Original Article

DOI: 10.7860/JCDR/2026/84836.24114

Pathology Section

Evaluation of Immunohistochemical Expression of CD68 and CD163 in Oral Squamous Cell Carcinoma and Oral Squamous Cell Carcinoma Arising from Oral Submucous Fibrosis: A Cross-sectional Study

DIVYA SHREE SANKAR[1], SANDHYA SUNDAR[2], DEEPAK PANDIAR[3]

ABSTRACT

Introduction: Oral Squamous Cell Carcinoma (OSCC) is a common malignancy with poor prognosis, the development of which is affected by the Tumour Microenvironment (TME). Tumour-Associated Macrophages (TAMs), particularly the M2 phenotype marked by CD 163, play a key role in tumour progression. Oral Submucous Fibrosis (OSMF)-induced OSCC shows distinct biological behaviour due to fibrosis-related alterations in macrophage polarisation.

Aim: To evaluate and compare the immunohistochemical expression of CD 68 and CD 163 and the CD 68/CD 163 macrophage ratio, in conventional OSCC and OSMF-induced OSCC.

Materials and Methods: This cross-sectional study was conducted between January 2020 and February 2025 in the Department of Oral Pathology and Microbiology at Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India. The study included 34 histopathologically confirmed OSCC cases (20 conventional OSCC and 14 OSMF-induced OSCC). Immunohistochemical expression of CD 68 and CD 163 was evaluated using percentage positivity, staining intensity and Immunoreactive Score (IRS). The CD 68/CD 163 ratio was calculated to assess macrophage subtype distribution. As data was non normally distributed (Shapiro-Wilk test), comparisons were performed using the Mann-Whitney U test and results were expressed as median {Interquartile Range (IQR)}.

Results: Conventional OSCC demonstrated significantly higher median CD 68 IRS {4.00 (2.00)} than OSMF-induced OSCC {3.50 (2.00)} (p=0.004). CD 163 IRS was also higher in conventional OSCC {6.00 (4.50)} compared to OSMF-induced OSCC {3.00 (2.00)} (p<0.001). Percentage positivity and staining intensity differed significantly for both markers (p<0.05). However, the CD 68/CD 163 ratio did not differ significantly between the groups (median IRS ratio: 1.00 vs 1.00; p=0.570).

Conclusion: Differential macrophage expression between conventional and OSMF-associated OSCC indicates variations in TME characteristics. However, the balance of macrophage subtypes remained comparable, warranting further investigation of their biological and prognostic relevance.

Keywords: Cancer, Disease, Immune cell infiltration Immunohistochemistry, Inflammatory biomarkers, Macrophage polarisation, Mortality, Oral malignancy, Risk stratification, Stromal modulation, Tumour microenvironment

INTRODUCTION

The OSCC is a solid tumour of oral epithelial cells that accounts for nearly 91% of all oral malignancies and is recognised as the second most common type, being a major cause for cancer related mortality in men across South-East Asia [1]. Metastasis contributes to nearly 90% of all cancer-associated deaths [2]. Although significant progress has been made in surgical approaches, chemotherapy and radiotherapy, the 5-year survival rate for OSCC has only shown a little improvement over the past decade [3]. One of the major factors, for the invasive nature of OSCC is its ability to invade the adjacent structures and spread to the cervical lymph nodes and is considered a major contributing factor responsible for its higher fatality rates and recurrence [4,5].

Among the established "Hallmarks of Cancer," increasing attention has been directed towards the TME because of its pivotal role in cancer initiation and progression [6]. The reactive stroma, which is also termed as TME is composed of various cells like fibroblasts, myofibroblasts, endothelial cells, pericytes, myocytes adipocytes, mesenchymal stem cells, neural cells, immune cells, inflammatory cells and other components of the extracellular matrix, which plays an important role in tumour behaviour, progression and facilitates the metastatic spread of the malignant cells [7]. Tumour Promoting Inflammation (TPI), which is largely driven by immune cells, plays a vital role in establishing and sustaining a microenvironment which in turn favours tumour initiation and progression [8]. Mantovani et al., (2009) proposed the concept of "tumour inflammation" as the seventh hallmark of cancer, highlighting the importance of the host microenvironment in establishing the disease progression [9]. Within the TME, macrophages constitute for nearly 30-50% of all the inflammatory cells and exhibit considerable heterogeneity [10]. They are broadly classified into two distinct phenotypes: M1, or classically activated macrophages and M2, or alternatively activated macrophages. Out of all these, the M2 macrophages are commonly considered as the TAMs [11].

M1 macrophages are mainly identified with proinflammatory activityand tumour suppressive functions and M2 macrophages are anti-inflammatory activity and they are associated with tumour growth and progression [12]. M1 phenotype is usually known by the representation of receptorslike CD80, CD86, CD64 and CD32 surface receptors. CD68 and CD11b are pan-macrophage markers, whereas CD204 and CD206 are more closely associated with the M2 macrophage phenotype [13]. M2 macrophages TAMs are a crucial tumour component in a number of tumour pathology pathways such as angiogenesis, metastasis and immune suppression [14].

This subset has a specialised marker that is commonly referred to as CD 163. It was particularly found that at later tumour progression stages, the TAMs are highly polarised into the M2 phenotype, an advanced form of tumouricidal activity and allow the development of a tumour growth and tumour neovascularisation promoting microenvironment [15,16].

The condition is called OSMF and it causes chewing areca nut, which can also be cancerous and is common in India and other South Asian countries (primarily) [17]. The first description was made by Schwartz in the year 1952, when he termed it as Atrophica idiopathica mucosae Oris in East Africa living patients of Indian causes. Later on, the condition was reported in India by Lal and Joshi SG and named OSMF [18]. It has been recorded to vary between 2-8 percent that OSMF progresses to OSCC. This development has been postulated to be mediated by mechanisms that cover collagen maturation, interplay between collagen and myofibroblasts and mast cells and fibrosis induced vasoconstriction that leads to epithelial hypoxia [19,20]. Moreover, the chronic inflammation of the oral mucosa caused by the continuous exposure to the alkaloids contained in quid, causes the activation of macrophages and T lymphocytes, which increases the levels of cytokines, including Interleukin-6 (IL-6), Tumour Necrosis Factor (TNF), Interferon-α (IFN-α) and transforming growth factor-β. Also, the mechanical injury that is produced by the coarse fibres of areca nut facilitates infiltration of these alkaloids into the subepithelial connective tissue leading to juxtaepithelial infiltration of inflammatory cells [21].

Carcinoma developing in relation to OSMF is considered a pathologically different entity, but its clinical progression and the biological behaviour is not properly described in the available literature. This loophole reveals the necessity to conduct more research to gain a better comprehension of the TME and the corresponding immune-related process in this condition. Thus, the objective of the study was to evaluate the immunohistochemical expression level of CD 68 and CD 163 and to compare the CD 68/CD 163 ratio in conventional OSCC and OSMF-induced OSCC.

MATERIALS AND METHODS

The present cross-sectional study was conducted between January 2020 and February 2025 in the Department of Oral Pathology and Microbiology at Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India. Following approval from the Institutional Ethical Committee (SRB/SDC/OPATH-2404/24/516), a total of 34 cases were retrieved from the departmental archives and the institutional electronic database. The study population was divided into two groups: Group-A comprised Conventional OSCC (n=20) and Group-B comprised OSMF-induced OSCC (n=14).

Sample size: The sample size was determined by the number of eligible and completed cases that were available in the institutional archives within the study period.

Inclusion criteria: Only histopathologically confirmed cases of OSCC were included in the study. Cases of OSCC arising in association with OSMF were also considered. Inclusion required the availability of adequate formalin-fixed, paraffin-embedded tissue blocks suitable for immunohistochemical evaluation. Additionally, only cases with complete clinicopathological data, including demographic information and details of the lesion, were selected for analysis.

Exclusion criteria: The OSCC cases associated with oral potentially malignant disorders other than OSMF were excluded from the study. In addition, cases with insufficient tissue or poorly preserved specimens that were unsuitable for immunohistochemical analysis were not included in the evaluation.

Study Procedure

Data were assessed on various parameters of both the groups (A and B), such as demographic variables (age and gender), habit history and co-morbidity conditions such as diabetes, hypertension, coronary heart disease and neurological problems. Clinical assessment included lesion characteristics such as site, duration, clinical presentation, associated symptoms, pain, degree of deviation of the mouth, presence of trismus and palpable fibrotic bands. Histopathological assessment focused on the presence of epithelial dysplasia, mitotic figures, pattern of invasion, keratinisation pattern, nature of hyalinisation, keratin pearl formation and nature of inflammatory cell infiltrate. Additional parameters assessed by Byrne's grading for OSMF [22], grading system for OSCC [23], presence of skeletal muscle invasion, lymphovascular invasion, perineural invasion and pathological TNM staging [24].

Immunohistochemistry: A total of 34 tissue blocks were immunohistochemically analysed with CD 68 and CD 163 antibodies according to the standardised protocols that have already been established [25]. Formalin-fixed- paraffin embedded tissue blocks were sectioned, trimmed to 3 µm and then mounted on poly-L-lysine-coated slides and incubated overnight at 50°C. The sections were immersed in xylene in three 10 minutes shifts to deparaffinise them. The recovery of the antigens was done by heating the slides in Ethylenediaminetetraacetic Aid (EDTA) buffer in a pressure cooker containing electrical power for 20 minutes, then cooling the slides at room temperature for 20 minutes. In order to block the endogenous activity of peroxidase, the sections were allowed to react with hydrogen peroxide for 30 minutes and then washed twice using three minutes of Tris-Buffered Saline (TBS). Then, primary antibodies (CD 68, mouse monoclonal antibody, Pathnsitu Biotechnologies Pvt., Ltd., India) and CD 163, Master Diagnosica, Granada, Spain) were used to incubate the sections for 60 minutes. Following which the secondary antibody is incubated for 30 minutes and three washes of TBS are done. The immune reaction was visualised by incubating with 3,3´-diaminobenzidine (DAB) substrate between 5-10 minutes. Lastly, the slides were counterstained with the Harris haematoxylin and dehydrated with the graded ethanol and xylene and then mounted on Dibutylphthalate Polystyrene Xylene (DPX) medium.

Scoring criteria and analysis: Each section was first analysed under low-power magnification (10x) to determine the areas with the highest macrophage concentration, which was indicated by the positive brown chromogen staining of both CD 68 and CD 163 markers. The evaluation of TAMs expressing CD 68 and CD 163 was performed by counting the number of positively stained cells in three High-Power Fields HPF) (40×) and the mean macrophage count per HPF was calculated.

Immunohistochemical evaluation was carried out independently by two experienced oral pathologists who were blinded to the group allocation and clinicopathological details of the cases. In cases of discrepancy, the slides were re-evaluated jointly and if consensus was not achieved, a third senior oral pathologist (also blinded to the study groups) was consulted and the final score was assigned based on majority agreement.

Immunoreactivity was assessed using the IRS system as described by Remmele W and Stegner HE (1987) [26]. For each case, [Table/Fig-1] the proportion of positively stained tumour cells (A) was assessed semi-quantitatively and assigned a score ranging from 0 to 4 based on the percentage of positive cells. Staining intensity

A (percentage of positive cells)	B (intensity of staining)	IRS score (multiplication of a and b)
0=no positive cells	0= no colour reaction	0= Negative
1 ≤10% of positive cells	1= mild reaction	1-3= mild
2= 10-50% positive cells	2=moderate reaction	4-8=moderate
3=51-80% positive cells	3=intense reaction	9-12=strongly positive
4 ≥80% of positive cells		
	Final IRS score (AXB): 0-12	

[Table/Fig-1]: Grading criteria for immunohistochemical evaluation of CD 68 and CD 163 expression using the Immunoreactive Score (IRS) system.

(B) was independently graded on a scale of 0 to 3 according to the strength of cytoplasmic staining. A single proportionality score and intensity score were assigned per specimen after evaluation of representative tumour areas.

The final IRS was calculated individually for each case by multiplying the proportionality score and intensity score (A×B), yielding a composite score ranging from 0 to 12. According to the original classification, IRS values of 0 and 1 were considered negative (as a score of one represents minimal immunoreactivity), 2-3 mild, 4-8 moderate and 9-12 strongly positive. As these parameters represent ordinal variables, proportionality scores, intensity scores and IRS values were summarised using median and Interquartile Range (IQR) for each study group. Relative distribution of macrophage subtypes in the TME was determined by calculation of the CD 68/ CD 163 ratio based on the case-wise IRS values.

STATISTICAL ANALYSIS

Results were tabulated in Microsoft Excel. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software version 26.0. Demographic data, clinical features, histopathological parameters and immunohistochemical results were analysed with the assistance of descriptive statistics and the Mann-Whitney U test. Continuous variables were represented by median and IQR, whereas categorical variables were reported in forms of frequencies and percentages. Intergroup comparisons were done using the Mann-Whitney U test since the data were not normally distributed. The level of agreement between observers was determined by Cohen's kappa (κ).

RESULTS

The demographic, clinical and lesional data of the patients in groups A and B were tabulated in [Table/Fig-2]. In both groups, a distinct male dominance was evident, with 80% of males in Group-A (n=16) and 85.7% in Group-B (n=12) cases showing a possible gender related vulnerability.The most common habit in Group-A was pan chewing 14 (70%), then gutkha chewing 2 (10%) and the combined habit of smoking and alcohol use 2 (10%). Also, 2 (10%) of patients had no history of habit. The frequent habits in Group-B were pan chewing 7 (50%) and the synergistic habit of smoking and alcohol consumption 3 (21.4%). The incidence of gutkha chewing and areca nut chewing were 2 (14.3%) and 2 (14.3%), respectively.

Variables	Category	Group-A (conventional OSCC) n (%)	Group-B (OSMF induced OSCC) n (%)
Gender	Male	16 (80%)	12 (85.7%)
	Female	4 (20%)	2 (14.3%)
Habit history	Pan chewing	14 (70%)	7 (50.0%)
	Gutkha chewing	2 (10%)	2 (14.3%)
	Smoking + Alcohol	2 (10%)	2 (14.3%)
	No habit history	2 (10%)	3 (21.4%)
Co-morbidities (diabetes, hypertension)	Present	9 (45%)	8 (57.1%)
	Absent	11 (55%)	6 (42.9%)
Site of lesion	Buccal mucosa	8 (40%)	9 (64.3%)
	Upper + Lower Alveolus region	4 (20%)	2 (14.3%)
	Floor of the mouth	1 (5%)	1 (7.1%)
	Lateral border of tongue + GBS	7 (35%)	2 (14.3%)
Laterality of lesion	Right	10 (50%)	9 (64.3%)
	Left	10 (50%)	5 (35.7%)
Lymph node involvement	Involved	11 (55%)	1 (7.1%)
	Not Involved	9 (45%)	13 (92.9%)
Extra Nodal Extension (ENE)	Positive	6 (30%)	1 (7.1%)
	Negative	14 (70%)	13 (92.9%)
Degree of dysplasia	Mild	Absent	4 (28.6%)
	Moderate	3 (15%)	4 (28.6%)
	Severe	17 (85%)	6 (42.8%)
Presence of mitotic figures	Present	17 (85%)	8 (57.2%)
	Absent	3 (15%)	6 (42.8%)
Type of epithelium-Hyperparakeratinised	Present	12 (60%)	8 (57.2%)
Hyperorthokeratinised	Present	8 (40%)	6 (42.8%)
Keratinisation pattern	Keratin pearls+ Islands	12 (60%)	6 (42.8%)
	Sheets +nests	5 (25%)	4 (28.6%)
	Cords	3 (15%)	4 (28.6%)
Lymphovascular invasion	Present	10 (50%)	0
	Absent	10 (50%)	14 (100.0%)
Perineural invasion	Present	12 (60%)	0
	Absent	8 (40%)	14 (100.0%)
Skeletal muscle invasion	Present	5 (25%)	6 (42.9%)
	Absent	15 (75%)	8 (57.1%)
Distant metastasis	Absent	20 (100%)	14 (100%)
Inflammation type	Chronic- Lymphocytes± Plasma cells	14 (70%)	10 (71.4%)
	Mixed (incl. neutrophils)	6 (30%)	4 (28.6%)
pTNM Staging	pT1N0	6 (30%)	6 (42.9%)
	pT2N0	0	2 (14.3%)
	pT2aN0	4 (20%)	2 (14.3%)
	pT2N2b	0	1 (7.1%)
	pT2bN0	4 (20%)	3 (21.4%)
	pT4aN0	3 (15%)	0
	pT4bN3	3 (15%)	0

[Table/Fig-2]: Demographic, clinical and Histopathological characteristics of conventional OSCC and OSMF-induced OSCC cases. Data are presented as frequency (number and percentage).

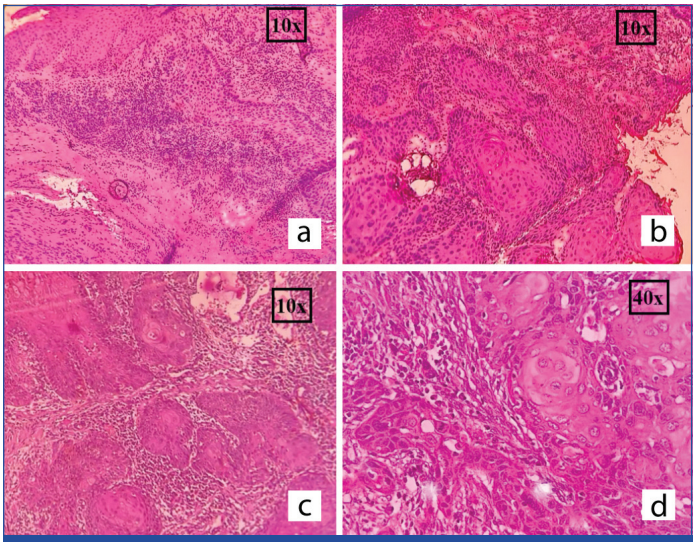
In Group-A, 9 (45%) of the patients were found to be experiencing co-morbidities, mostly diabetes and hypertension. In Group-B, a co-morbid condition was found in 8 (57.1%) of patients, such as diabetes, hypertension, coronary heart disease and neurological disorders, which could have contributed to the progression of the disease and the inability to heal tissues. In Group-A, the buccal mucosa was the most commonly involved site 8 (40%), followed by the lateral border of the tongue and the gingivobuccal sulcus 7 (35%). In Group-B, the buccal mucosa was the predominant site of involvement 9 (64.3%), consistent with the habitual placement of quid in this region and right-sided lesions observed more frequently 9 (64.3%) than left-sided lesions 5 (35.7%).

In Group-B, clinically, all patients presented with pain and trismus and fibrous bands were also consistently observed, reflecting the classic fibrotic nature of OSMF. Mouth deviation was observed in 13 (92.9%) cases, of which moderate deviation was the most prevalent, 6 (42.9%), followed by mild 5 (35.7%) and severe 3 (21.4%) forms. Additionally, radiating pain, suggestive of possible neural involvement, was reported in 6 (42.9%) of patients.

The ulceroproliferative nature of the lesion, epithelial dysplasia and presence of fibrous bands were seen in 14 (100%) cases, highlighting the precancerous or malignant potential of the lesion. In Group-A, severe dysplasia was observed in 17 (85%) of cases, followed by moderate dysplasia in 3 (15%). In Group B, severe dysplasia was present in 6 (42.8%) of cases, while mild and moderate dysplasia were equally distributed at 4 (28.6%) each, suggesting that a substantial portion of the cohort was at high risk for malignant transformation. Overall, these findings highlight a clear association between high-risk oral habits, particularly pan and

areca nut chewing and advanced clinical features indicative of oral potentially malignant disorders.

The histopathological and staging features [Table/Fig-3a-d] of both groups included lymph node involvement in 11 (55%) of cases in Group-A, with extranodal extension observed in 6 (30%). In Group-B, a significant proportion of patients, 11 (78.6%), exhibited advanced OSMF, followed by moderately advanced 3 (21.4%), indicating chronic progression and fibrotic transformation of the oral mucosa.



[Table/Fig-3]: a,b) Depicts Haematoxylin and Eosin (H&E) stained sections of Oral Squamous Cell Carcinoma (OSCC) arising in the background of Oral Submucous Fibrosis (OSMF) (10X). These sections demonstrate fibrous connective tissue characterised by dense collagen bundles with extensive areas of hyalinisation. Additionally, malignant epithelial cells are seen infiltrating the connective tissue stroma; c) (10X); and d) (40X) show H&E stained sections of conventional OSCC. These images reveal characteristic features such as epithelial keratin pearl formation and disruption of the basement membrane continuity, indicative of tumour invasion.

Considering the epithelial characteristics, hyperparakeratinisation was observed in 12 (60%) of Group-A cases, whereas hyperorthokeratinisation was observed in 8 (40%). In group B, hyperparakeratinised was present in 8 (57.2%) of cases, while hyperorthokeratinised pattern was noted in 6 (42.8%) of cases. This indicated the variability in differentiation of epithelium. In Group-B, the hyalinisation pattern was predominantly dense collagenous in nature accounting for 10 (71.4%) of cases, highlighting the fibrotic characteristics of OSMF. Keratin pearl formation was observed in all cases 14 (100%), indicating tumour differentiation and histological confirmation of this pathology. In Group-A, inflammation was predominantly chronic with lymphoplasmacytic infiltrates seen in 14 (70%) of cases, while mixed inflammatory infiltrates, including neutrophils, were observed in 6 (30%). In Group-B, chronic lymphocytic inflammation was present in 10 (71.4%) of cases, with mixed inflammatory infiltrates noted in 4 (28.6%) cases, suggesting varying immune responses. In Group-A, mitotic figures were observed in 17 (85%) of cases. In Group-B, high mitotic activity existed in 8 (57.1%), which is an indication of cellular proliferation, a characteristic of malignancy.

In Group-A, Byrne's grading score of 7/16 was the most common, with 6 (30%) of cases. The skeletal muscle invasion was found in 5 (25%) and perineural and lymphovascular invasion in 12 (60%) and 10 (50%), respectively. In Group-B, the Bryne's score fell between 6/16 and 11/16. The skeletal muscle invasion was observed in 6 (42.9%) of the cases, reflecting deeper tissue infiltration, in a subset of patients. But no lymphovascular and perineural invasion was observed in all the cases, which is promising, as their occurrence usually indicates a worse prognosis.

According to pathological TNM (pTNM) staging, the majority of cases in Group-A were in the pT1N0 stage {6 (30%)}, followed by pT2aN0 and pT2bN0 stages {4 (20%) each}. In Group-B, pT1N0 was observed in 6 (42.9%) of cases, pT2N0 in 8 (57.1%) and pT2N2b in 1 (7.1%), indicating nodal involvement. These staging results suggest that most patients were diagnosed before significant regional spread, offering better chances for early diagnosis and curative treatment in Group-B.

Immunohistochemical analysis revealed [Table/Fig-4] that for CD 68, the median percentage of positive cells was significantly lower in Group-A (OSMF-induced OSCC) (Median=2; IQR=0.75; range: 1-3) compared to OSCC (Median=2; IQR=1; range: 2-3) (Mann–Whitney U=26.0; p=0.043; r=0.48). Staining intensity was also significantly reduced in OSMF-induced OSCC (Median=2; IQR=1; range: 1-2) relative to OSCC (Median=2; IQR=0; range: 2-3) (U=24.0; p=0.017; r=0.52). Correspondingly, the IRS score demonstrated significantly lower values in OSMF-induced OSCC (Median=3.5; IQR=2; range: 1-4) compared to OSCC (Median=4; IQR=2; range: 4-9) (U=15.0; p=0.004; r=0.70).

IHC marker	Groups	Median	MIN-MAX	IQR	Effect size	p-value
CD 68 percentage of positive cells	OSMF-induced OSCC	2	1-3	0.75	0.48	0.043*
	OSCC	2	2-3	1		
CD 68 Intensity of staining	OSMF-induced OSCC	2	1-2	1	0.52	0.017*
	OSCC	2	2-3	0		
CD 68 IRS score	OSMF-induced OSCC	3.5	1-4	2	0.7	0.004*
	OSCC	4	4-9	2		
CD 163 percentage of positive cells	OSMF-induced OSCC	2	1-2	0.75	0.79	<0.001*
	OSCC	3	2-4	0.75		
CD 163 Intensity of staining	OSMF-induced OSCC	2	1-2	0	0.52	0.017*
	OSCC	2	2-3	1.0		
CD 163 IRS score	OSMF-induced OSCC	3	2-4	2	0.85	<0.001*
	OSCC	6	4-12	4.5		

[Table/Fig-4]: Comparative immunohistochemical analysis of CD 68 and CD 163 expression in conventional OSCC and OSMF-induced OSCC. Values are expressed as median (interquartile range, IQR). Intergroup comparison was performed using the Mann-Whitney U test.
A p-value ≤0.05 was considered statistically significant

Similarly, CD 163 expression showed highly significant differences between the groups. The median percentage of positive cells was lower in OSMF-induced OSCC (Median=2; IQR=0.75; range: 1-2) than in OSCC (Median=3; IQR=0.75; range: 2-4) (U=10.5; p<0.001; r=0.79). Staining intensity was significantly decreased in OSMF-induced OSCC (Median=2; IQR=0; range: 1-2) compared to OSCC (Median=2; IQR=1; range: 2-3) (U=24.0; p=0.017; r=0.52). The IRS score for CD 163 exhibited the most pronounced difference, with substantially lower values in OSMF-induced OSCC (Median=3; IQR=2; range: 2-4) relative to OSCC (Median=6; IQR=4.5; range: 4-12) (U=7.5; p <0.001; r=0.85).

Interobserver reliability analysis using Cohen's kappa [Table/Fig-5] demonstrated excellent agreement for CD 68_GpI (κ=0.913), CD 68_GpII (κ=0.898), CD 163_GpI (κ=0.922) and CD 163_GpII (κ=0.806), with all p-values <0.001, indicating high reproducibility of immunohistochemical scoring.

The analysis of the CD 68/CD 163 ratio [Table/Fig-6] showed that the median percentage ratio (A) was 1.00 (IQR=0.25; range: 0.50-1.00) in conventional OSCC and 1.00 (IQR=0.00; range: 0.50-3.00) in OSMF-induced OSCC (p=0.398). The median staining intensity ratio (B) was 1.00 (IQR=0.00; range: 0.66-1.00) in conventional OSCC and 1.00 (IQR=0.37; range: 0.50-2.00) in OSMF-induced OSCC (p=0.744). Equally, the conventional OSCC median IRS ratio was 1.00 (IQR=0.25; range: 0.33-1.00) and the OSMF-

induced OSCC median IRS was 1.00 (IQR=0.87; range: 0.50-2.00) (p=0.570).

Variables	Cohen's kappa	p-value
CD 68 A	0.913	<0.001
CD 68 B	0.898	<0.001
CD 163 A	0.922	<0.001
CD 163 B	0.806	<0.001

[Table/Fig-5]: Interobserver reliability analysis for immunohistochemical scoring of CD 68 and CD 163 using Cohen's kappa statistics.

Ratios	Group	Median (IQR)	Min-Max	p-value	Effect size
Ratio A	OSCC	1(0.25)	0.5-1	0.39	0.20
	OSMF-induced OSCC	1 (0.00)	0.5-3		
Ratio B	OSCC	1 (0.00)	0-1	0.74	0.08
	OSMF-induced OSCC	1(0.37)	0.5-2		
Ratio IRS	OSCC	1 (0.25)	0.33-1	0.57	0.15
	OSMF-induced OSCC	1(0.87)	0.5-2		

[Table/Fig-6]: Comparison of CD 68/CD 163 ratio (percentage positivity ratio, staining intensity ratio and IRS ratio) between conventional OSCC and OSMF-induced OSCC. Data are expressed as median (interquartile range, IQR). Statistical analysis was performed using the Mann-Whitney U test, with p≤0.05 considered statistically significant.

DISCUSSION

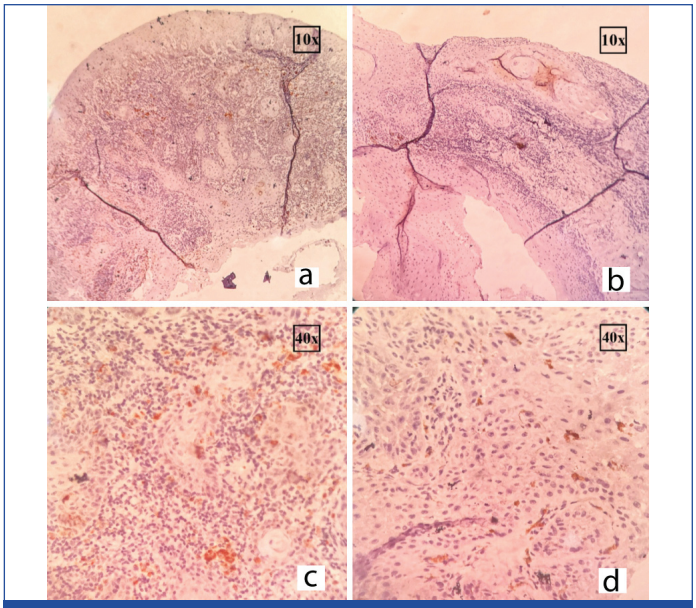
The OSCC is a multifaceted malignant neoplasm in the head and neck region with a 5-year survival rate of about 50% [27]. In OSCC, the TME is highly enriched with a range of immune cells, but it is mainly composed of macrophages. These macrophages are plastic and can be polarised to either M1 or M2 phenotypes. It is primarily the M2 subtype, which is commonly referred to as TAMs, that is of critical importance in regulating the TME [28].

The malignant transformation rate of OSMF into OSCC is believed to involve collagen maturation, interaction with myofibroblasts and mast cells and fibrosis-induced vascular constriction leading to epithelial hypoxia [20,29]. Prolonged exposure of the oral mucosa to alkaloids present in the quid leads to persistent inflammation, which subsequently activates macrophages and T lymphocytes, causing elevated levels of cytokines such as IL-6, TNF, IFN-α and transforming growth factor-β. In addition, the mechanical trauma induced by the coarse fibres of areca nut promotes the penetration of these alkaloids into the subepithelial connective tissue, resulting in juxtaepithelial infiltration of inflammatory cells [30].

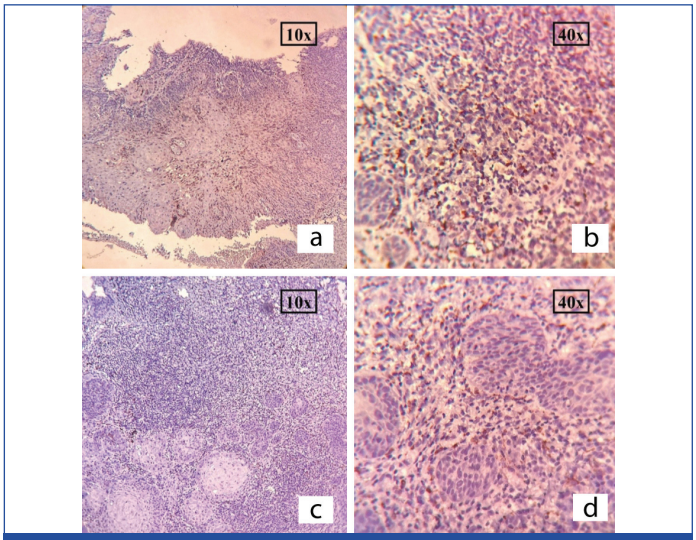
Previous studies have reported malignant transformation rates of 5.5% [31] and 3.75% [32] in OSMF patients. The primary aetiological factor in OSMF and its malignant transformation is attributed to the carcinogenic alkaloids and related compounds present in areca nut, which initiate a cascade of cellular and molecular changes in the oral mucosa [33]. Similarly, in the present study, a prominent male predominance was evident, comprising 85.7% of cases, suggesting gender-related susceptibility. Pan chewing emerged as the most prevalent deleterious habit (50.0%), along with synergistic smoking and alcohol consumption (21.4%). Gutkha and areca nut chewing were equally reported (14.3% each), underscoring the aetiological significance of areca nut containing alkaloids in the development and malignant transformation of OSMF.

The gingivobuccal complex has been reported as the most common site of involvement [34]. Buccal mucosa was also the most common in our study (64.3%), which is associated with quid placement as a habit. OSMF-induced OSCC histopathologically has been defined as a unique, better-differentiated and reduced nodal metastasis condition, which may be explained by fibrotic stromal barriers limiting tumour invasion [18,34]. In the present study, most cases were well-differentiated (50%) and early-stage (pT1N0 and pT2N0), supporting previous observations.

Irfan A et al., demonstrated that increased TAM density correlates with tumour size and nodal metastasis [35], consistent with evidence linking higher CD 163 expression to aggressive tumour behaviour [36,37]. Comparative immunohistochemical analysis [Table/Fig-7-9a-d] in the present study showed that both the median percentage positivity, intensity of staining and IRS of the CD 68 and CD 163 were significantly lower in OSMF-induced OSCC than conventional OSCC [Table/Fig-10,11], The results suggest that there is a decreased infiltration of overall macrophages, including M2-polarised macrophages in OSMF-associated tumours. The reduced macrophage density could indicate the changed stromal architecture and fibrosis peculiar to OSMF, which could possibly impact on the immune cell recruitment in the TME.

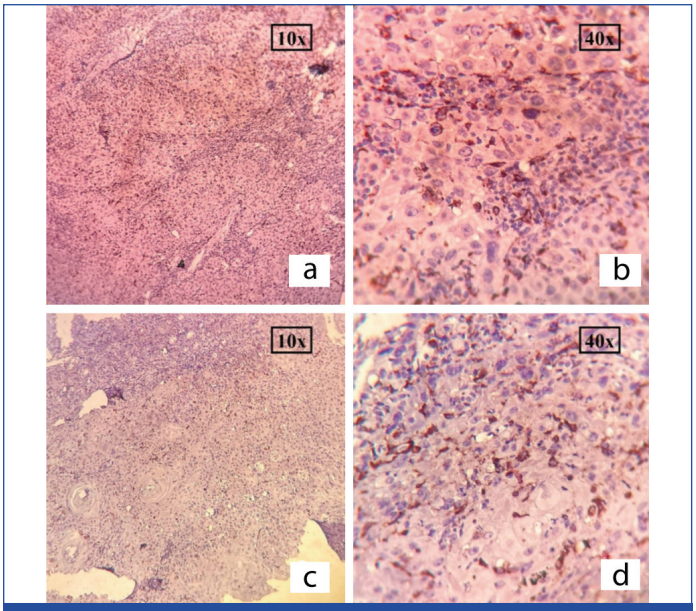


[Table/Fig-7]: a,b) Immunohistochemically-stained sections demonstrating positive expression of CD 68 and CD 163 markers at the tumour front in OSMF-induced OSCC. The staining highlights the presence of Tumour-Associated Macrophages (TAM) expressing these markers in the peritumoural region; c,d) Immunohistochemically-stained sections of conventional OSCC, displaying positive CD 68 and CD 163 expression with notably increased activity localised at the tumour front. This enhanced expression indicates a significant presence of these macrophage markers in the invasive tumour area.

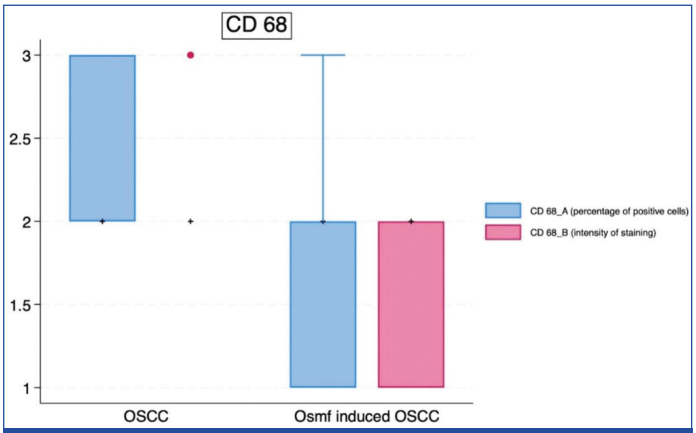


[Table/Fig-8]: a) (10X); b) (40X) shows immunohistochemically-stained sections demonstrating positive expression of CD 68 marker at the tumour front in conventional OSCC. The staining highlights the presence of Tumour-Associated Macrophages (TAM) expressing these markers in the peritumoural region; c) (10X); d) (40X) depicts immunohistochemically-stained sections of conventional OSCC, displaying positive CD 163 marker expression with notably increased activity localised at the tumour front. This enhanced expression indicates a significant presence of these macrophage markers in the invasive tumour area.

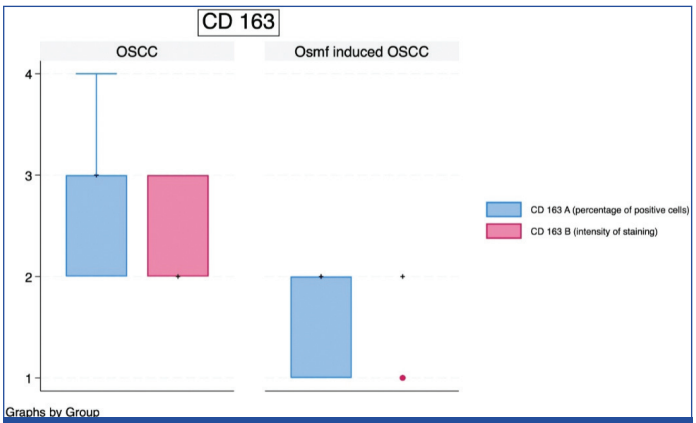
Regarding macrophage polarisation, the CD 68/CD 163 ratio [Table/Fig-12] was close to one in both groups and no statistically significant difference was observed between conventional OSCC and OSMF-



[Table/Fig-9]: a) (10X); b) (40X) shows immunohistochemically-stained sections demonstrating positive expression of CD 68 marker at the tumour front in OSMF-induced OSCC. The staining highlights the presence of Tumour-Associated Macrophages (TAM) expressing these markers in the peritumoural region. c) (10X); and d) (40X) depicts immunohistochemically-stained sections of OSMF-induced OSCC, displaying positive CD 163 marker expression with notably increased activity localised at the tumour front. This enhanced expression indicates a significant presence of these macrophage markers in the invasive tumour area.

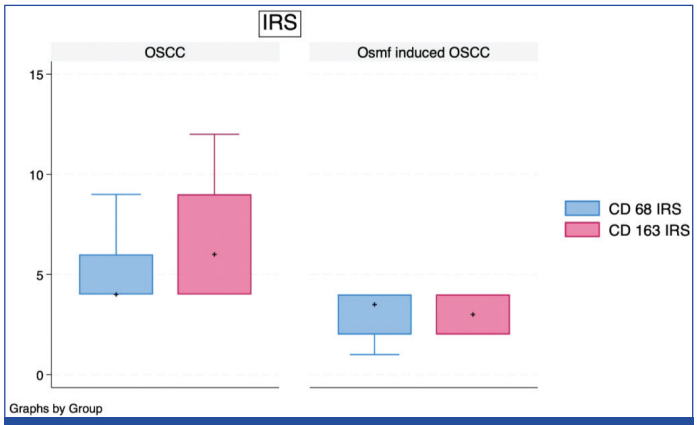


[Table/Fig-10]: Box-and-whisker plot illustrating the distribution of CD 68 expression in conventional OSCC and OSMF-induced OSCC. The central line represents the median, the box indicates the Interquartile Range (IQR) and the whiskers denote the minimum and maximum values. Intergroup comparison was performed using the Mann-Whitney U test.



[Table/Fig-11]: Box-and-whisker plot illustrating the distribution of CD 163 expression in conventional OSCC and OSMF-induced OSCC. The central line represents the median, the box indicates the Interquartile Range (IQR) and the whiskers denote the minimum and maximum values. Intergroup comparison was performed using the Mann-Whitney U test.

induced OSCC. Even though the conventional OSCC showed a slightly lower ratio value in some parameters, the difference was not significant. It implies that the overall macrophage density varies across the types of tumours, but the percentage ratio of the total amount of macrophages (CD 68) and M2 macrophages (CD 163) is quite similar. Mukherjee A et al., and Takabatake K et al., have also made similar observations and highlighted the role of absolute TAM density but not ratio in predicting tumour aggressiveness and metastatic potential [38,39]. Together, these results reveal the idea of the possibility of quantitative differences in macrophage infiltration, but not skew polarisation, in the cause of the specific TME phenotype in OSMF-related OSCC.



[Table/Fig-12]: Box-and-whisker plot depicting the distribution of Immunoreactive Score (IRS) for CD 68 and CD 163 in conventional OSCC and OSMF-induced OSCC. The central line represents the median, the box denotes the Interquartile Range (IQR) and the whiskers indicate the minimum and maximum values. Statistical comparison between groups was carried out using the Mann-Whitney U test.

Limitation(s)

The cross-sectional design of the study limited the ability to establish temporal or causal relationships. The relatively small sample size, based on the availability of eligible cases, may have affected the statistical power of the analysis. As this was a single-institution study, the findings may not be generalisable to the broader population.

CONCLUSION(S)

The present study demonstrates significant differences in CD 68 and CD 163 expression between conventional OSCC and OSMF-associated OSCC, reflecting variations in TME characteristics. However, the relative balance of macrophage subtypes, as assessed by the CD 68/CD 163 ratio, remained comparable between the groups. These findings emphasise the importance of evaluating individual macrophage markers in understanding tumour biology.

REFERENCES

[1] Tan Y, Wang Z, Xu M, Li B, Huang Z, Qin S, et al. Oral squamous cell carcinomas: State of the field and emerging directions. Int J Oral Sci. 2023;15(1):44.
[2] Gupta GP, Massagué J. Cancer metastasis: Building a framework. Cell. 2006;127(4):679-95. Doi: 10.1016/j.cell.2006.11.001. PMID: 17110329.
[3] Hu Y, He MY, Zhu LF, Yang CC, Zhou ML, Wang Q, et al. Tumor-associated macrophages correlate with the clinicopathological features and poor outcomes via inducing epithelial to mesenchymal transition in oral squamous cell carcinoma. J Exp Clin Cancer Res. 2016;35(1):12.
[4] Markwell SM, Weed SA. Tumor and stromal-based contributions to head and neck squamous cell carcinoma invasion. Cancers. 2015;7(1):382-406.
[5] Varsha BK, Radhika MB, Makarla S, Kuriakose MA, Kiran GS, Padmalatha GV. Perineural invasion in oral squamous cell carcinoma: Case series and review of literature.J Oral Maxillofac Pathol. 2015;19(3):335-41.
[6] Babu S, Manavalan MJ, Krishnan M. Tumor microenvironment in oral squamous cell carcinoma: Implications for novel therapies.Oral Oncol Rep. 2024;12:100666.
[7] de Visser KE, Joyce JA. The evolving tumor microenvironment: From cancer initiation to metastatic outgrowth. Cancer Cell. 2023;41(3):374-403.
[8] Pennacchiotti G, Valdés-Gutiérrez F, González-Arriagada WA, Montes HF, Parra JMR, Guida VA, et al. SPINK7 expression changes accompanied by HER2, P53 and RB1 can be relevant in predicting oral squamous cell carcinoma at a molecular level. Sci Rep. 2021;11(1):6939. Doi: 10.1038/s41598-021-86208-z. PMID: 33767253; PMCID: PMC7994578.
[9] Hema SK, Gayathri R, Veeraraghavan VP, Selvaraj J, Ramani P. Pro-inflammatory mediator and cancer-associated chemokine CXCL8 as a prognostic indicator of tumor aggressiveness, angiogenesis, and disease progression with focus on therapeutic implications and recurrence monitoring. Oral Oncol Rep. 2025;13:100723. Available from: https://doi.org/10.1016/j.oor.2025.100723.

[10] Zou Z, Lin H, Li M, Lin B. Tumor-associated macrophage polarization in the inflammatory tumor microenvironment. Front Oncol. 2023;13:1103149. Doi: 10.3389/fonc.2023.1103149. PMID: 36816959; PMCID: PMC9934926.
[11] Buldak N, Veeraraghavan VP, Mony U. Revolutionizing precision medicine in oral squamous cell carcinoma: Harnessing 3D in vitro models for translation. Oral Oncology Reports [Internet]. 2024;11:100622. Available from: https://doi.org/10.1016/j.oor.2024.100622.
[12] Chohan MH, Perry M, Laurance-Young P, Salih VM, Foey AD. Prognostic role of CD68+ and CD163+ tumour-associated macrophages and PD-L1 expression in oral squamous cell carcinoma: A meta-analysis. Br J Biomed Sci. 2023;80:11065.
[13] Cendrowicz E, Sas Z, Bremer E, Rygiel TP. The role of macrophages in cancer development and therapy. J Cancers. 2021;13(08):1946.
[14] Ferrisse TM, de Oliveira AB, Palaçon MP, Silva EV, Massucato EM, de Almeida LY, et al. The role of CD68+ and CD163+ macrophages in immunopathogenesis of oral lichen planus and oral lichenoid lesions. Immunobiology. 2021;226(3):152072.
[15] Surendran LS, Joseph AP, Pillai VBR, Somaraj V, Sudhakar S, Mohan A. Prognostic implications of tumor-associated macrophages in oral squamous cell carcinoma: A systematic review and meta-analysis. J Orofac Sci. 2025;17:88-100.
[16] Kalogirou EM, Tosios KI, Christopoulos PF. The role of macrophages in oral squamous cell carcinoma. Front Oncol. 2021;11:611115.
[17] Veerappan P, Krishnan RP, Pandiar D, Ramani P, Jayaraman S. Prognostic role of tumour stroma ratio in oral squamous cell carcinoma arising in the background of oral submucous fibrosis: A retrospective study. J Clin Diagn Res. 2025;19(7).
[18] Joshi SG. Submucous fibrosis of the palate and pillars.Indian J Otolaryngol Head Neck Surg. 1953;4(1):01-04.
[19] Rangaswamy S, Chikkalingaiah RG, Sanjeevarayappa PN, Govindraju P. Carcinoma arising in the background of oral submucous fibrosis.Ann Maxillofac Surg. 2019;9(2):247-52.
[20] Ray JG, Ranganathan K, Chattopadhyay A. Malignant transformation of oral submucous fibrosis: Overview of histopathological aspects. Oral Surg Oral Med Oral Pathol Oral Radiol. 2016;122(2):200-09.
[21] Sadat SA, Shaikh MH, Tajin F, Akter R, Shuvo SA, Ahmed H. Oral submucous fibrosis associated oral squamous cell carcinoma: A case report with review of literature. J Oral Maxillofac Surg Cases. 2022;8(1):100246.
[22] Alexander AJ, Ramani P, Sherlin HJ, Gheena S. Quantitative analysis of copper levels in areca nut plantation area–a role in increasing prevalence of oral submucous fibrosis: An in vitro: study. Indian J Dent Res. 2019;30(2):261-66.
[23] Wagner VP, Webber LP, Curra M, Klein IP, Meurer L, Carrad VC, et al. Bryne's grading system predicts poor disease-specific survival of oral squamous cell carcinoma: A comparative study among different histologic grading systems. Oral Surg Oral Med Oral Pathol Oral Radiol. 2017;123(6):688-96.
[24] More CB, Gupta S, Joshi J, Varma SN. Classification system for oral submucous fibrosis. Journal of J Indian Acad Oral Med Radiol. 2012;24(1):24.
[25] Almangush A, Mäkitie AA, Triantafyllou A, de Bree R, Strojan P, Rinaldo A, et al. Staging and grading of oral squamous cell carcinoma: An update. Oral Oncol. 2020;107:104799.
[26] Remmele W, Stegner HE. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Pathol. 1987;8:138-40.
[27] Srinivasan S, Balasubramaniam A. Risk stratification of submandibular salivary gland involvement in oral squamous cell carcinoma based on histopathological parameters: A 15-year retrospective study.J Oral Maxillofac Pathol. 2024;28(2):261-67.
[28] Kai K, Moriyama M, Haque AR, Hattori T, Chinju A, Hu C, et al. Oral squamous cell carcinoma contributes to differentiation of monocyte-derived tumor-associated macrophages via PAI-1 and IL-8 production. Int J Mol Sci. 2021;22(17):9475.
[29] Jason D AS, Krishnan M, Santhosh Kumar MP, Lakshmanan S. Survivin in the malignant transformation of oral potentially malignant disorders to oral squamous cell carcinoma: A systematic review.Cuest Physioter. 2025;54(3).
[30] Wang L, Tang Z. Immunopathogenesis of oral submucous fibrosis by chewing the areca nut. J Leukoc Biol. 2022;111(2):469-76.
[31] Nayanar G, Reddy MGS, Kakodkar P, Dony EC. Malignant transformation rate of oral submucous fibrosis: A systematic review and meta-analysis. Oral Surg. 2023;16:220-27. https://doi.org/10.1111/ors.12771.
[32] Kujan O, Mello FW, Warnakulasuriya S. Malignant transformation of oral submucous fibrosis: A systematic review and meta-analysis. Oral Dis. 2021;27(8):1936-46.
[33] Divya B, Vasanthi V, Ramadoss R, Kumar AR, Rajkumar K. Clinicopathological characteristics of oral squamous cell carcinoma arising from oral submucous fibrosis: A systematic review.J Cancer Res Ther. 2023;19(3):537-42.
[34] Irfan A, Fahim A, Jalil V, Allah NU, Anjum A, Mustafa R, et al. Relationship of microvascular density and tumor-associated macrophages with orofacial squamous cell carcinoma progression.Oral Surg Oral Med Oral Pathol Oral Radiol. 2024;138(4):532-42.
[35] Zhao S, Zhang Y, Meng X, Wang Y, Li Y, Li H, et al. INHBA+ macrophages and Pro-inflammatory CAFs are associated with distinctive immunosuppressive tumor microenvironment in submucous Fibrosis-Derived oral squamous cell carcinoma. BMC Cancer. 2025;25(1):857.
[36] Pamuk S, Ertugrul B, Kasarci G, Bireller S, Ergen A, Cakmakoglu B, et al. Focusing on tumor and it's microenvironmental immune members for head and neck cancer patients. Pathol Res Pract. 2024; 263:155575.
[37] Mukherjee A, Spadigam A, Dhupar A. Tumor-associated macrophages: Harbingers of aggressiveness in oral squamous cell carcinoma. J Oral Maxillofac Pathol. 2021;25(1):46-50.
[38] Takabatake K, Tianyan P, Arashima T, Chang A, Kawai H, Eain HS, et al. Refining the role of tumor-associated macrophages in oral squamous cell carcinoma. Cancers. 2025;17(17):2770. https://doi.org/10.3390/cancers17172770.
[39] Kumar AT, Knops A, Swendseid B, Martinez-Outschoom U, Harshyne L, Philp N, et al. Prognostic significance of tumor-associated macrophage content in head and neck squamous cell carcinoma: A meta-analysis.Front Oncol. 2019;9:656.

PARTICULARS OF CONTRIBUTORS:
1. Postgraduate Resident, Department of Oral and Maxillofacial Pathology, Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India.
2. Associate Professor, Department of Oral Pathology and Microbiology, Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India.
3. Associate Professor, Department of Oral Pathology and Microbiology, Saveetha Dental College and Hospitals, Chennai, Tamil Nadu, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Sandhya Sundar,
162, Poonamallee High Road, Velappanchavadi, Chennai-600077,
Tamil Nadu, India.
E-mail: sandhyas.sdc@saveetha.com

PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Nov 14, 2025
- Manual Googling: Apr 14, 2026
- iThenticate Software: Apr 16, 2026 (2%)

ETYMOLOGY: Author Origin

EMENDATIONS: 7

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. Yes

Date of Submission: **Oct 25, 2025**
Date of Peer Review: **Dec 03, 2025**
Date of Acceptance: **Apr 18, 2026**
Date of Publishing: **Aug 01, 2026**