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Salivary EGF: EGFR ratio as potential early diagnostic biomarker for oral cancer detection in tobacco chewers: a preliminary cross-sectional study

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Abstract

Background Tobacco chewing and smoking are major risk factors for oral cancer, with Oral Squamous Cell Carcinoma (OSCC), comprising approximately 90% of cases, especially in South-Central Asia. This study examines the early diagnostic value of salivary Epidermal Growth Factor (EGF) and Epidermal Growth Factor Receptor (EGFR) for OSCC in long-term chewers of tobacco.

Methods This study assessed salivary EGF and EGFR concentrations by Enzyme-Linked Immunosorbent Assay (ELISA), in three groups: Healthy Controls (HC, $n=41$), Tobacco Chewers with a 10-years history of tobacco use (TC, $n=41$), and newly diagnosed OSCC patients (with tobacco chewing history of more than 10 years) with no prior history of neoplasms or anticancer treatment (OSCC, $n=19$). All participants were over the age of 35.

Results Salivary EGF levels were significantly reduced, while EGFR levels were elevated in tobacco chewers (TC) and newly diagnosed OSCC patients compared to healthy controls (HC), with no significant difference observed between TC and OSCC groups. Notably, the EGF: EGFR ratio demonstrated better diagnostic performance for OSCC in TC compared to EGF or EGFR alone. ROC analysis highlighted the potential of these biomarkers as diagnostic tools for OSCC in TC.

Conclusion Salivary EGF: EGFR ratio may serve as an early diagnostic biomarker for OSCC in TC. Reduced EGF and elevated EGFR levels suggest their role in disease progression, warranting further large-scale studies for clinical validation.

Keywords Oral squamous cell carcinoma, ELISA, Early diagnostic biomarker, Salivary EGF and EGFR

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Introduction

About two-thirds of global oral cancer cases occur in low- and middle-income countries in South Asia, especially India, where oral cancer makes up 10.2% of all cancer cases, second only to breast cancer [1–3]. The incidence and mortality of oral cancer in India increased by 100–200% between 1990 and 2019 [4]. In India, the cumulative risk of oral cancer is 1 in 65 individuals [5]. Oral Squamous Cell Carcinoma (OSCC) develops on the mucosal epithelium of the oral cavity (mostly at the buccal mucosa, tongue, or floor of the mouth), which accounts for approximately 90% of oral malignancies [6, 7]. Risks to oral cancer are dominated by tobacco, both smoked and smokeless, and heavy alcohol consumption [8]. Recent research has established that tobacco chewing serves as an independent etiological factor for oral cancer [9]. According to the World Health Organization (WHO), tobacco use causes over eight million deaths annually, and about 80% of the 1.3 billion tobacco users worldwide live in low- and middle-income countries [10]. In regions such as Southeast Asia and the Western Pacific Islands, where oral cancer incidence is high, the main risk factors include the use of smokeless tobacco and areca nut products, including betel quid [8, 11]. Over 83% of chewing tobacco users are concentrated in South Asia, with approximately 185.8 million in India and 25.7 million in Bangladesh alone [12]. Current tobacco usage stands at 32.8% in India, comprising 12.6% smokers and 24.7% chewing tobacco users [13]. Nearly 9 in 10 (88%) of all oral cancer cases caused by chewing tobacco and areca nut use occurred in South-Central Asia and more than 95% (96.4%) occurred in low- and middle-income countries [11]. Long-term tobacco chewing significantly contributes to oral carcinogenesis and oral cancer-related deaths among individuals [14]. The situation is exacerbated by the late diagnosis of the disease, which significantly worsens the prognosis [15].

Any oral mucosal abnormality that is associated with a statistically increased risk of developing oral cancer is known as Oral Potentially Malignant Disorder (OPMD) [16]. Patients with OPMDs have a higher likelihood of developing invasive oral carcinomas compared to those with healthy mucosa [17–20]. OPMDs include Oral Leukoplakia (OL), Oral Erythroplakia (OE), Oral Sub-Mucosal Fibrosis (OSMF), and Oral Lichen Planus (OLP). However, at the time of diagnosis, most patients with OPMDs show no symptoms [20]. Patients with OL face a potential malignancy risk of 1–30% and even clinically normal-appearing oral mucosa may show molecular abnormalities [21]. OL on the floor of the mouth progresses more frequently to OSCC followed by tongue and lip [22]. OL has been defined as "A predominantly white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" [21, 23]. Oral cancer is currently diagnosed through a visual examination of oral lesions, followed by a biopsy for confirmation [24]. However, these techniques are insufficient for 'early' diagnosis, which is crucial for successful treatment, better prognosis, and survival. Overall, the five-year survival for early, localized cancers exceeds 80% and falls to less than 20% when regional lymph nodes are involved [25]. Thus, investigation for early diagnostic biomarkers that could be used for large-scale screening may improve survival and reduce morbidity, prevent disfigurement, and reduce overall cost of treatment.

Multiple Salivary biomarkers including miRNA-21 [26], miRNA-184 [26], miRNA-375 [27], Prion Protein (PrPC) [28] have potential as early diagnosis markers of OSCC in OPMDs. Based on the previous studies, various cytokines, such as Interleukin-6 (IL-6), Interleukin-1β (IL-1 β), Tumor Necrosis Factor-α (TNF-α), Interleukin-1α (IL-1α), and Interleukin-8 (IL-8), have been suggested to be OSCC biomarkers in individuals with OPMDs [29]. However, there remains a gap in studies specifically targeting tobacco chewers regarding the early diagnostic capabilities of these salivary biomarkers for OPMD progression to OSCC. To date, no study has evaluated the potential of salivary biomarkers for the progression of OPMDs, such as oral leukoplakia, to OSCC specifically in tobacco chewers.

The salivary glands synthesize numerous cytokines or growth factors, which play an important role in maintaining oral homeostasis [30, 31]. EGF is a biologically active growth factor synthesized and secreted by the salivary glands which protects the oral mucosa against various injuries and maintains its integrity [32–34]. Aberrant activation and upregulation of oncogenic signalling pathways, such as the EGFR, PI3K/AKT/mTOR, JAK/STAT, MET, Wnt/β-catenin, and RAS/RAF/MAPK pathways, drive the progression of OSCC [35]. EGF functions through binding to its membrane receptor, EGFR, which is involved in the proliferation, differentiation, survival, and death of OSCC cells, and activation of multiple downstream signalling pathways including the RAS/RAF/MEK/ERK1/2 pathway [36]. The EGF/EGFR signalling pathway has been implicated in the pathogenesis of a variety of tumours including OSCC [37]. EGFR is frequently overexpressed in the saliva of OSCC and pre-malignant oral lesions [38]. On the other hand, low salivary EGF levels have been found in oral cancer patients [39]. Some studies have reported the association between salivary and tumoral EGF/EGFR levels in oral cancer patients [40–43]. However, the simultaneous detection of salivary EGF and EGFR levels as diagnostic biomarkers for oral cancer, in tobacco chewers with OL and OSCC patients has not been reported. In this study, we showed that the salivary EGF: EGFR ratio could be

suggested as a potential biomarker in the early detection of OSCC in tobacco chewers.

Materials and methods

Comparative gene expression analysis of EGF and EGFR at mRNA level in head and neck squamous cell carcinoma (HNSC) and normal tissue

To perform a thorough comparison of gene expression levels between HNSC tissues and normal tissues, we obtained gene expression plots for both EGF and EGFR from GEPIA2 [44] by selecting the cancer type "Head and Neck Squamous cell carcinoma". GEPIA2 is a widely used online tool that facilitates comprehensive gene expression analysis in diverse cancer and normal tissues. The tool utilizes data from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) databases, ensuring a robust and reliable dataset for our analyses (http://gepia2.cancer-pku.cn/#degenes).

Sample size estimation

The minimum required sample size was determined to be 34 participants per group using the one-way ANOVA function in G*Power 3.1.9.7 software, with assumptions of $\alpha = 0.05$, $\beta = 0.05$, and an effect size of 0.4 [45, 46]. A high power (95%) and large effect size (0.4) were chosen to ensure the detection of small but clinically important differences, while maintaining a feasible sample sizes across groups. Although the OSCC group had fewer participants than required, the final sample sizes of 41, 41, and 19 in HC, TC, and OSCC groups were considered adequate for analysis.

Patients and saliva samples collection

The study received approval from the Institutional Ethics Committee (IEC) of Bharati Vidyapeeth (Deemed to be University), Pune, Maharashtra, India. Participants were recruited from Bharati Medical Hospital, Pune, Maharashtra, India, and provided written informed consent as per the Institutional Ethics Committee (IEC) guidelines. Informed consent was obtained from all participants, which included 41 healthy controls (HC) with no significant illness in the recent past, 41 individuals with a history of tobacco chewing for over 10 years (TC), and 19 patients diagnosed with OSCC. In this study, we defined oral cancer as any malignant neoplasm occurring in intraoral or buccal cavity 143–145 (ICD-9) [1, 47]. Eligible OSCC patients were: (1) aged over 35 years, (2) with biopsy-confirmed OSCC, (3) having no prior history of neoplasms, and (4) with no history of surgical or non-surgical treatment (including chemotherapy or radiotherapy). Unstimulated saliva samples (Saliva sample donors were asked to abstain from eating/drinking from the morning, where drinking water was permitted) were collected from all subjects in the morning between 9:00 and 11:00 a.m., 10 min after rinsing their mouths with Milli-Q water. Saliva was collected via the spitting method into 50 mL sterile tubes. The samples were then transferred to 1.5 mL sterile microtubes and centrifuged at 5,000 rpm for 10 min. The supernatant, separated from the cellular component, was aliquoted and stored at -80 °C within 60 min of collection until further investigation (Fig. 1).

Quantification of EGF and EGFR in saliva of HC, TC, and OSCC patients

Saliva samples were thawed on ice and centrifuged at 3,000 rpm at 4 °C for 10 min before analysis. Salivary EGF and EGFR levels were quantified using commercially available sandwich ELISA kits: Human EGF (AssayMax™, Assaypro, USA) and Human EGFR (Booster Biological Technology Co., Ltd., USA) (Fig. 1). The assays were performed following the manufacturer's protocols. Each sample was analyzed in a singlet, with concentration values averaged across replicates. Absorbance was measured at 450 nm using a BMG Labtech microplate reader, and EGF and EGFR concentrations were determined by plotting standard curves generated from the absorbance readings.

Statistical analysis

All the obtained data was analysed using Graph Pad Prism 9 (GraphPad Software Inc., La Jolla, CA, USA), with $p < 0.001^{***}$, $p < 0.01^{**}$, and $p < 0.05^{*}$ were considered to be statistically significant, whereas $p > 0.05$ considered non-significant. The Kruskal–Wallis test was used to compare levels of salivary EGF, EGFR, and EGF: EGFR ratio between the three study groups: HC (Healthy Control), TC (Tobacco Chewers), and OSCC (Newly diagnosed OSCC patients). For pairwise comparisons between these groups, post hoc (Dunn's test) was applied. Sensitivity, specificity, receiver operating characteristic (ROC) curve, and area under the curve (AUC) were calculated for each biomarker to assess diagnostic performance derived at the threshold (Youden index J) [48–50]. ROC curves for each biomarker were then plotted using MedCalc 23.0.9 (Ostend, Belgium) with $p < 0.0001^{****}$, $p < 0.001^{***}$, $p < 0.01^{**}$, and $p < 0.05^{*}$ were considered to be statistically significant, whereas $p > 0.05$ considered non-significant. By comparing sensitivity against specificity, the AUC was computed to quantify overall discriminative ability of salivary EGF, EGFR, or EGF: EGFR ratio among the groups. An ROC/AUC value > 0.7 was considered to indicate an acceptable/significant discriminatory power (Fig. 1).

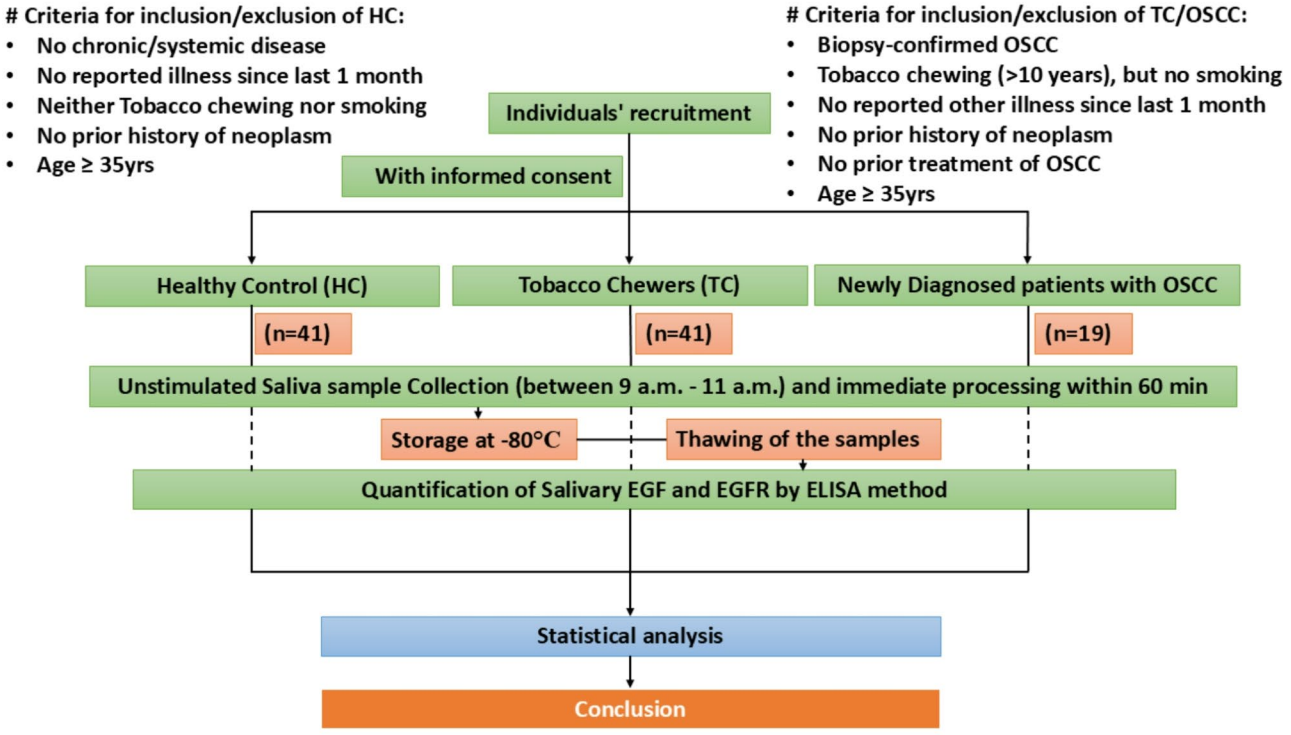


Fig. 1 Study design flowchart

Results

Downregulation of EGF and upregulation of EGFR mRNA expression in head and neck squamous cell carcinoma (HNSC): database analysis

OSCCs represents 80–90% of head and neck squamous cell carcinoma (HNSC) cases. EGFR is overexpressed in 80–90% of HNSC, including OSCC, and this overexpression is associated with poorer prognosis and reduced treatment efficacy [51]. Using the GEPIA2 tool for gene expression analysis, we found that EGF mRNA expression was downregulated in HNSC patients compared to normal tissues, whereas EGFR mRNA expression was upregulated in HNSC relative to normal tissue (Fig. 2).

Participant individual characteristics

Based on previous study findings and analyses by the GEPIA2 tool with TCGA and GTEx data using EGF/EGFR expression levels and their roles in HNSC, along with the established impact of tobacco in HNSC (especially OSCC) carcinogenesis, we designed a preliminary clinical study involving three groups: healthy individuals, tobacco chewers, and newly diagnosed OSCC patients. The characteristics of study cohorts are given in Table 1.

Decreased expression of salivary EGF in TC and OSCC

Compared to healthy controls (HC), who had a median EGF level of 5.4 ng/mL (interquartile range [IQR] = 4.495), significantly lower EGF levels were detected in unstimulated saliva samples from tobacco chewers (TC) and OSCC patients. Specifically, TC samples had a median EGF level of 3.385 ng/mL (IQR = 2.13), while OSCC samples had a median of 3.720 ng/mL (IQR = 2.625) (Fig. 3A). Statistical analysis revealed that EGF levels in TC (HC vs. TC, Dunn's multiple comparisons tests, $p < 0.001$) and OSCC (HC vs. OSCC, Dunn's multiple comparisons tests, $p < 0.05$) were significantly lower compared to HC. However, no significant difference in salivary EGF levels was found between TC and OSCC groups (TC vs. OSCC, Dunn's multiple comparisons test, $p > 0.05$). The result suggested that reduced salivary EGF levels could be indicative of tobacco-related changes in OSCC carcinogenesis.

Increased expression of salivary EGFR in TC and OSCC

Compared to healthy controls (HC), who had a median EGFR level of 1.0436 ng/mL (interquartile range [IQR] = 1.345), higher EGFR levels were detected in unstimulated saliva samples from both tobacco chewers (TC) and OSCC patients. samples had a median EGFR level of 2.0442 ng/mL (IQR = 5.5042), while OSCC samples had a median of 1.9838 ng/mL (IQR = 5.0738) (Fig. 3B). Statistical analysis revealed that EGFR levels in OSCC patients were significantly higher than those in HC (HC vs. OSCC, Dunn's multiple comparisons test,

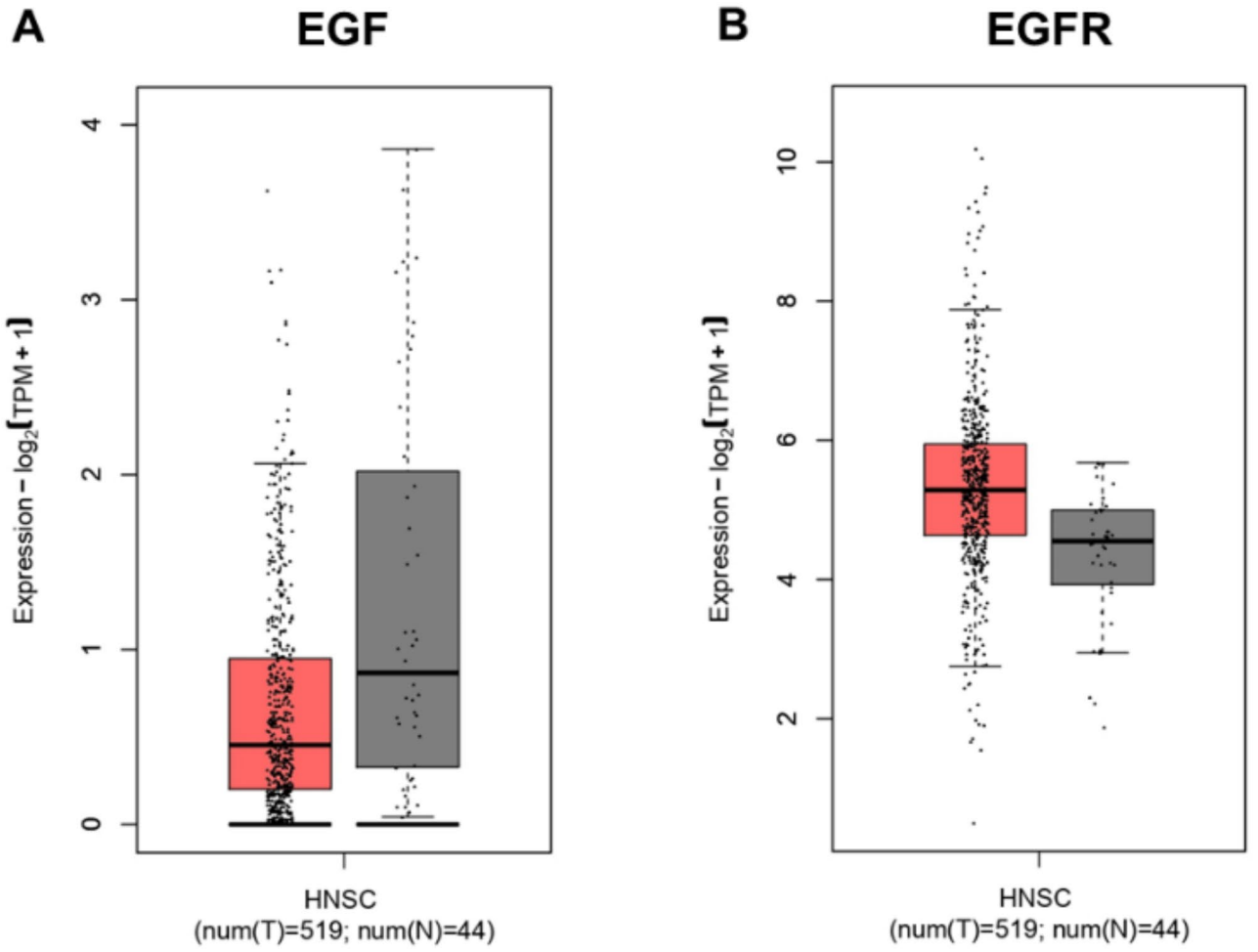


Fig. 2 Gene expression patterns of EGF and EGFR across normal (grey box) and HNSC tumour tissues (red box) as determined by the GEPIA2 platform

Table 1 Main characteristics of recruited individuals in the study

Variable	HC	TC	OSCC
Number	41	41	19
Age-average (range)	48 years (35–61 years)	47.5 years (35–60 years)	50 years (35–65 years)
Sex (Men/Women)	Men (25) Women (16)	Men (32) Women (9)	Men (14) Women (5)
Tobacco chewing habit	No	More than 10 years	More than 10 years
Smoking habit	No	No	No
Site of Lesion (OL or OSCC)	NA	Tongue/ Floor of oral cavity[#]	Tongue/ floor of the oral cavity
Previous malignancy	No	No	No
Any illness at the time of sample collection (fever, major infection, or cough)	No	No	No

Notes:

1. NA-Not Applicable

2. # All TC were recalled for clinical evaluation, and upon re-examination, OL was detected in all the cases

$p < 0.05$). In contrast, the increase in EGFR levels in TC compared to HC approached but did not reach statistical significance (HC vs. TC, Dunn's multiple comparisons tests, $p = 0.138$). No significant difference in salivary EGFR levels was observed between the TC and OSCC groups (TC vs. OSCC, Dunn's multiple comparisons tests, $p = 0.808$). This result suggested that tobacco exposure may initiate the secretion of EGFR in saliva where it may contribute to OSCC carcinogenesis.

Decreased ratio of salivary EGF: EGFR in TC and OSCC

Results of salivary EGF and EGFR levels in the study and given the critical roles of EGFR (receptor) and EGF (its ligand) in the EGFR signalling pathway, prompted us to evaluate the statistical significance of the salivary EGF: EGFR ratio among the HC, TC, and OSCC groups. Compared to healthy controls (HC), who had a median EGF: EGFR ratio of 4.69 (interquartile range [IQR] = 6.9998), significantly lower EGF: EGFR ratios were detected in unstimulated saliva samples from tobacco chewers (TC) and OSCC patients. Specifically, TC samples had

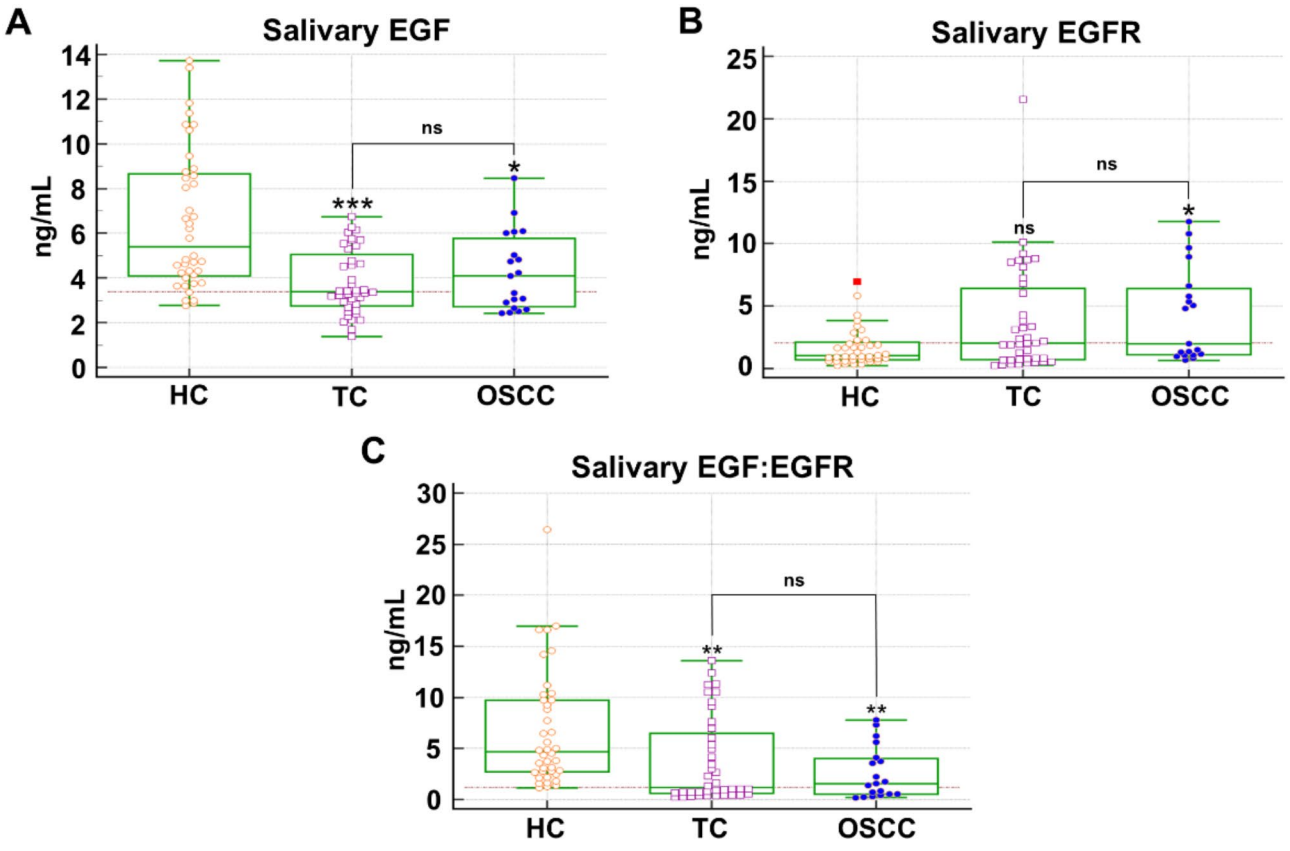


Fig. 3 Salivary EGF (**A**), EGFR (**B**) and EGF: EGFR (**C**) levels in HC, TC, and OSCC. $p < 0.001$***, $p < 0.01$**and $p < 0.05$* were considered to be statistically significant, whereas $p > 0.05$ considered non-significant (ns)

a median EGF: EGFR ratio of 1.18 (IQR = 5.8754), while OSCC samples had a median of 1.55 (IQR = 3.5767) (Fig. 3C). Statistical analysis revealed that the EGF: EGFR ratio in TC (HC vs. TC, $p < 0.01$) and OSCC (HC vs. OSCC, Dunn's multiple comparisons test $p < 0.01$) were significantly lower compared to HC. However, no significant difference in salivary EGF: EGFR ratio was found between TC and OSCC groups (TC vs. OSCC, Dunn's multiple comparisons test, $p > 0.05$). The results suggested that alterations in the EGF: EGFR ratio may indi cate an early disruption in the balance between EGF and EGFR in tobacco chewers, potentially contributing to the development of OSCC.

Salivary EGF and EGFR as diagnostic biomarkers for oral cancer in tobacco chewers: insights from ROC analysis

Marker performance was assessed using sensitivity, specificity, the Youden Index, and the Area Under the Receiver Operating Characteristic (ROC) curve (AUC)., the latter quantifying how well a biomarker distinguishes between two states (here, within HC, TC, and OSCC). Sensitivity reflects the ability to detect true positives, specificity measures true negatives, and the Youden Index balances the two (J = Sensitivity + Specificity – 1), highlighting overall test effectiveness.

We analyzed the ROC curves to evaluate the diagnostic power of salivary biomarkers EGF and EGFR; and the EGF: EGFR ratio, for the transition to OSCC in TC across three comparisons: ND vs. HC, TC vs. HC, and ND vs. TC (Table 2; Fig. 4A, B and C). Salivary EGF, with an AUC exceeding 0.7, effectively differentiated HC from TC and ND OSCC cases, though it did not distinguish between TC and ND, suggesting its potential as an early diagnostic biomarker for transition to OSCC in TC. Similarly, Salivary EGFR showed strong discrimination between HC and OSCC (AUC > 0.7), though it was less effective in distinguishing TC from HC (0.6 < AUC < 0.7). However, Salivary EGFR could not differentiate TC from OSCC, underscoring its potential diagnostic utility for transition to OSCC in TC. EGF: EGFR ratio achieved perfect specificity (100%) for TC vs. HC with AUC = 0.719, J = 0.500, $P < 0.001$, outperforming EGF and EGFR. Moreover, its significance surpassed that of EGF alone in differentiating OSCC from HC, where it achieved an AUC of 0.779 with a higher level of statistical significance ($P < 0.0001$). Additionally, the EGF: EGFR ratio demonstrated a good balance of performance, with a moderate sensitivity of

Table 2 Salivary EGF, EGFR, EGF: EGFR cut-offs for the 3 combinations of the groups (HC, TC, and OSCC)

Comparison	Cut-offs	Sensitivity	Specificity	Youden index J	AUC	Significance level *P* (Area = 0.5)
EGF (TC vs. HC)	≤ 3.46	60.00%	87.50%	0.475	0.787	< 0.001
EGF (OSCC vs. HC)	≤ 3.34	50%	90%	0.4	0.742	< 0.001
EGF (OSCC vs. TC)	> 2.28	100%	15%	0.150	0.540	> 0.05
EGFR (TC vs. HC)	> 1.84	60.98%	70.73%	0.317	0.632	< 0.05
EGFR (OSCC vs. HC)	> 0.43	47.37%	95.12%	0.425	0.721	< 0.01
EGFR (OSCC vs. TC)	> 0.82	94.74%	34.15%	0.289	0.579	> 0.05
EGF: EGFR (TC vs. HC)	> 0.87	50%	100%	0.500	0.719	< 0.001
EGF: EGFR (OSCC vs. HC)	> 0.54	57.89%	87.50%	0.454	0.779	< 0.0001
EGF: EGFR (OSCC vs. TC)	> 0.11	100%	20%	0.200	0.549	> 0.05

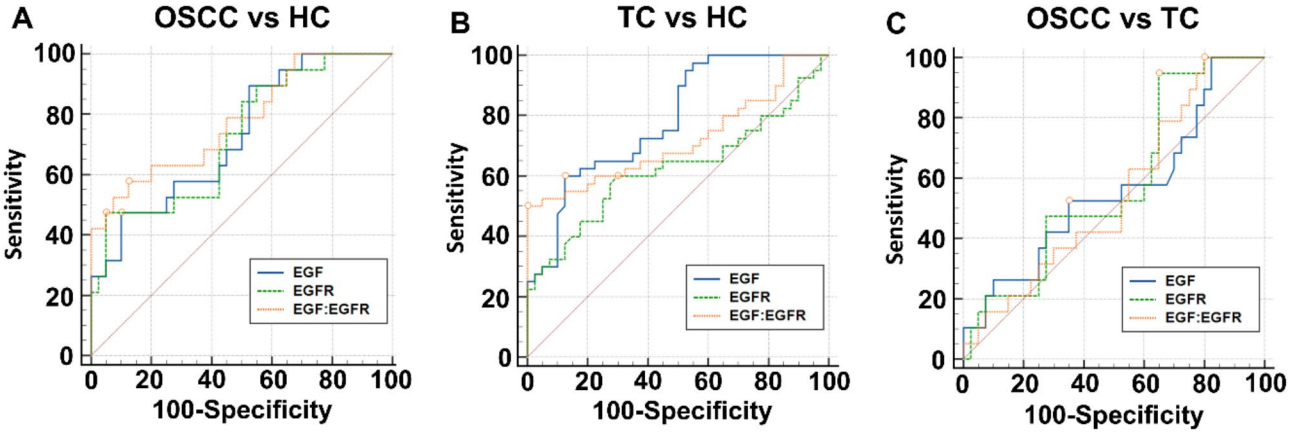


Fig. 4 ROC (Receiver Operator Characteristic) curve analysis for diagnostic power of salivary biomarkers, EGF, EGFR, EGF: EGFR, (**A**) OSCC vs. HC, (**B**) TC vs. HC (**C**) OSCC vs. TC. The small hollow orange circles typically indicate the parameter values corresponding to the Youden index on the respective ROC curve

57.89% and a high specificity of 87.5% for distinguishing OSCC from HC, surpassing the performance of EGF and EGFR alone. Thus, this data showed that salivary EGF: EGFR seems to be the best marker among the three (salivary EGF, EGFR, and EGF: EGFR) to predict the progression of tobacco chewer to OSCC.

Post-Hoc power analysis for sample size justification

As the OSCC group had fewer participants than the calculated requirement, a post hoc power analysis was performed using the one-way ANOVA function in G*Power 3.1.9.7 software to justify the final sample sizes of 41, 41, and 19 subjects in the HC, TC, and OSCC groups, respectively [45, 46]. This calculation derived from pooled standard deviations of 2.31 ng/mL for salivary EGF, 3.31 ng/mL for salivary EGFR, and 5.04 for the EGF: EGFR ratio. The achieved power was over 99% for salivary EGF and 84% for both salivary EGFR and the EGF: EGFR ratio, confirming that the study maintained sufficient statistical power, as all values remained above the well accepted 80% threshold.

Discussion

Histologic characteristics of OPMD (as detected in primary or secondary oral examination) are not consistently predictive of their clinical progression [52–54]. Thus, there is a significant need to identify biomarkers that can improve early diagnostic accuracy and guide treatment strategies [54]. Direct and continuous contact between saliva and the oral cancer lesion makes saliva a simpler tertiary mode of oral examination yet more powerful and directly relevant to oral pathology than serum testing [54–56]. The most frequently amplified genes observed in oral cancer cases include CCND1, MYC, BCL-2, and EGFR, with EGFR as one of the regulators of the other three [54]. EGFR is necessary for cell proliferation and survival and dysregulation of EGFR, involving either its ligands or the receptors themselves, has been linked to various diseases, including glioblastoma, and cancers of the breast, skin, lung and oral cavity [54, 57]. The EGF concentration in saliva is higher (about 10 times) than that in serum and directly relevant to the pathophysiology of the oral cavity [58]. Even asymptomatic normal-like mucosa could have molecular abnormalities [21]. As long-term tobacco chewing is a major risk factor and sufficient cause for OSCC [14], alterations in salivary EGF

and EGFR levels may reflect early molecular changes. Identifying the determinants of neoplastic progression is essential in oral carcinogenesis research. This study aims to assess whether salivary levels of Epidermal Growth Factor (EGF) and its receptor-Epidermal Growth Factor Receptor (EGFR) could serve as early diagnostic markers for the development of OSCC in individuals who have been chewing tobacco for more than 10 years. It has been shown that nicotine induces overexpression and activation of EGFR in Leuk-1 (an oral pre-cancerous cell line) and DOK (an oral normal keratinocyte cell line) cells to induce migration [59]. This study evaluated whether EGF/EGFR could help in predicting the likelihood of OSCC in chronic tobacco chewers, aiding in early targeted intervention strategies. Salivary EGF/EGFR levels were compared with OSCC patients and healthy individuals. Recently, tobacco chewing in OL patients has been associated with higher expression of EGFR and WNT pathways, which may indicate their potential risk for progression to OSCC [60]. EGFR regulates the survival, proliferation, and differentiation of epithelial cells as well as tumours of epithelial cell origin. More than 90% of head and neck squamous cell carcinoma show high expression of EGFR. On the other hand, the role of EGF in oral cancer has always been controversial. Previously a clinical study indicated that EGF may have a protective role while in vitro studies have shown its association with oral cancer progression such as stemness, epithelial-mesenchymal transition (EMT), and migration [42, 61]. In our study, an inverse association was observed between EGF and EGFR levels in tobacco chewers and OSCC patients. We found that salivary EGF levels were quite low in tobacco chewers and OSCC patients, compared to healthy individuals. ROC analysis indicates that salivary EGF levels effectively serve as a good diagnostic biomarker for the OSCC in TC. On the other hand, EGFR levels were significantly higher in saliva samples of tobacco chewers and OSCC patients compared to healthy ones. The partial diagnostic capability of salivary EGFR was validated through ROC analysis, confirming its utility as a diagnostic factor. It was interesting to find that the EGF and EGFR levels of tobacco chewers were almost similar to those of OSCC patients as also supported by ROC analysis. Based on this observation all the tobacco chewers enrolled in the study were called for oral examination at Bharati Hospital. Surprisingly all of them were diagnosed with different grades of OL. A couple of studies have reported low EGF levels in the saliva of OSCC patients [40, 42].

It has also been reported that patients with tumours on the tongue and floor of the mouth have low salivary EGF levels, whereas those with tumours at other sites have high salivary EGF levels [40]. It has been suggested that the tongue and floor of the mouth could be sites more sensitive to the reduction of EGF levels in saliva, considering that oral mucosa could be more permeable to carcinogens than other sites in the body [40]. Further, EGF plays an important role in maintaining tissue homeostasis, as it regulates epithelial cell proliferation, growth, and migration. In addition, it has an effect on angiogenesis for the nutritional support of tissues. EGF protected squamous cell carcinoma (A431) and human oral squamous cell carcinoma (SCC4 and SCC25) against cisplatin-induced cytotoxicity through increased IL-1β expression [62]. High levels of EGF in saliva, before and during radiation treatment are associated with less mucosal damage [63]. Thus, low or depleted EGF levels could hamper wound healing and tissue generation of oral mucosa thereby contributing to the development of unresolved wounds leading to OSCC. On the other hand, some studies have shown no correlation between salivary EGF levels and OL development [64]. Similar EGF levels have been observed in OL patients and healthy controls [65]. However, some in vitro studies have shown the role of EGF in promoting oral cancer growth. In a study, EGF increased the invasiveness of OSCC cell lines (HSC-3, SAS, and Ca9-22) by regulating MMP1 expression [66]. In another, EGF induced metastasis of OSCC through EMT transition and generated cancer stem-like cell properties in oral cancer cells by promoting the Warburg effect [67]. In clinical studies, high expression of EGF is associated with an aggressive grade of the oral lesion [39, 40]. Based on the findings of this study, along with supporting in vitro and clinical studies, EGF may serve as a marker for both early development (indicated by reduced salivary levels) and progression to OSCC in tobacco chewers.

Several researchers have reported increased EGFR levels in OSCC. It has also been reported that patients with tumours on the tongue and floor of the mouth have high EGFR levels in situ, whereas those with tumours at other sites have low EGFR levels [68]. Amplification of the EGFR gene is observed in approximately 15.4–31.2% of OSCC cases [69]. EGFR functions as a tyrosine kinase receptor and is activated upon ligand binding [70]. Overexpression of EGFR results in a poor prognosis for oral cancer [71–74]. Its activation is associated with stemness acquisition, cell proliferation, cell adhesion, malignant phenotype acquisition, inhibition of apoptosis, and thus increased metastatic potential in oral cancer cells [75–78]. Moreover, patients with EGFR overexpression have worse 5-year survival and higher chances of recurrence as compared to EGFR-negative patients [79]. In OSCC, EGFR expression is downregulated in high-grade invasive cells compared to that of low-grade invasive cells [80]. However, our study revealed high EGFR levels in tobacco chewers, who were later re-evaluated and diagnosed with OL. Previously, it has been reported that EGFR and Her-2

levels were elevated in the oral cavity of patients using betel quid [74]. This may indicate that overexpression of EGFR could be associated with early oral carcinogenesis. Indeed, the changes in epidermal growth factor receptor gene copy number are associated with oral carcinogenesis [81]. Thus, EGFR might still be useful as a diagnostic marker, although for this, it is necessary to establish which is the best method to evaluate EGFR status [68].

The reduction in salivary EGF and overexpression in salivary EGFR are influenced by tobacco smoking [82]. In OSCC, despite low EGF levels, it is important to understand how EGFR-mediated oncogenic signals get activated. Although EGF is the most widely studied ligand for EGFR, the latter can be activated by several other ligands which include Transforming Growth Factor-α (TGF-α), Amphiregulin (AREG), Epigen (EPGN), Beta-Cellulin (BTC), Heparin Binding-EGF (HB-EGF), and Epiregulin (EPR) [83]. EGFR ligands generally fall into two classes: high affinity or low affinity. Ligands with high affinity, such as EGF, TGF-α, HB-EGF, and BTC, bind with dissociation constants (K_d) ranging from 1 to 100 nM, whereas low-affinity ligands, including AR, EPR, and EPG, exhibit K_d values exceeding 100 nM [84]. It has been suggested that the affinity of EGFR for its ligands depends on the tissue and pathological condition [85]. High-affinity ligands tend to drive EGFR toward intracellular degradation, while low-affinity ligands tend to promote its recycling to the cell surface [86]. Therefore, partly, higher EGFR levels on the cell surface and in saliva may result from low salivary EGF levels. Depending on the specific ligand that binds to EGFR, different cellular pathways can be activated that can induce transcription of genes responsible for inhibition of apoptosis, cell growth, angiogenesis, cell adhesion, cell motility, and invasion [85, 87]. Lower salivary EGF levels in tobacco chewers and OSCC patients may be hypothesized to indicate sustained EGFR-mediated signalling in tobacco chewers with premalignant lesions like OL which have high EGFR expression. This is consistent with our findings, which indicate the diagnostic ability of salivary EGF levels for OSCC in tobacco chewers, and we found all TC to have OL upon re-evaluation.

ROC curve analysis showed that the EGF: EGFR ratio is a more reliable biomarker than (EGF and EGFR alone) for progression to OSCC in TC. It reiterated the previous suggestions that to attain high predictive power of salivary biomarkers must be evaluated and validated in panels [88, 89]. Although EGF: EGFR showed high specificity with balanced with moderate sensitivity to improve diagnostic accuracy, we should extend the biomarker panel to include multiple EGFR ligands along with EGF along with EGFR. This broader approach will enable the development of a more robust and reliable diagnostic model. Additionally, contrary to OSCC vs. HC and TC vs. HC groups, the evaluated biomarkers including the EGF: EGFR ratio showed very high sensitivity and low specificity in OSCC vs. TC analysis. When used in populations already at high risk of OSCC (e.g., Tobacco chewers) the number of 'false positive cases' is reduced, making the high sensitivity of EGF: EGFR more practical for screening purposes (SNOUT-Sensitive Test Negative Rules Out).

In future studies, it will be highly important to evaluate the in-situ activation of EGFR signalling in tobacco chewers to validate the proposed mechanism. In line with this, IL-1β has been shown to activate EGFR in oral premalignant (DOK) and OSCC (TW2.6 and OC3) cell lines through the regulation of CXCL1 and CXCR2 chemokines [90]. Moreover, EGFR inhibitors (EGFRi) upregulate IL-6 and IL-8, which promote tumor progression via STAT3-mediated signalling, while their antagonists sensitize oral cancer cells to EGFRi [91]. Thus, salivary EGFR and its ligands along with previously proposed IL-6, IL-8, and IL-1β [88, 92–94] markers could be evaluated for a potential theranostic panel for OSCC in future clinical studies.

Limitations of the study

Despite the significant potential of salivary EGF: EGFR as an early detection biomarker in tobacco chewers, our study has certain limitations. One key limitation is the cross-sectional design, which does not allow for tracking the progression of OSCC in tobacco chewers over time. Without longitudinal follow-up, we are unable to determine whether altered EGF and EGFR levels can predict future OSCC development in high-risk individuals. Additionally, while we established ROC-based cut-offs for biomarker levels, the lack of prospective data prevents us from accurately calculating positive predictive value (PPV) and negative predictive value (NPV) for OSCC risk in tobacco chewers.

Another limitation in the study is the small sample size and recruitment of participants from a single tertiary care hospital in Pune, India, which limits the broader applicability of our findings. A larger multicentric study involving diverse populations across India can enhance the robustness and generalizability of our results. Furthermore, this study has focused on analyzing only salivary EGF and EGFR levels in OSCC patients, identified based upon biopsy-confirmed histological diagnosis. Further limitations of the study include, recording of critical clinical parameters such as tumor staging, lymph node involvement, distant metastases, and cancer-specific survival. These factors play a crucial role in disease progression and prognosis, and their inclusion can provide a more comprehensive understanding of the clinical relevance of the salivary biomarkers.

To overcome these limitations, future studies are warranted to incorporate larger, multicenter cohorts with longitudinal follow-up of tobacco chewers to assess the diagnostic value of salivary biomarkers in OSCC development. Additionally, integrating detailed clinical staging and prognostic parameters would lead to thorough evaluation of the role of EGF: EGFR in different stages of OSCC and its potential utility in guiding clinical decision-making. These steps would significantly enhance the translational potential of these biomarkers for early OSCC detection and risk stratification in high-risk populations.

Conclusion

In the present study, salivary EGF: EGFR ratios were significantly modulated in tobacco chewers and patients of OSCC as compared to the healthy controls, however, the ratios were similar in both the tobacco chewers and OSCC patients. The EGF level was downregulated with simultaneous increase in the levels of EGFR, which could be considered to be a critical salivary biomarker as EGF: EGFR ratio for the early diagnosis of OSCC in tobacco chewers. Timely detection and treatment can increase the survival rate of OSCC and reduce morbidity and mortality rates. The results of the present study paves way for future studies on salivary EGF: EGFR ratio as a non-invasive salivary biomarker for early diagnosis and monitoring for OSCC in long-term tobacco chewers.

Abbreviations

AREG	Amphiregulin
BTC	Beta-Cellulin
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFRi	Epidermal Growth Factor Receptor inhibitor
ELISA	Enzyme-Linked Immunosorbent Assay
EPGN	Epigen
EPR	Epiregulin
GTEx	Genotype-Tissue Expression
HB-EGF	Heparin Binding-EGF
HC	Healthy Control
HNSC	Head and Neck Carcinoma
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
OL	Oral Leukoplakia
OPMD	Oral Potentially Malignant Disorder
OSCC	Oral Squamous Cell Carcinoma
PrPC	Prion Protein
TC	Tobacco Chewers
TCGA	The Cancer Genome Atlas
TGF-α	Transforming Growth Factor-α
TNF-α	Tumor Necrosis Factor-α

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Author contributions

RKG: Conceptualization, Resources, Supervision, Methodology, Formal analysis, Writing - original draft, review & editing; RSK: Resources, Investigation, Methodology; PR: Investigation, Methodology, formal analysis, Writing - original draft; AC: Investigation, Methodology, Formal analysis, Writing- original draft; SG: Investigation, Methodology.

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Data availability

All raw data supporting the findings of this study will be made available upon reasonable request. For access, please contact Dr. Ruchika Kaul-Ghanekar (ruchika.kaulghanekar@gmail.com) or Amol Chaudhary(amolchaudhary1988@gmail.com).

Declarations

Ethics approval and consent to participate

The present study is completed after approval from the Institutional Ethics Committee Bharati Vidyapeeth (Deemed to be University), Pune (Maharashtra), India, with IRB Number: IRB-1223/DUHS/Approval/2019/34. All procedures performed in studies involving human participants were under the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent in writing from all the research participants was obtained according to Helsinki's declaration.

Consent for publication

The current study does not contain any images or videos related to any research participant. The consent for publication is obtained from all research participants included in the current study.

Competing interests

The authors declare no competing interests.

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