN MET	HISTOLOGY GRADE	% CD 68+	IHC GRADE
1	2673/23	31 F	WLE + Neck dissection	R-BM	2.5X1.5CM	0.3CM	+	+	-	+	MDSCC	54	2
2	2757/23	49 M	COMMANDO + PMML FLAP	GBS+BM	1.5X1CM	1.4CM	+	+	MND+	+	MDSCC	25	1
3	2841/23	46 M	COMMANDO + PMML FLAP	L-BM	3.5x1CM	1CM	+	+	-	-	MDSCC	1	0
4	2846/23	38 M	BITE RESSECTION+ SEG MANDIBULECTOMY	R-BM	3x1.5	1.2CM	+	+	-	+	PDSCC	18	1
5	2918/23	46 M	COMPLETE RESSECTION +SEG MANDIBULECTOMY	L-BM	2.5x1.5	1CM	-	+	-	-	WDSCC	28	1
6	2985/23	60 F	COMMANDO + NECK DESSECTION	L-BM	2x2	0.6CM	+	+	-	+	MDSCC	26	1
7	3106/23	32 M	HEMIGLOSSECTOMY+NECK DISSECTION	TONGUE	2X2CM	2CM	-	-	-	+	MDSCC	6	0
8	3729/23	61 M	WLE + Neck dissection	L-BM	2.5x2	0.5CM	+	+	-	-	MDSCC	8	0
9	3793/23	71 M	L-HM	GBS+BM	3.5x2.5cm	1CM	+	+	-	+	MDSCC	14	1
10	3866/23	35 M	BITE RESECTION	L-BM	3.8X3.8X1.5CM	1.3cm	+	+	-	+	MDSCC	23	1
11	3916/23	60 F	WLE+NECK DISSECTION	R-BM	2x1.5cm	1.1cm	+	+	MND+	+	PDSCC	28	1
12	3933/23	50 F	WLE+NECK DISSECTION	L-BM	2X2cm	1CM	+	+	-	+	MDSCC	51	2
13	4203/23	46 M	WLE+NECK DISSECTION	TONGUE	3.5X2CM	1.2CM	+	+	-	+	MDSCC	21	1
14	4228/23	53 F	WLE+HM	L-BM	3.5X2.5X2CM	1.8CM	+	+	-	+	MDSCC	23	1
15	4285/23	61 M	R-HEMIGLOSSECTOMY	TONGUE	2X2X2CM	1.2CM	+	+	-	-	MDSCC	18	1
16	4306/23	54 M	R-HEMIGLOSSECTOMY	TONGUE	2X0.7X0.3cm	1CM	+	+	-	-	MDSCC	10	1
17	1054/24	64 M	WLE+HM	L-BM	3.5X3CM	1CM	+	+	-	+	MDSCC	27	1
18	1084/24	45 M	BITE RESECTION+NECK DISSECTION	R-BM	4.5X3.5CM	2cm	+	+	-	-	MDSCC	22	1
19	1091/24	61 F	COMMANDO RESECTION	GBS+BM	2.5X1.5CM,	1.2CM	+	+	MND+	+	MDSCC	52	2
20	1226/24	33 M	WLE+NECK DISSECTION	TONGUE	2x2cm	0.5cm	-	+	-	-	MDSCC	9	0
21	1378/24	48 F	WLE	TONGUE	3X1.8X1CM	1.1cm	+	+	-	+	PDSCC	26	1
22	1425/24	49 M	WLE+HM	TONGUE	3.5X2X1.5CM	1.5cm	+	+	-	+	MDSCC	21	1
23	1584/24	50 M	WLE+NECK DISSECTION	TONGUE	1.5X1CM	0.7cm	-	+	-	-	WDSCC	3	0
24	1610/24	37 M	WLE+HM	L-BM	2.5X2CM	2cm	+	+	-	-	MDSCC	9	0
25	1637/24	54 M	COMMANDO RESECTION	GBS+RMT	3.5X1.5X0.8CM	0.5CM	-	+	-	-	MDSCC	5	0
26	1653/24	66 F	WLE+HM	GBS+RMT	3.5X2CM	0.5CM	+	+	MND+	+	PDSCC	11	1
27	3610/24	48 F	COMPLETE RESECTION+ SEG MANDIBULECTOMY	GBS+ BM	3.5X2CM	0.5CM	+	+	-	+	MDSCC	56	2
28	3618/24	30 M	WLE+ NECK DISSECTION	TONGUE	2.5X2CM	1.3CM	+	+	MAXI+	+	MDSCC	26	1
29	3752/24	53 M	WLE+HM	ALVEOLUS+	3X2X1.5CM	2CM	+	+	-	+	MDSCC	30	1

				BM									
30	3875/24	57 M	WLE+NECK DISSECTION	TONGUE	3X2CM	1CM	+	+	-	-	WDSCC	17	1
31	3934/24	51 F	WLE+NECK DISSECTION	R-BM	1X1CM,	0.5CM	+	+	-	+	MDSCC	26	1
32	4051/24	49 M	WLE+NECK DISSECTION	R-BM	1X1CM	0.6CM	+	+	-	-	PDSCC	2	0
33	4098/24	60 M	WLE+HM	ALVEOLUS+BM	4.5X4CM	0.5CM	+	+	MND+	+	MDSCC	45	1
34	4112/24	45 F	WLE+NECK DISSECTION	TONGUE	3X2X1CM	1.1CM	+	+	MND+	+	MDSCC	58	2
35	4270/24	44 M	HM+WLE+NECK DISSECTION	ALVEOLUS+BM	2.5X2CM	0.5cm	+	+	-	+	MDSCC	18	1
36	4406/24	59 M	NEAR TOTAL GLOSSECTOMY+ SEG MAND	TONGUE	3.5X1.5CM	1.1CM	+	+	-	-	WDSCC	4	0
37	4426/24	40 M	R- BITE RESECTION	R-BM	1X1CM	1.8CM	+	+	MND+	+	MDSCC	22	1
38	4487/24	70 F	WLE+ MAXILLECTOMY	HARD PALATE	3X3CM	0.5CM	+	+	MAXI+	+	MDSCC	42	1
39	4508/24	70 M	WLE+ SEG MANDIBULECTOMY	TONGUE + FOM5X3CM		1.3CM	+	+	MND+	+	MDSCC	38	1
40	4528/24	48 F	WLE+ NECK DISSECTION	L-BM 2x2cm		0.5CM	+	+	-	+	PDSCC	1	0
41	4673/24	58 M	WLE+ SEG MANDIBULECTOMY	TONGUE + FOM 3X2CM		1.1CM	+	+	-	+	MDSCC	16	1
42	4692/24	29 M	COMMANDO + PMML FLAP	L-BM 5X4.5CM		1.4CM	+	+	-	+	MDSCC	25	1
43	4728/24	50 M	COMMANDO +PMML FLAP	ALVEOLUS+BM 2X2CM		0.5CM	+	+	-	+	MDSCC	31	1
44	4730/24	52 F	WLE+NECK DISSECTION	R-BM 2X1CM		0.5CM	+	+	-	+	MDSCC	22	1
45	4737/24	44 M	WLE+NECK DISSECTION	TONGUE 2.5X2.5CM		1.5CM	-	+	-	-	MDSCC	9	0
46	4756/24	48 F	WLE+ HEMI-MANDIBULECTOMY	GBS+BM 4CM		1CM	+	+	-	-	MDSCC	18	1
47	5041/24	52 M	WLE+ HEMI MANDIBULECTOMY	GBS+RMT 1.5X1.5CM		1.4CM	+	+	-	-	MDSCC	17	1
48	5042/24	50 M	WLE+ HEMI-MANDIBULECTOMY	ALVEOLUS+ BM 2X1CM		1.1cm	+	+	MND+	-	MDSCC	15	1
49	5324/24	47 M	WLE+NECK DISSECTION	R-BM 3X2CM		1.5CM	+	+	-	-	MDSCC	5	0
50	5463/24	46 M	WLE+ HEMI-MANDIBULECTOMY	ALVEOLUS+ BM 4.5X2.5CM		2CM	+	+	-	+	MDSCC	25	1
51	5481/24	50 F	COMMANDO RESECTION	L-BM 1.4x0.8cm		1CM	+	+	-	+	MDSCC	59	2
52	6167/24	52 M	WLE+L-MARGINAL MANDIBULECTOMY	L-BM 2x1.5cm		0.6CM	+	+	-	+	PDSCC	3	0
53	6169/24	46 M	L-HM+ L-RND	L-BM 3X1.5CM		1.5cm	+	+	MND+	+	PDSCC	4	0
54	6208/24	52 M	HM+WLE+PMML FLAP RECONSTRUCTION	GBS+RMT 1.5X1CM		0.7CM	+	+	MND+	+	MDSCC	9	0
55	6280/24	45 M	WLE+ HEMIMANDIBULECTOMY	R-BM 2X1.5CM		1CM	+	+	MND+	+	MDSCC	19	1
56	6282/24	52 M	WLE+ NECK DISSECTION	L-BM 2x2cm		0.7CM	+	+	-	+	PDSCC	2	0