Short Communications

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Impact of Tobacco Consumption on Salivary Protein Profiles: A MALDI-TOF Mass Spectrometry Study

Manish S, Ramya Ramadoss*, Nitya Krishnasamy, Sandhya S, Suganya Panneer Selvam, Hemashree K

Abstract

Objective: To investigate the impact of tobacco consumption on salivary protein profiles and identify proteomic alterations associated with tobacco use, using advanced mass spectrometry techniques. **Methods:** This cross-sectional study involved 100 adults aged 18–60, divided into two groups: 50 tobacco users (smokers and smokeless users) and 50 non-users. Participants were selected based on strict inclusion criteria, excluding individuals with systemic diseases, ongoing medications, or cancer history to minimize confounding factors. Unstimulated saliva samples were collected under standardized conditions, centrifuged, and stored at -80°C. Proteins were extracted using trichloroacetic acid (TCA) precipitation, quantified via Bradford assay, and analyzed with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Bioinformatics tools, including Cytoscape and heatmap analysis, were utilized to identify protein interaction networks and significant proteomic alterations. Statistical significance was set at $p < 0.05$. **Results:** Tobacco users exhibited significant proteomic alterations compared to non-users, with marked upregulation of inflammatory proteins such as TRML1, MAFB ($p < 0.05$), and NINJ1, indicating chronic inflammation. Oxidative stress markers, including mitochondrial proteins NU4M and GSAS1, were significantly elevated ($p < 0.01$), reflecting tobacco-induced cellular stress. DNA repair proteins like O6C70 and PP4RL showed increased expression ($p < 0.05$), suggesting an adaptive response to smoking-induced DNA damage. Cancer-related proteins TWST1 and CD82 were upregulated ($p < 0.05$), highlighting processes linked to carcinogenesis, such as epithelial-mesenchymal transition (EMT). Cytoscape analysis revealed highly interconnected protein networks in smokers, with central nodes involving JUNB, EGR1, and FOSB, indicating systemic biological disruptions. In contrast, non-smokers displayed modular and compartmentalized networks reflecting stable cellular processes. Heatmap analysis further emphasized significant differences in protein expression patterns, with smokers showing elevated levels of stress-related proteins while non-smokers exhibited a balanced proteomic profile associated with immune homeostasis and metabolic regulation. **Conclusion:** Tobacco consumption significantly alters salivary protein profiles, promoting chronic inflammation, oxidative stress, and DNA repair dysregulation, all of which may contribute to oral carcinogenesis. These findings emphasize the utility of MALDI-TOF MS in detecting salivary biomarkers for early diagnosis of tobacco-related oral diseases and provide insights into the molecular pathways disrupted by tobacco use.

Keywords: Salivary proteomics- tobacco-induced inflammation- MALDI-TOF mass spectrometry

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Introduction

Tobacco use is known to significantly alter the oral environment, with direct effects on the composition and functionality of saliva [1]. Saliva, being a biofluid that plays a key role in maintaining oral health, serves as an excellent medium for assessing systemic and localized biochemical changes [2, 3].

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is an advanced analytical technique widely used for proteomic analysis due to its high sensitivity, accuracy, and speed. MALDI-TOF MS combines matrix-assisted laser desorption/ionization (MALDI) with time-of-flight (TOF) mass spectrometry to analyze and identify biomolecules, particularly proteins, peptides, and other macromolecules, based on their mass-to-charge (m/z) ratio. In this technique, the sample is mixed with a matrix compound, co-crystallized, and then irradiated with a laser. The matrix absorbs the laser energy, facilitating the ionization of the sample without significant fragmentation. The ions are then accelerated into a TOF analyzer, where their flight times are measured to determine their m/z ratio.

The sensitivity and precision of MALDI-TOF MS allow for the detection of various protein changes, potentially shedding light on the biological mechanisms

*Department of Oral Biology, Saveetha Dental College and Hopsitals, Chennai, India. *For Correspondence: drramya268@gmail.com*

influenced by tobacco and offering biomarkers for the early detection of oral pathologies [4, 5]. MALDI-TOF MS is particularly suitable for salivary proteomic profiling because it requires minimal sample preparation, accommodates complex biological samples, and enables high-throughput analysis.

In tobacco users, significant alterations in the salivary proteome have been documented. Key proteins such as pro-inflammatory cytokines, antioxidants, and antimicrobial peptides are often found to be deregulated. Proteins like albumin, lactoferrin, and immunoglobulins, which play vital roles in maintaining oral immunity and tissue repair, tend to show reduced expression [6]. Concurrently, tobacco exposure increases the levels of pro-inflammatory proteins like interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), promoting a pro-inflammatory environment. Salivary enzymes such as matrix metalloproteinases (MMPs) also tend to be elevated, which is linked to tissue degradation and potential tumor progression in the oral cavity [6, 7].

The changes in the salivary proteomic profile have significant implications for the development of oral diseases, particularly oral cancer, periodontal disease, and other tobacco-related pathologies [8]. The upregulation of inflammatory and degradative proteins suggests an ongoing state of oxidative stress and tissue damage, which may contribute to carcinogenesis or the progression of pre-existing conditions. Furthermore, reduced levels of protective proteins impair the mouth's natural defenses, leaving it more susceptible to infections and chronic inflammation, which are known risk factors for oral cancer and periodontal disease [9].

Utilizing MALDI-TOF MS for proteomic profiling in tobacco users has a broader significance beyond the identification of individual protein changes. This technique not only aids in the discovery of biomarkers for early diagnosis but also provides insights into the molecular pathways disrupted by tobacco use. Understanding these changes at the proteomic level could lead to the development of targeted interventions aimed at mitigating the harmful effects of tobacco on oral health. Moreover, MALDI-TOF MS stands out as a non-invasive, high-throughput tool that can monitor disease progression and response to treatment in tobacco-related conditions, thereby paving the way for personalized therapeutic approaches.

Materials and Methods

Study Design and Participants

This study involved two distinct groups: tobacco users and non-tobacco users (control group), with each group comprising at least 50 participants to ensure statistical relevance. The inclusion criteria focused on adults aged 18–60 with a history of regular tobacco use (smoking or smokeless) for a minimum of one year for the experimental group, while non-users served as controls. Individuals with systemic diseases, ongoing medication affecting saliva, or a history of cancer were excluded to eliminate confounding factors. All participants provided informed consent, and the study was conducted with ethics committee approval.

Saliva Collection and Protein Extraction

Unstimulated whole saliva samples were collected from participants after ensuring they had refrained from eating, drinking, or smoking for at least an hour prior to collection. The saliva was gathered over 5–10 minutes by spitting into sterile tubes. To maintain sample integrity, saliva was placed on ice and processed within 30 minutes. After centrifugation to remove debris, the supernatant was aliquoted and stored at -80°C. Protein extraction from the saliva samples was performed using standard techniques, such as trichloroacetic acid (TCA) precipitation. Protein concentrations were determined using the Bradford or BCA assay to standardize protein input for the proteomic analysis [6].

MALDI-TOF MS Analysis

The extracted proteins were subjected to MALDI-TOF mass spectrometry. A matrix, such as α-Cyano-4-hydroxycinnamic acid (CHCA), was used for co-crystallization with the protein samples. Each sample was spotted onto the MALDI plate, and the resulting spectra were generated in the mass range of 500–20,000 Da, depending on the matrix and instrument settings. MALDI-TOF MS (Bruker Daltonics UltrafleXtreme MALDI TOF-TOF) allowed for the identification of protein peaks, revealing changes in the salivary proteome between tobacco users and non-users. The spectra from each group were compared to detect significant differences in protein expression, with data acquired in triplicate to ensure accuracy [9, 10].

Results

String Data

The salivary protein profile of smokers shows key biological disruptions related to chronic inflammation, immune dysregulation, oxidative stress, and DNA damage. Proteins such as TRML1, MAFB, and NINJ1 point to an inflammatory state, while proteins like O6C70 and PP4RL suggest activation of DNA repair mechanisms in response to smoking-induced damage. Additionally, mitochondrial stress is indicated by proteins like NU4M and GSAS1, signaling oxidative damage. Cancer-related proteins, including TWST1, CD82, and PTTG, suggest involvement in processes like epithelial-mesenchymal transition (EMT) and tumor suppression, underscoring an increased risk of oral cancer in smokers. Collectively, these proteins highlight smoking's harmful effects on inflammation, oxidative stress, DNA repair, and carcinogenesis pathways (Figure 1).

In contrast, non-smokers' saliva reflects normal biological functions and immune homeostasis. Proteins such as UFM1, EBPL, and UT1 indicate regular metabolic activity, while MCAF1 and DMTF1 suggest intact cell cycle control and genomic integrity. Immune-related proteins like CXL17, CF122, and CD320 point to a well-regulated immune system without signs of chronic inflammation. Proteins like LRRN3 and DYLT1 are involved in normal neural and tissue maintenance,

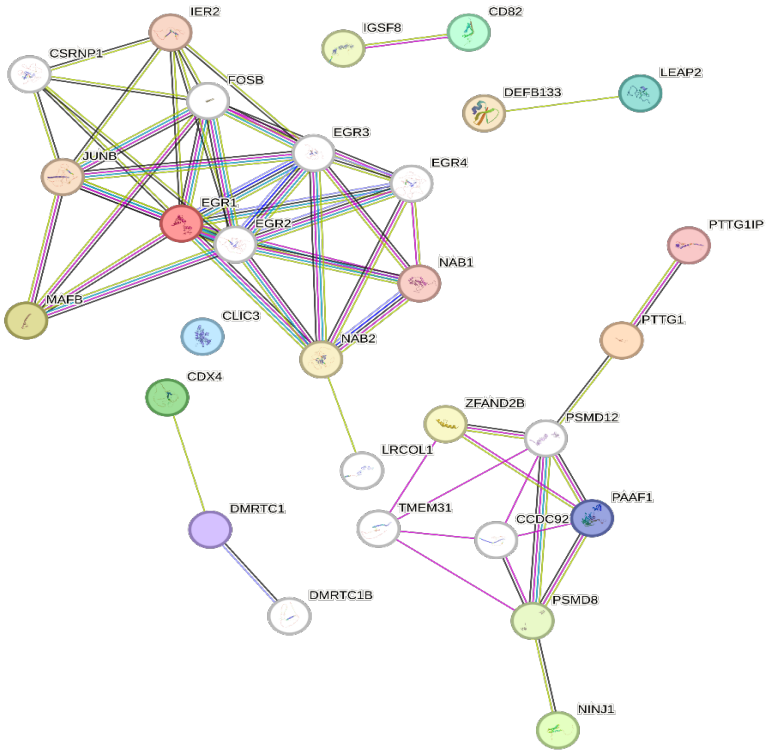

Figure 1. String Analysis of Salivary Proteins in Smokers Showing Inflammatory, Oxidative Stress, DNA Repair, and Cancer Pathway Alterations

with no markers of oxidative stress, DNA damage, or cancer-related changes, indicating an overall stable biological state (Figure 2).

Cytoscape Analysis

The Cytoscape network for smokers demonstrates a more complex and interconnected structure. Transcription factors like JUNB, EGRs, and FOSB play central roles, indicating widespread interactions linked to inflammatory and stress responses, likely induced by exposure to smoke-related toxins. Clusters around PTTG1 and PSM family proteins show increased connectivity, suggesting alterations in proteasome activity and cell cycle regulation. Additionally, more extensive cross-talk between protein clusters suggests that smoking induces widespread and systemic effects on protein interactions and biological processes (Figure 3).

In non-smokers, the Cytoscape network reveals

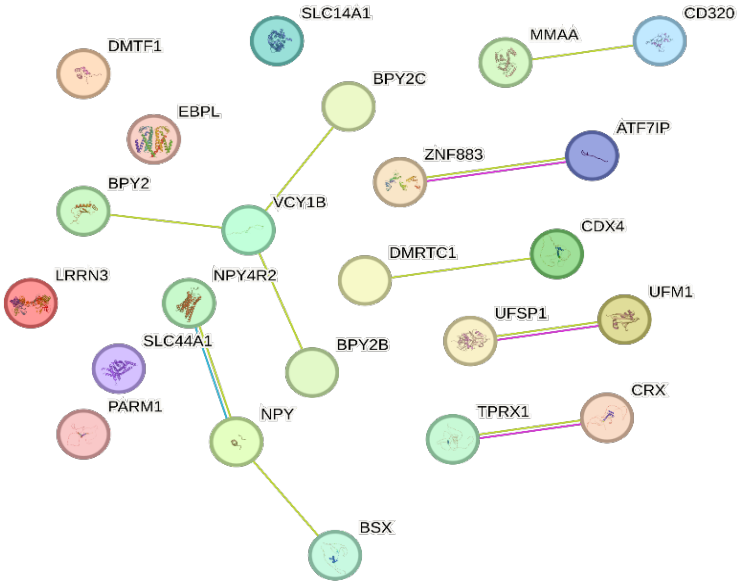

Figure 2. String Analysis of Salivary Proteins in Non-Smokers Indicating Normal Metabolic, Immune, and Neural Function

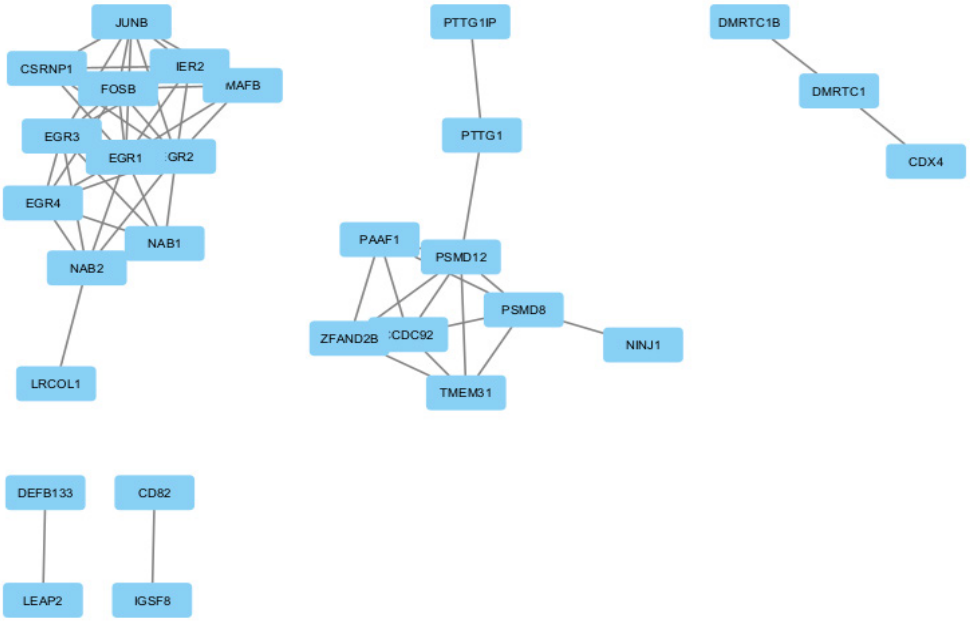

Figure 3. Cytoscape Network of Salivary Proteins in Smokers Highlighting Altered Inflammatory and Stress-Response Pathways

distinct, compartmentalized clusters, such as those involving BPY2 family members, UFM1, and CDX4. These clusters suggest the maintenance of regulatory networks without significant external influence, such as smoking. Fewer interconnections between clusters indicate a modular and stable interaction network, reflective of normal biological function without the systemic stress seen in smokers (Figure 4).

Heatmap Analysis

The heatmap for smokers shows elevated expression levels of proteins involved in key biological processes like inflammation (TRML1, MAFB), DNA repair (O6C70, PP4RL), oxidative stress (NU4M, GSAS1), and cancer-related pathways (TWST1, CD82). The color gradient, shifting from blue (low expression) to red (high expression), highlights the upregulation of these proteins, emphasizing the impact of smoking on cellular stress and damage (Figure 5).

In non-smokers, the heatmap demonstrates balanced expression levels of proteins involved in cellular and metabolic functions (UFM1, EBPL), immune homeostasis (CXL17, CD320), and cell cycle regulation (MCAF1, DMTF1). The absence of significant protein upregulation reflects a stable biological environment, devoid of smoking-related stress or damage (Figure 6).

Discussion

This study provides a comprehensive analysis of the

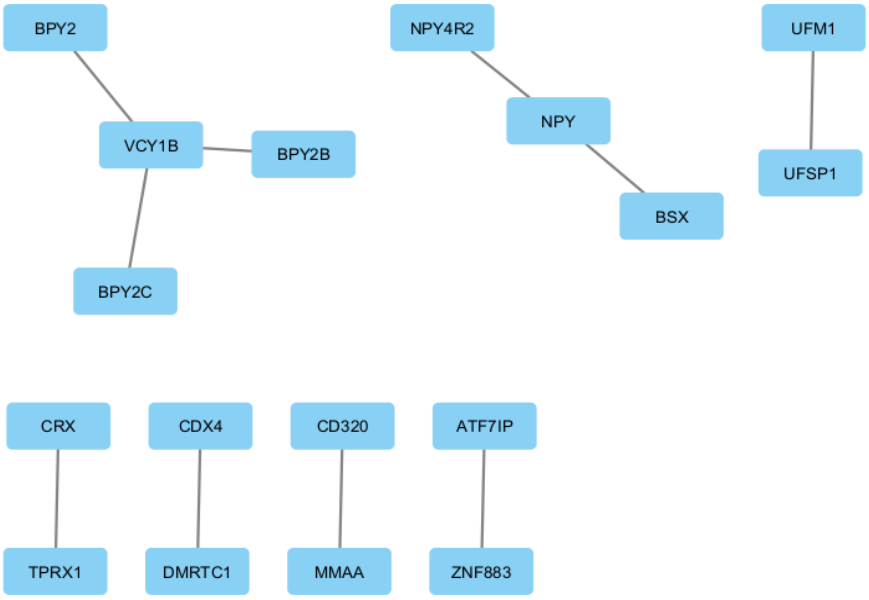

Figure 4. Cytoscape Network of Salivary Proteins in Non-Smokers Indicating Stable and Modular Protein Interactions

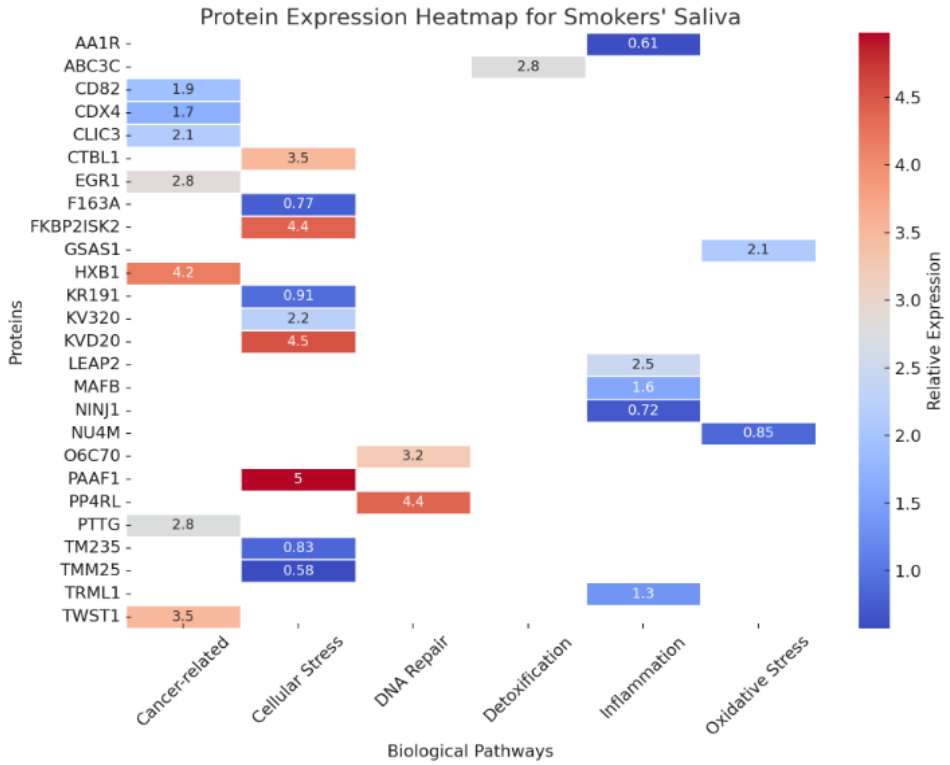

Figure 5. Heatmap of Protein Expression in Smokers' Saliva Highlighting Inflammatory, Oxidative, and Cancer Pathway Alterations

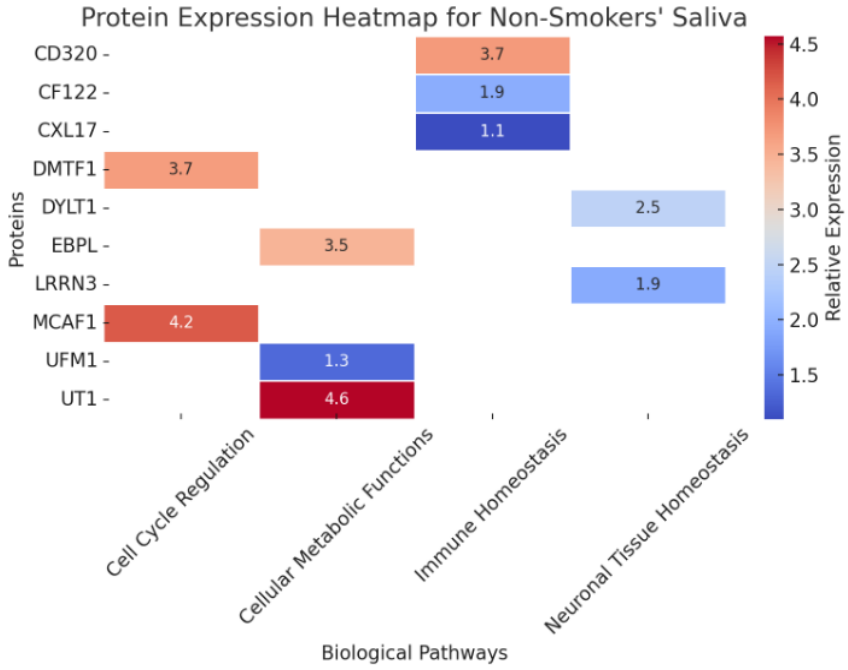

Figure 6. Heatmap of Protein Expression in Non-Smokers' Saliva Indicating Normal Metabolic and Immune Functions

salivary proteomic alterations in tobacco users, utilizing MALDI-TOF MS to detect significant molecular changes. Tobacco smoking is well-known to contribute to various oral pathologies, including cancer, periodontal disease, and chronic inflammation [11]. The proteomic shifts identified in smokers' saliva reflect the extensive impact of tobacco exposure on biological processes such as immune regulation, oxidative stress, inflammation, and DNA repair.

The upregulation of inflammatory proteins like TRML1, MAFB, and NINJ1 in smokers' saliva is consistent with previous studies linking smoking to a pro-inflammatory state in the oral cavity. These proteins, involved in immune response activation, suggest that chronic tobacco exposure triggers immune pathways that contribute to tissue damage and disease progression.

Elevated levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, reported in earlier studies, further indicate that smoking induces sustained inflammation, a precursor to carcinogenesis [12,13].

Increased expression of oxidative stress-related proteins, such as NU4M and GSAS1, highlights the oxidative damage induced by smoking. This observation aligns with existing literature on the role of reactive oxygen species (ROS) in cellular stress and tissue degradation [14]. The elevated levels of mitochondrial-associated proteins support the idea that smoking disrupts cellular energy balance, leading to oxidative stress and damage to oral tissues [15].

Furthermore, the presence of DNA repair proteins, including O6C70 and PP4RL, indicates an active response to tobacco-induced DNA damage. Studies have shown that tobacco toxins, such as nicotine and polycyclic aromatic hydrocarbons, are significant sources of DNA damage, resulting in the formation of DNA adducts and necessitating upregulation of DNA repair mechanisms. The upregulation of these proteins points to the oral epithelium's response to mitigate damage, yet the chronic exposure to tobacco toxins likely overwhelms these repair pathways, contributing to a heightened cancer risk [16].

Additionally, the identification of proteins such as TWST1, CD82, and PTTG, which are associated with epithelial-mesenchymal transition (EMT), tumor suppression, and cell cycle regulation, suggests that tobacco exposure may drive cellular dysregulation. Studies have highlighted the role of CD82, a metastasis suppressor, in cancer, and its altered expression in the saliva of smokers could indicate impaired tumor suppression due to smoking. The proteomic profile of non-smokers, by contrast, reflected homeostatic balance, with proteins involved in metabolic activity and immune regulation expressed at normal levels. The absence of oxidative stress markers and DNA repair proteins in non-smokers underscores the detrimental impact of tobacco on oral biology [17, 18].

The use of Cytoscape and heatmap analyses further validated these conclusions, demonstrating more complex protein interaction networks in smokers. Proteins like JUNB, FOSB, and EGR, known for their roles in stress response and inflammation, were more interconnected in smokers, suggesting that tobacco induces systemic changes affecting multiple pathways [13,14]. These findings align with previous studies showing that chronic tobacco exposure disrupts signaling networks, leading to widespread effects on oral and systemic health.

In summary, this study underscores the importance of salivary proteomic analysis in understanding the molecular changes induced by tobacco use. The detection of specific proteins related to inflammation, oxidative stress, DNA repair, and cellular regulation in smokers' saliva points to significant disruptions in biological processes critical to oral health. By identifying these proteomic alterations, the study offers potential biomarkers for the early diagnosis of tobacco-related oral diseases and highlights the utility of MALDI-TOF MS in uncovering molecular signatures that may not be detectable through conventional methods

Limitations and future perspective

The limitations of this study include a relatively small and homogenous sample size, which limits the generalizability of the findings across diverse populations and smoking behaviors. Additionally, the influence of confounding factors such as diet, oral hygiene, and existing health conditions was not fully controlled, potentially affecting the salivary proteomic profile. MALDI-TOF MS, while powerful, may have limited sensitivity for detecting low-abundance proteins compared to more advanced methods like LC-MS/MS. Future research should focus on larger, more diverse cohorts, consider longitudinal designs to track changes over time, and integrate multiple proteomic techniques to capture a broader spectrum of protein alterations. The application of bioinformatics and machine learning for biomarker validation and personalized therapeutic strategies could also enhance the clinical relevance of salivary proteomics, offering promising avenues for early detection and targeted treatment of tobacco-related oral diseases.

In conclusion, this study highlights the profound impact of tobacco use on the salivary proteome, revealing significant alterations in proteins related to inflammation, oxidative stress, DNA repair, and cancer pathways. By employing MALDI-TOF MS, we identified key proteins such as TRML1, MAFB, and CD82, which are deregulated in smokers, offering potential biomarkers for early detection of tobacco-related oral pathologies. The findings underscore the utility of salivary proteomics as a non-invasive tool for monitoring the harmful effects of tobacco and guiding personalized interventions.

While the study contributes valuable insights into the molecular mechanisms underlying tobacco-induced oral diseases, future research should address its limitations by expanding the sample population, incorporating other proteomic techniques, and controlling for confounding variables. The growing application of proteomic technologies in clinical diagnostics and personalized medicine presents exciting opportunities for early detection, targeted treatment, and monitoring of disease progression in tobacco users, ultimately advancing oral health care for at-risk populations.

Author Contribution Statement

Manish S- analysis, data collection. Dr. Ramya Ramadoss- manuscript review, conceptualization, formal analysis. Dr. Nitya. K- manuscript writing, soft ware. Sandhya Sundar- manuscript review. Dr. Suganya Panneer Selvam-manuscript review. Dr. Hema Shree. K.- software, resources, review.

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Approval

This study was approved by the Institutional Review

Board of Saveetha Dental College, ensuring compliance with ethical standards and guidelines.

References

1. Balasubramaniam A, Arumugham MI. Salivary oxidative stress level among tobacco chewers and smokers: A comparative Study. J Adv Pharm Technol Res. 2022;13(Suppl 1):S21-S25. https://doi.org/10.4103/japtr.japtr_116_22.
2. Ravikumar D, Ramani P, Gayathri R, Hemashree K, Prabhakaran P. Physical and chemical properties of saliva and its role in Early Childhood caries - A systematic review and meta-analysis. J Oral Biol Craniofac Res. 2023;13(5):527-538. https://doi.org/10.1016/j.jobcr.2023.05.011.
3. Suresh C, Veeraraghavan V, Jayaraman S, Gayathri R, Kavitha S. Awareness about the significance of acid– base balance of saliva in maintaining oral health. J Adv Pharm Technol Res. 2022;13(Suppl 1):S325-9. https://doi.org/10.4103/japtr.japtr_402_22
4. Petrušić N, Posavac M, Sabol I, Mravak-Stipetić M. The Effect of Tobacco Smoking on Salivation. Acta Stomatol Croat. 2015;49(4):309-15. https://doi.org/10.15644/asc49/4/6.
5. Ploypetch S, Roytrakul S, Jaresitthikunchai J, Phaonakrop N, Krobthong S, Suriyaphol G. Salivary proteomics of canine oral tumors using MALDI-TOF mass spectrometry and LC-tandem mass spectrometry. PLoS One. 2019;14(7):e0219390. https://doi.org/10.1371/journal.pone.0219390.
6. K HS, R G, Ramani P, Veeraraghavan VP. Longitudinal study on salivary IL-6 trajectories in postoperative OSCC patients after chemotherapy and radiotherapy. J Stomatol Oral Maxillofac Surg. 2024;125(4S):101909. https://doi.org/10.1016/j.jormas.2024.101909.
7. Lisetska IS. The influence of smoking on the biochemical indicators of oral fluid in teenagers and young adults who smoke. Ukr J Perinatol Pediatr. 2022;37-41. https://doi.org/10.15574/pp.2022.92.37.
8. Hsiao YC, Lin SY, Chien KY, Chen SF, Wu CC, Chang YT, et al. An immuno-MALDI mass spectrometry assay for the oral cancer biomarker, matrix metalloproteinase-1, in dried saliva spot samples. Anal Chim Acta. 2020;1100:118-130. 10.1016/j.aca.2019.12.006
9. Lazari LC, Zerbinati RM, Rosa-Fernandes L, Santiago VF, Rosa KF, Angeli CB, et al. MALDI-TOF mass spectrometry of saliva samples as a prognostic tool for COVID-19. J Oral Microbiol. 2022;14(1):2043651. https://doi.org/10.1080/20002297.2022.2043651.
10. Wang H, Chen H, Fu Y, Liu M, Zhang J, Han S, et al. Effects of Smoking on Inflammatory-Related Cytokine Levels in Human Serum. Molecules. 2022;27(12):3715. https://doi.org/10.3390/molecules27123715.
11. Szabo YZ, Slavish DC. Measuring salivary markers of inflammation in health research: A review of methodological considerations and best practices. Psychoneuroendocrinology. 2021;124:105069. https://doi.org/10.1016/j.psyneuen.2020.105069.
12. Lee KT, Guo ZL, Teng NC, Hsu KC, Chen IH, Lee CY, et al. Salivary Pro-Inflammatory Markers and Smoking Status Influences the Treatment Effectiveness of Periodontal Disease Patients with Hypertension. Int J Environ Res Public Health. 2021;18(14):7364. https://doi.org/10.3390/ijerph18147364.
13. Yang Z, Harrison CM, Chuang GC, Ballinger SW. The role of tobacco smoke induced mitochondrial damage in vascular dysfunction and atherosclerosis. Mutat Res. 2007;621(1-2):61-74. https://doi.org/10.1016/j.mrfmmm.2007.02.010.
14. Khariwala SS, Ma B, Ruszczak C, Carmella SG, Lindgren B, Hatsukami DK, et al. High Level of Tobacco Carcinogen-Derived DNA Damage in Oral Cells Is an Independent Predictor of Oral/Head and Neck Cancer Risk in Smokers. Cancer Prev Res (Phila). 2017;10(9):507-513. https://doi.org/10.1158/1940-6207.CAPR-17-0140.
15. Tang MS, Lee HW, Weng MW, Wang HT, Hu Y, Chen LC, et al. DNA damage, DNA repair and carcinogenicity: Tobacco smoke versus electronic cigarette aerosol. Mutat Res Rev Mutat Res. 2022;789:108409. https://doi.org/10.1016/j.mrrev.2021.108409.
16. Reddi KK, Zhang W, Shahrabi-Farahani S, Anderson KM, Liu M, Kakhniashvili D, et al. Tetraspanin CD82 Correlates with and May Regulate S100A7 Expression in Oral Cancer. Int J Mol Sci. 2024;25(5):2659. https://doi.org/10.3390/ijms25052659.
17. Vu T, Jin L, Datta PK. Effect of Cigarette Smoking on Epithelial to Mesenchymal Transition (EMT) in Lung Cancer. J Clin Med. 2016;5(4):44. https://doi.org/10.3390/jcm5040044.