RESEARCH ARTICLE

Salivary VOCs as a Diagnostic Marker for OSCC by Gas Chromatography-Mass Spectrometry Analysis: An Observational Study

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ABSTRACT

Background: Head and neck carcinoma is the sixth most common malignancy globally, with oral squamous cell carcinoma (OSCC) being the most prevalent subtype. While histopathological examination remains the diagnostic gold standard, it is invasive and time-consuming. Metabolomic profiling, particularly analysis of volatile organic compounds (VOCs), offers a promising non-invasive diagnostic approach.

Objectives: To identify and compare salivary VOCs between OSCC patients and healthy controls, aiming to explore their potential as diagnostic biomarkers.

Study Design and Setting: A case-control study involving OSCC patients and age- and sex-matched healthy controls.

Participants: A total of 75 subjects were enrolled, including 35 histopathologically confirmed OSCC patients and 40 healthy controls.

Methods: Unstimulated saliva samples were collected and VOCs were extracted using a ZSM-5/PDMS-coated film. Extracts were condensed with 100 µL methanol, and 1.0 µL aliquots were analyzed using gas chromatography–mass spectrometry (GC-MS). Statistical comparisons were performed using the Chi-square test, with significance set at $p < 0.05$.

Results: Ten VOCs demonstrated statistically significant differences between OSCC and control groups. These included two acids, three alcohols, three ketones, and two alkanes. Notably, butanoic acid, pentanoic acid, and 4-methyl were significantly elevated in OSCC patients ($p \leq 0.001$). VOC expression patterns also correlated significantly with age, gender, pTNM staging, and histopathological grade.

Conclusion: Salivary VOC profiling using GC-MS reveals distinct metabolomic signatures in OSCC patients.

Clinical significance: These findings suggest that specific VOCs may serve as non-invasive biomarkers for early diagnosis, prognosis, and therapeutic monitoring of OSCC.

Keywords: Oral Squamous cell carcinoma, Volatile organic compounds, saliva, Gas chromatography mass spectroscopy.

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INTRODUCTION

Head and neck squamous cell carcinoma [HNSCC] is the 6th highly occurring cancer with Oral squamous cell carcinoma [OSCC] being the most predominant. India is considered as the world core for OSCC and posing a malicious health challenge[1] where around 77,000 novel reports and 52,000 mortality of OSCC are being declared annually[2].The concernment of OSCC is significantly progressive in India as about 70% are reported in the enhanced stages as reported by American Joint Committee on Cancer[3].Though visual and screening tests which are widely used now for diagnosis, their efficacy is limited by both knowledge and expertise[4]. Histopathological examination has remained the gold standard, but it is invasive and often tedious for the patients. There is a claim for modest, non-invasive, and integral need for novel diagnostic and prognostic biomarkers. Metabolomics is a surfacing adjunct to explore the thorough profile of VOCs, which can be detected using GCMS. They act as chemical fingerprint, capable of delineating metabolic changes in the biological system, thereby ascertaining pathologies. Carcinogenesis is related to a transformed metabolism, escalated aerobic glycolysis which produces oxidative stress[5]. The term VOCs covers a varied class of chemicals like aldehydes, alcohols, aliphatic, aromatic, and chlorinated type of hydrocarbons. They are basically

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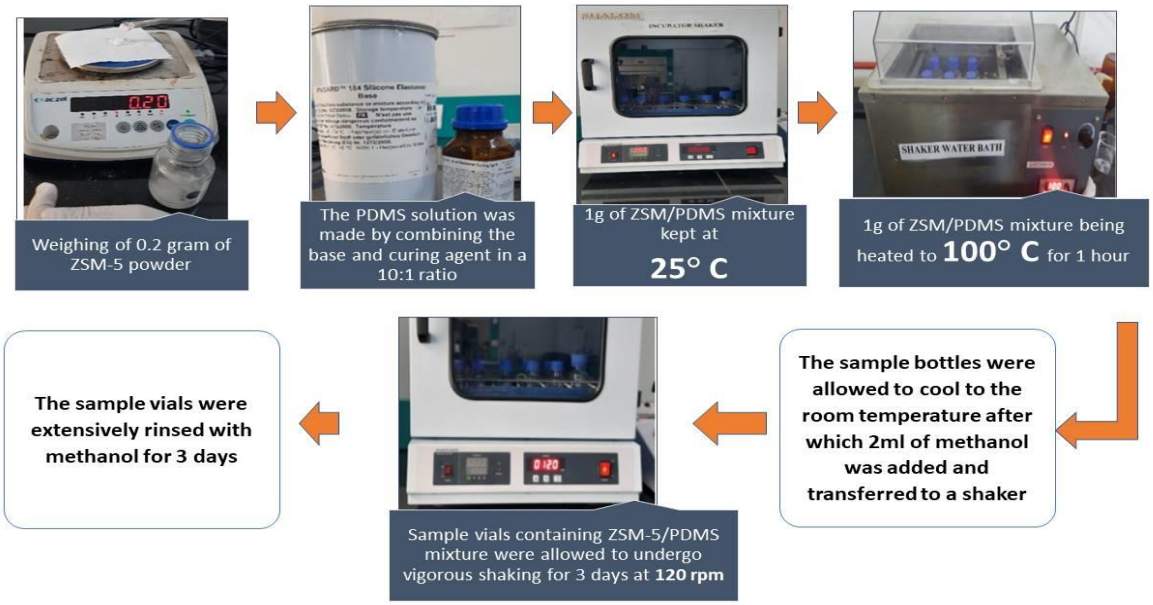


Figure 1: Steps in pre-extraction- Preparation of ZSM-5/PDMS film

carbon-based compounds having shallow vapour pressure and elevated boiling point. Considering the increased number of hydrocarbons and chemicals detected in variable arrays, which plays a viable role as cancer biomarker of research[6]**.** VOCs are considered as incredible non-invasive biomarkers for early cancer detection, as it can be measured from bio-samples like breath, blood, urine, faeces, saliva and sweat[7].

VOCs have already portrayed clinical prospects as biomarkers for cancers involving various organs like lung, GIT, breast and prostate. However, VOCs expression in OSCC has been reported meagrely in literature and a major proportion of these studies have analysed in breath. Saliva a near available bio- fluid containing a range of analytes which can alter with towering alterations based on health representing resourceful systemic information. VOCs reach from blood to saliva through docile diffusion, hence salivary VOCs can highlight metabolic alterations in retaliation to inflammation, degeneration, necrosis, and cancer. The dysplastic cells in the oral mucosa are continuously shed into the saliva owing to its proximity which also simplifies the sampling of cells from occult areas.

Objectives: This present study was undertaken to understand OSCC induced metabolic adaptations in cells and to undertake a comparative analysis VOC profile and its alterations between OSCC and healthy controls saliva samples using GC-MS analysis.

METHODS

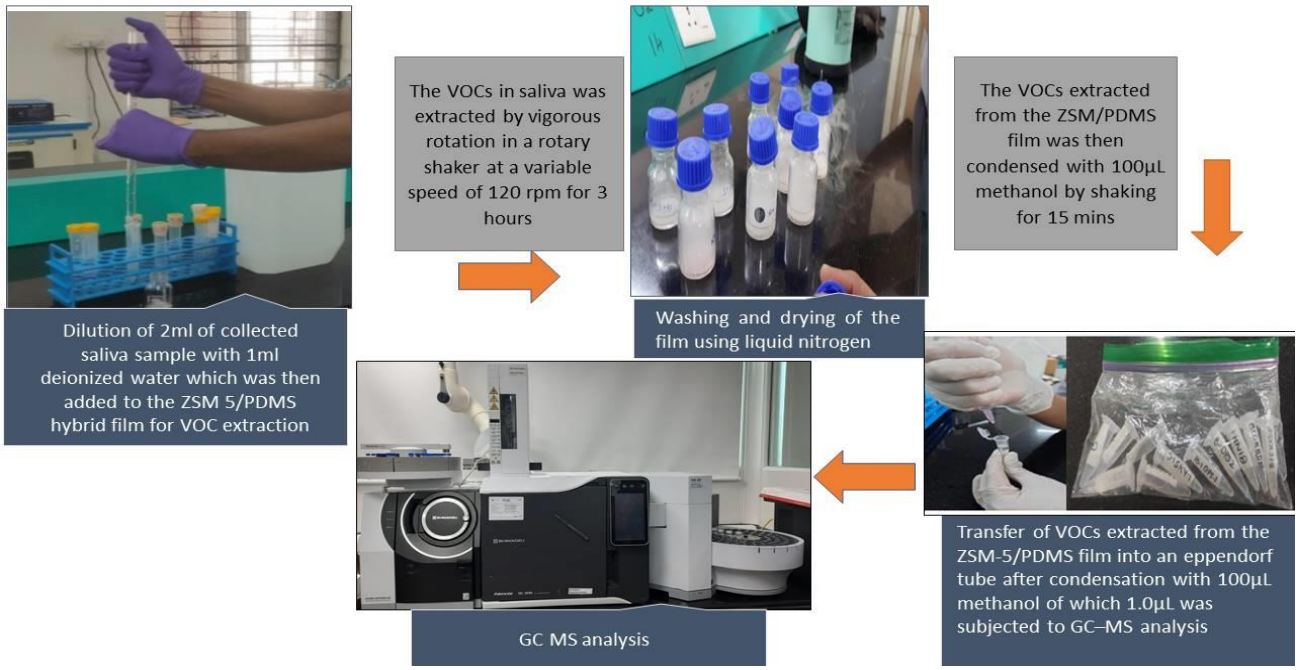
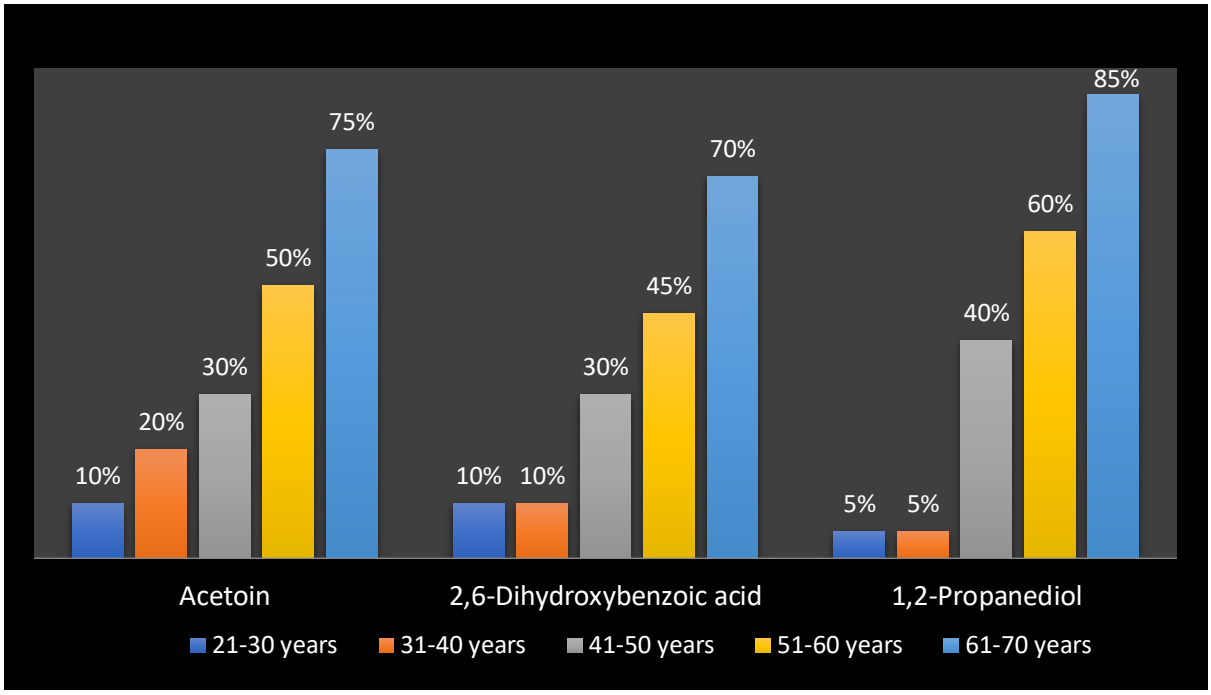
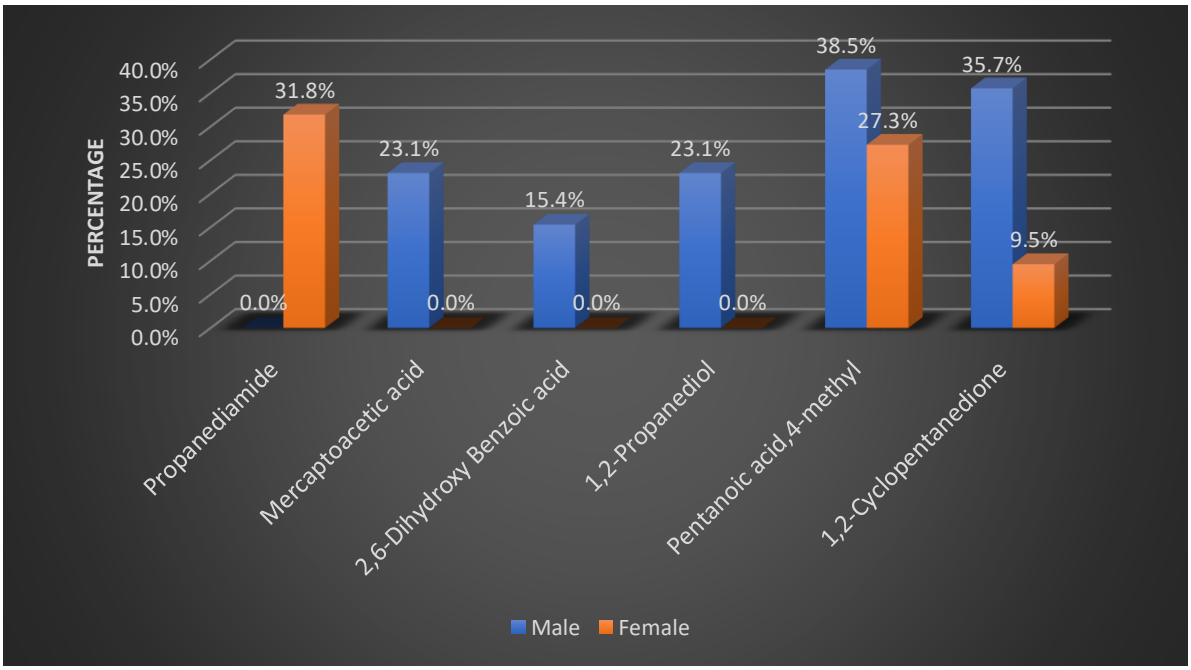


Figure 2: Pre-extraction followed by GC-MS analysis



Graph 1: Comparison of VOC profile among different age groups in OSCC subjects



Graph 2: Comparison of VOC profile among gender in OSCC group

Study design and setting

A case-control observational study was conducted in an institutional setting to investigate salivary volatile organic compounds (VOCs) as potential diagnostic biomarkers in oral squamous cell carcinoma (OSCC). The study was conducted after the approval of the institutional ethics committee IRB: EC- 2021/RS/95.

Participants: A total of 75 participants were included, comprising 35 histopathologically confirmed OSCC cases and 40 healthy, age- and gender-matched control subjects. All participants underwent a thorough oral clinical examination prior to inclusion. Written informed consent was obtained from all the participants prior to the study

Inclusion criteria

Cases: Patients above 18 years with histopathological confirmation of OSCC

Controls: Age and gender matched control subjects without H/O hypertension, diabetes or thyroid disorders and use of related medications or any deleterious habits

Exclusion criteria

Subjects with systemic conditions affecting the flow and nature of saliva were excluded

Variables

Independent (Exposure) was the group status of the subjects (OSCC vs Healthy Control)

Table 1: List of significant VOCs in OSCC

S. No	Type of VOCs	VOCs	Cases	Controls	Odds ratio	P value
1.	Acids	Butanoic acid	77.1%	25%	10.125	<0.001
		Phosphonic acid	20%	12.5%	1.750	0.377
		Pentanoic acid,3-methyl	2.9%	2.5%	1.147	0.924
		Formic acid	11.4%	2.5%	5.032	0.122
		Pentanoic acid,4-methyl	31.4%	2.5%	17.87	<0.001
		Pentanoic acid	14.3%	7.5%	2.056	0.342
2	Alcohols	3-Furanmethanol	14.3%	0%	0	0.013
		1,2-Propanediol	8.6%	0%	0	0.059
		n-Tridecan-1-ol	0%	15%	0	0.017
3	Ketones	2-Propanone,1-Hydroxy	48.6%	2.5%	36.83	<0.001
		2-Piperidinone	88.6%	87.5%	1.107	0.887
		2-Hydroxy Gamma Butyrolactone	14.3%	2.5%	6.500	0.061
		2,5-Dimethylfuran-3,4(2H, 5H)-dione	28.6%	10%	3.600	0.039
		1,2-Cyclopentanedione	20%	0%	0	0.059
4	Aldehydes	2,5-Dihydroxybenzaldehyde	2.9%	2.5%	1.147	0.924
5	Acid amide	Propanediamide	20%	15%	1.417	0.568
		Propanamide,2-hydroxy	8.6%	0%	0	0.059
6.	Alkanes	Dodecane,1-chloro	0%	30%	0	<0.001
		Butane,1,4-bis(9,10-dihydro-9-methylanthracen-10-yl)	11.4%	2.5%	5.032	0.122
8	Ester	Diethyl Phthalate	11.4%	5%	1.161	0.842

Dependent (outcome) variables were Salivary VOCs (types, concentration via GC-MS)

Control/Confounding vairbales: these variables were accounted for to reduce bias. Age, Gender, Systemic conditions, Histopathology, pTNM stage, Time of sample collection, Habits

Data sources and measurement

Saliva Sample Collection: 2 ml un-stimulated saliva was collected in 10 mL sterile glass vial was collected between 8.00 am to 11.00 and immediately placed in dry ice present in cold storage box and kept at a temperature of -20°C. The extracted samples were transported using the cold storage box for further analysis

Pre-extraction procedures: For the Preparation of "Zeolite Socony Mobil-5/Polymethyl Siloxane (ZSM 5/PDMS) hybrid film", a 50ml glass container was used as a support container. The PDMS solution was made by combining the base and curing agent in a 10:1 ratio and then mixing it with "ZSM-5"to produce a 20 wt. % "ZSM-5" mixture in a "PDMS" matrix before solidification. 1g of this substance was kept in a glass container at 25°C for three hours before being heated to 100°C for an hour. To eliminate the unreacted PDMS monomers, the sample vials containing "ZSM-5/PDMS film" were extensively rinsed with methanol and shaken for three days at 120 rpm [Fig 1].

Sample analysis: The 2ml of collected saliva was diluted with 1ml deionized water and added to the "ZSM 5/PDMS" hybrid film. The VOCs in saliva was then extracted using a rotary shaker with a variable speed of 120 rpm by vigorous shaking of the glass extraction bottle. The extraction container was carefully cleaned with clean water before being dried with liquid nitrogen. VOCs isolated from the ZSM-5/PDMS film were condensed with 100µL methanol, of which 1.0µL was subjected to "GC–MS "analysis [Fig 2].

GCMS Analysis: The GCMS machine (SHIMADZU GCMS- QP 2020NX -KYOTO, Japan) was used in at 70 eV in electron ionization mode. The interphase temperatures of the ion source were kept at 200°C and 230°C. Delineation was done using an HP- INNOWAX capillary column of 30m length with 0.320 mm inner diameter and 0.25 m film thickness. The mobile phase was 99.99 percent ultrahigh helium gas, flowing at 1.78 ml/min. The temperature of the GC injection was fixed at 230°C. The retention time was then noted from 5 min 6 seconds to 27 minutes time frame. Data acquisition was done in a complete scan mode.

The primary dependent variable in this study was the presence and profile of salivary volatile organic compounds (VOCs), as detected by gas chromatography–mass spectrometry (GC–MS). These compounds were identified and quantified using a SHIMADZU GCMS-QP 2020NX system, and matched against the National Institute of Standards and Technology (NIST) database (version 23).

The main independent variable was the disease status of the participants—categorized as either OSCC cases (n=35) or healthy controls (n=40). Disease status for OSCC patients was confirmed via histopathological examination, while healthy controls were verified to be free of systemic illness or deleterious oral habits.

Salivary VOC levels were the key exposure variables, measured through a standardized process involving un-stimulated saliva collection, ZSM-5/PDMS film-based extraction, methanol condensation, and GC–MS analysis. The GC–MS analysis produced retention times and mass spectra, which were interpreted to identify specific compounds.

Demographic variables such as age and gender were collected through clinical records and used to match controls to cases. These variables were also used in subgroup analyses.

Clinical variables included pTNM staging and histopathological grade for OSCC patients, collected from clinical pathology reports. These were used to assess associations between VOC profiles and disease severity.

Sample size calculation

Sample size estimation was done using "G*power, version 3.0.1(Franz Faul universitas, Kiel, Germany)." A sample size of 75 subjects yielded 80% power to attain significant differences, with significance level at 0.05.

Quantative variables

In this study the vaolume of slaiva collected, the concentration of VOCs, the temperature and retention time in GC- MS are some of the quantative variables which were standardized.

RESULTS

Participants

A total of 75 individuals were enrolled in this case-control observational study, comprising 35 histopathologically confirmed oral squamous cell carcinoma (OSCC) cases and 40 healthy controls. All participants met the predefined eligibility criteria. There were no dropouts or missing data.

Descriptive Data

The mean age of OSCC cases was 52.71 ± 12.75 years, and for controls, it was 47.85 ± 6.05 years. The majority of OSCC cases belonged to the 61–70 age group (34.2%), whereas most controls were within the 41–50 age group (35%). On comparing the VOC profiles between different age groups, cases revealed 3 statistically significant compounds: Ketones, organic acids, and alcohols all of which were higher in the 61-70 age group while in controls no significant changes were found. (graph 1) Among OSCC cases, females were more prevalent, in contrast to the control group, where an equal distribution of males and females was observed. 6 metabolites were established to be statistically noted ($p \leq 0.05$) in group OSCC among which 5 of them being more frequent in males than females, they were organic acids, ketones, alcohol, and amides. There were no notable differences in VOCs comparing the genders in controls (graph 2).

Clinical Characteristics of OSCC Cases

NM staging: 4 patients were in stage I, 2 in stage II, 16 in stage III, and 13 in stage IV.

Histological differentiation: 10 were well differentiated, 20 moderately differentiated, and 5 poorly differentiated.

Outcome Data: VOC Profiles

Salivary volatile organic compounds (VOCs) were analyzed using GC-MS. VOCs detected in OSCC cases were compared with healthy controls.

Ten VOCs showed statistically significant differences ($p \leq 0.05$) between cases and controls.

In OSCC cases, significantly elevated compounds included:

Butanoic acid (77.1% vs 25%, $p < 0.001$, OR: 10.125)

Pentanoic acid, 4-methyl (31.4% vs 2.5%, $p < 0.001$, OR: 17.87)

2-Propanone, 1-Hydroxy (48.6% vs 2.5%, $p < 0.001$, OR: 36.83)

In contrast, two VOCs were significantly more common in controls:

n-Tridecan-1-ol (15% vs 0%, $p = 0.017$)

Dodecane, 1-chloro (30% vs 0%, $p < 0.001$)

(Table 1 provides a detailed list of significant VOCs with corresponding p-values and odds ratios.)

VOC profiles by tumor grade

Oxalic acid, Boronic acid, and Tridecanoic acid were predominant in well-differentiated tumors.

Diethyl Phthalate and 2-Hydroxy gamma butyrolactone were present across all moderately differentiated cases.

Propanamide, 2-hydroxy was observed in both Stage I and II, while 2-Hydroxy gamma butyrolactone was confined to Stage II.

Main Results Summary

The case group showed a distinct VOC profile with multiple compounds significantly associated with OSCC status, tumor stage, age, and gender. The chi-square test confirmed statistically significant associations between several VOCs and OSCC presence.

DISCUSSION

More than 90% of all HNCs are squamous cell carcinoma that emerge from mucosal lining of the oral cavity are the most malignant neoplasm. Among them is oral cancer with a mortality rate of more than 50%[8].. Visual and screening tests which are widely used for diagnosis requires both knowledge and physician experience. Histopathological examination has remained the gold standard for diagnosing dysplastic changes causes physical impairment and faces the challenge of inaccurate sampling caused by tumor heterogeneity[9].The vulnerability to carcinogens induces accelerating accumulation of alterations in the squamous cells of the head or neck, leading to cellular oxidative stress and outflow of cancer-specific VOCs into the blood, which are flushed into the bloodstream and reach the alveolus, salivary glands, kidneys and from there they are pushed out via sweat, breath, saliva, urine, and feces[10]. Corporal mechanisms assigned to produce cancer, such as oxidative stress or the action of cytochrome P450 enzymes, seem to be responsible to produce a varied class of VOCs that could be used as non-invasive diagnostic markers[11]. VOCs are also transferred from blood to saliva mainly via passive diffusion and saliva is the natural real time pool closest to the primary carcinoma site. Several VOCs are final yields of reactive oxygen species (ROS) interactions with cardinal cellular components resulting in lipid peroxidation, protein oxidation or DNA impairment. The terminal products of these chemical reactions can serve as characteristic biomarkers for OSCC[12.]

A study By "Calenic et al[13] and Conte et al[14]" stated a phenomenon of age-allied escalation of oxidative stress, with the defense mechanisms against ROS decreasing during age advancement which is in accordance with this study, as all the significant VOCs were observed to be elevated predominantly in subjects in the age group between 61-70 years in OSCC cases.

In the present study 10 VOCs were statistically significant in OSCC patients like Organic fatty Acids (33.3%), Ketones (33.3%), Alcohol (16.6%), Alkanes (8.3%) and Acid amides (8.3%). Free fatty acids production i.e.: lipogenesis appear to be upregulated which is during carcinogenesis to pillar the tumor development and critical for the formation of cellular components which plays a pivotal role in cell signaling pathways in neoplasms[15]**.** The percentage of patients with Butanoic acid was seen to gradually increase from healthy controls to further to OSCC group in this study. Butanoic acid ($p \leq 0.001$) was seen to be highly significant amongst the acids in VOCs. Butanoic acid is a vital extracellular metabolite observed in periodontopathic bacteria, thought to play an important role in the disease progression of periodontium as it destroys gingival tissues and modulates local immunity at gingival sites[16]. It has been also reported that butanoic acid promotes the migration of normal as well as neoplastic epithelial cells[17]. In addition to Butanoic acid, Pentanoic acid-4-methyl, 3-Furanmethanol, 2-Propanone,1-hydroxy, 2-hydroxy gamma butyrolactone and 1,2-Cyclopentanedione also followed a similar trend giving these VOCs strong potential to serve as early biomarkers in OSCC. 2 - Propanone-1-hydroxy ($p \leq 0.001$) was found to be elevated amongst the group of ketones in cases. Ketone production is also linked to greater fatty acid oxidation rates, which have been reported in malignancies[18]. Ketones function as chemo attractants which enhances the migration and growth of cancer cells promoting the growth of the primary tumor[19]. Tumor cells also exhibit varied glucose metabolism called as the "Warburg effect" in which their energy production shifts from the Krebs cycle to glycolysis hence explaining the appearance of ketonic VOCs in this study. However, as the concentration of ketone in human fluids or breath always fluctuates with certain physiological activities such as fasting, exercising, and eating, some experts advise against using ketone as a biomarker[20]. 2 Alcohols were found to be raised in OSCC group which is thought to be attributed to the cytochrome p450 enzymatic action[21]. Acid amides were also found to be noticeably ($p \leq 0.03$) present in OSCC group thereby, however there is lack of any previous literature to validate this finding. Finally, 10 VOCs were found to be statistically significant on comparison between OSCC and healthy controls and butanoic acid, Pentanoic acid, 4-methyl was the most significant VOC seen ($p \leq 0.001$) in OSCC patients. The results of this analysis were in line with study done by Raman, et al (2013)**[22].**

Limitations of the study

This study had some potential sources of bias as explained. *Selection Bias:*Participants were selected from an institutional setting, which may not represent the general population. Also, although age and gender matching was attempted, other confounding factors (like nutritional status or undiagnosed comorbidities) could influence salivary VOC profiles and were not controlled for.

Information Bias (Measurement Bias): The collection and analysis of VOCs rely heavily on the accuracy and consistency of GC–MS protocols. Any variations in saliva collection time, storage conditions, or handling could influence the VOC composition. Additionally, manual preparation of the ZSM-5/PDMS films introduces room for procedural variability.

CONCLUSION

The current study proposed salivary VOC profiling of OSCC and its comparison with healthy controls can initiate discovery of clinically useful biomarkers which has the prospect to be utilized as a diagnostic, prognostic, and remedial response marker. This study was conducted for the purpose of standardization process to evaluate the potential of VOCs as a marker using GCMS analysis. These VOCs might constitute tumor specific candidate biomarkers for oral cancer. However, further studies with large sample size should be initiated to establish protocols for its usage in a clinical setting in near future.

Regulations and Ethics

Ethics approval and patient consent to participate – obtained from IRB

Patient Consent for publication - obtained

Animal Studies - NA

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