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In Silico Prediction of Salivary Biomarkers Associated with Nicotine Metabolism

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ABSTRACT

Background: Nicotine metabolism varies significantly among individuals and influences addiction risk, treatment response, and disease susceptibility. Current biomarkers for nicotine exposure rely primarily on blood or urine samples, limiting their utility in large-scale or point-of-care applications. Saliva offers a non-invasive alternative, but protein-based salivary biomarkers for nicotine metabolism remain underexplored.

Objective: To identify candidate salivary biomarkers associated with nicotine metabolism using a stepwise in silico bioinformatics approach integrating gene function, secretion potential, salivary expression, protein interaction networks, and miRNA regulation.

Methods: Genes involved in nicotine metabolism were curated from public databases (KEGG, CTD, GeneCards). Enrichment analysis was performed to identify relevant biological processes and pathways. Candidate proteins were filtered based on secretion potential (SignalP, SecretomeP), salivary expression (SalivaDB, Human Protein Atlas), and protein-protein interaction centrality (STRING, Cytoscape). miRNA regulators were predicted using TargetScan and miRTarBase.

Results: Three high-confidence candidate proteins (GSTP1, FMO3, and UGT2B10) were identified as biologically relevant, secreted, and experimentally detected in saliva. Protein-protein interaction analysis highlighted GSTP1 and UGT2B10 as central nodes. Several regulatory miRNAs, including hsa-miR-155 and hsa-miR-27b, were predicted or validated to target these proteins, adding a regulatory dimension to biomarker selection.

Conclusion: This study proposes a set of salivary proteins and miRNAs as potential non-invasive biomarkers for nicotine metabolism. The findings provide a strong basis for experimental validation and highlight the utility of bioinformatics in accelerating biomarker discovery for personalized and public health applications

Keywords: *Nicotine metabolism, salivary biomarkers, bioinformatics, in silico, miRNA*

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1. INTRODUCTION

Nicotine is the principal addictive compound in tobacco, and its metabolism significantly influences individual susceptibility to tobacco-related diseases, dependence levels, and responsiveness to cessation therapies [1,2]. Once absorbed into the body, nicotine undergoes complex metabolic processes primarily in the liver, involving a range of enzymes such as cytochrome P450s (notably CYP2A6), flavin-containing monooxygenases, and UDP-glucuronosyltransferases [3,4]. The metabolic byproducts, particularly cotinine and trans-3′-hydroxycotinine, serve as reliable indicators of nicotine exposure and metabolism rate [5]. However, most current methods for monitoring these metabolites rely on blood or urine samples, which, although effective, are invasive and can be impractical for large-scale screening or continuous monitoring [6].

In recent years, saliva has emerged as a promising non-invasive biofluid for diagnostic purposes. It is easily accessible, collection is minimally invasive, and its composition reflects various physiological and pathological states [7]. Saliva contains a diverse array of biomolecules, including proteins, peptides, nucleic acids, and metabolites, many of which are derived from blood or produced locally by salivary glands. The potential of salivary diagnostics has been explored in various contexts, from infectious diseases to cancer and metabolic disorders [8,9]. However, the discovery and validation of specific salivary biomarkers that accurately reflect nicotine metabolism remain underexplored.

In the era of computational biology, in silico approaches have become powerful tools for biomarker discovery. These methods leverage vast amounts of biological data and predictive algorithms to identify candidate molecules that can be experimentally validated, significantly reducing the time and cost of early-stage research. Particularly for salivary biomarkers, integrating data from public gene and protein databases with secretome prediction tools and salivary expression profiles can yield valuable insights into which nicotine-related molecules are likely to be present in saliva [10,11].

This study aims to systematically identify potential salivary biomarkers associated with nicotine metabolism using an in silico bioinformatics pipeline. By combining gene pathway analysis, protein secretion prediction, and salivary proteome cross-referencing, we seek to generate a list of candidate biomarkers that can be used for future experimental validation. This work not only contributes to the foundational knowledge of nicotine metabolism but also supports the development of non-invasive, saliva-based diagnostics for personalized health monitoring and tobacco control programs.

2. LITERATURE REVIEW

Nicotine is a psychoactive alkaloid found predominantly in tobacco products, and it serves as the primary agent responsible for tobacco addiction. Once absorbed, nicotine undergoes extensive metabolism, primarily in the liver, through enzymatic reactions involving cytochrome P450 enzymes, most notably CYP2A6. The major metabolites, including cotinine and trans-3′-hydroxycotinine, are widely used in clinical and forensic settings to assess nicotine exposure [3,4]. However, the rate of nicotine metabolism varies significantly across individuals due to genetic polymorphisms, environmental influences, and co-exposures. These variations impact not only addiction potential but also the efficacy of nicotine replacement therapies and the risk of developing smoking-related diseases such as cancer, cardiovascular disease, and chronic obstructive pulmonary disease (COPD) [5]. Understanding this interindividual variability has become a key focus in the development of personalized interventions for tobacco use disorder.

Traditionally, nicotine and its metabolites have been measured in biological fluids such as blood, plasma, or urine. While these methods are well-established and analytically robust, they are often invasive, require trained personnel for sample collection, and may not be suitable for frequent or point-of-care testing [6]. In recent years, saliva has emerged as a viable alternative matrix for nicotine biomonitoring. Saliva offers several advantages, it is easy to collect non-invasively, requires minimal processing, and reflects the free (unbound) fraction of nicotine, which is pharmacologically active [7,8]. Moreover, nicotine enters saliva by passive diffusion, making salivary nicotine concentrations closely correlated with plasma levels. These attributes make saliva particularly attractive for large-scale epidemiological studies, community-based screenings, or real-time exposure monitoring in clinical and occupational settings [9,11].

Beyond measuring nicotine and its metabolites, there is growing interest in identifying salivary proteins and regulatory molecules that may serve as biomarkers for nicotine metabolism or its systemic effects. Saliva contains a rich array of biomolecules, including enzymes, cytokines, growth factors, and microRNAs, many of which are actively secreted by salivary glands or derived from plasma transudation [10]. Several studies have shown that smoking alters the composition of the salivary proteome, with effects observed in oxidative stress markers, detoxification enzymes, and inflammatory mediators. However, few studies have specifically focused on host-derived proteins that reflect an individual's metabolic processing of nicotine. Most existing salivary biomarker studies emphasize exposure assessment, rather than metabolism profiling or functional stratification, leaving a gap in the field [7-11].

Recent advances in bioinformatics, saliva proteomics, and microRNA profiling have opened new possibilities for salivary biomarker discovery. By leveraging computational tools and public databases, researchers can now predict and prioritize salivary biomarkers based on secretion signals, protein interaction networks, tissue expression profiles, and regulatory pathways. These tools are particularly valuable in identifying candidate markers before undertaking costly and time-consuming experimental validation. This paradigm shift, from solely empirical discovery to computational pre-screening followed by focused validation, has the potential to accelerate the development of diagnostic tools for nicotine metabolism and beyond. Despite this promise, the current literature remains limited, highlighting the need for integrative studies that bridge bioinformatics predictions with salivary diagnostics for nicotine-related applications.

3. METHODS AND RESULTS

This study employed a stepwise in silico bioinformatics pipeline to identify potential salivary biomarkers associated with nicotine metabolism. Each analytical stage, ranging from gene mining and pathway enrichment to secretion prediction, salivary expression filtering, network analysis, and regulatory element prediction, was designed to progressively refine a list of candidate proteins based on both biological relevance and salivary detectability. Given the computational nature of this research, and to maintain clarity and coherence, the methods and their corresponding results are presented together in each subsection. This integrative approach allows for immediate interpretation of analytical outcomes, facilitates logical progression through the pipeline, and reflects standard practice in bioinformatics studies.

3.1. Data Collection

To initiate the biomarker discovery process, we first compiled a comprehensive list of genes known to be involved in nicotine metabolism. This was accomplished through systematic searches of several publicly available biological databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG) [12], the Comparative Toxicogenomics Database (CTD) [13], and GeneCards [14]. The keyword "nicotine metabolism" was used in combination with filters for human-specific pathways, enzymes, and associated genes. KEGG pathway maps such as "Drug metabolism – cytochrome P450" and "Metabolism of xenobiotics by cytochrome P450" were particularly useful for identifying enzymatic components of nicotine biotransformation, including CYP2A6, FMO3, UGT1A9, and other relevant genes.

The search strategy emphasized genes with established roles in phase I and phase II metabolism of nicotine, as well as those associated with nicotine response, transport, or regulation. To ensure biological relevance and minimize false positives, we included only genes with documented experimental evidence in the context of nicotine exposure or metabolism in human tissues. In addition to KEGG and CTD, GeneCards provided integrated functional annotations, including protein expression, disease relevance, and subcellular localization, which aided in prioritizing genes with potential diagnostic value.

Each identified gene was documented along with its official symbol, full name, primary function, and source database. This initial list formed the foundation for downstream bioinformatics analyses. The curated dataset of nicotine metabolism-related genes is summarized in Table 1, which provides an overview of their known biological roles and database origins. These genes were then subjected to enrichment analysis and secretion prediction in subsequent steps.

Table 1. Nicotine Metabolism-Related Genes [12-14]

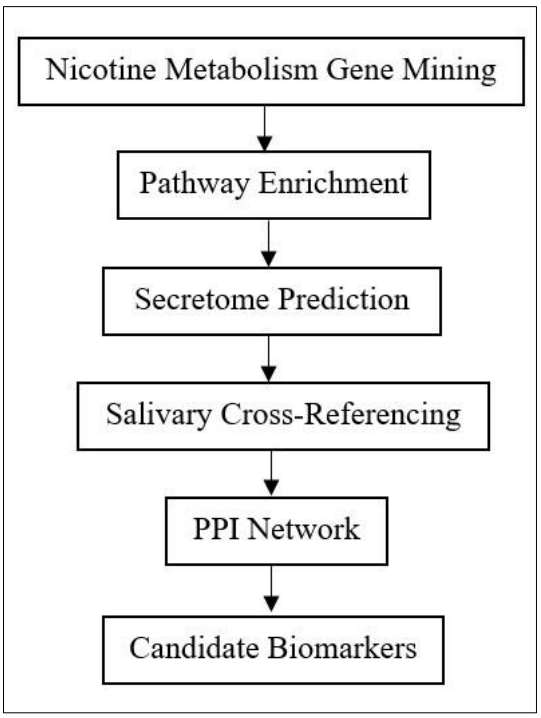
Gene Symbol	Full Name	Function	Source Database(s)
CYP2A6	Cytochrome P450 Family 2 Subfamily A Member 6	Primary enzyme responsible for nicotine C-oxidation	KEGG, GeneCards
FMO3	Flavin Containing Monooxygenase 3	Catalyzes N-oxidation of nicotine metabolites	GeneCards
UGT2B10	UDP Glucuronosyltransferase Family 2 Member B10	Involved in glucuronidation of nicotine-derived compounds	CTD, KEGG
CYP2B6	Cytochrome P450 Family 2 Subfamily B Member 6	Alternative pathway for nicotine metabolism	KEGG
NR1I2	Nuclear Receptor Subfamily 1 Group I Member 2	Regulates expression of detoxification enzymes	CTD, GeneCards
GSTP1	Glutathione S-Transferase Pi 1	Conjugates nicotine metabolites with glutathione	CTD
ALDH2	Aldehyde Dehydrogenase 2 Family Member	Oxidizes nicotine aldehydes	GeneCards
NAT2	N-Acetyltransferase 2	Catalyzes acetylation of arylamine and hydrazine drugs	GeneCards
CES1	Carboxylesterase 1	Hydrolyzes ester-containing nicotine derivatives	KEGG
CYP1A2	Cytochrome P450 Family 1 Subfamily A Member 2	Involved in metabolism of aromatic compounds	GeneCards

Table 1 presents a curated list of genes that play key roles in the metabolic processing of nicotine in humans. These genes were identified through comprehensive searches across multiple biological databases, including KEGG, CTD, and GeneCards, with selection criteria based on their known enzymatic or regulatory involvement in nicotine biotransformation. The list includes major phase I enzymes such as CYP2A6 and CYP2B6, which catalyze the oxidative metabolism of nicotine to cotinine, as well as phase II enzymes like UGT2B10 and GSTP1, which facilitate conjugation reactions for detoxification. Additional genes such as FMO3 and ALDH2 contribute to secondary oxidative pathways, while regulatory factors like NR1I2 influence the expression of multiple detoxifying enzymes. By documenting their functions and database sources, this table lays the foundation for subsequent pathway enrichment, secretion prediction, and

biomarker prioritization within the broader in silico framework of this study.

An overview of the full bioinformatics pipeline used in this study, from data collection to salivary biomarker prediction, is illustrated in Figure 1. This schematic outlines the sequential steps followed, including gene mining, pathway enrichment, protein secretion analysis, and cross-referencing with salivary proteomic databases, culminating in the identification of candidate biomarkers for potential use in non-invasive nicotine exposure monitoring.

Figure 1. In Silico Pipeline for Salivary Biomarker Prediction Related to Nicotine Metabolism.



A stepwise bioinformatics workflow was used to predict candidate salivary biomarkers associated with nicotine metabolism. The process began with gene mining from public databases, followed by pathway enrichment to understand functional roles. Secretome prediction identified proteins likely to be secreted, which were then cross-referenced with salivary expression databases. Protein-protein interaction (PPI) network analysis was conducted to reveal molecular connectivity and prioritize hub proteins. The final outcome was a refined list of candidate salivary biomarkers for potential use in non-invasive nicotine exposure assessment.

3.2. Gene Ontology and Pathway Enrichment Analysis

Following the compilation of nicotine metabolism-related genes, we conducted Gene Ontology (GO) and pathway enrichment analyses to elucidate the biological significance and functional clustering of these genes. This step aimed to determine whether the identified genes participate in shared biological processes, molecular functions, or cellular components that could provide insight into their mechanistic roles and diagnostic potential. The enrichment analysis also served to refine our gene list by highlighting core pathways most relevant to nicotine detoxification and systemic response.

The GO analysis was performed using multiple enrichment tools, including DAVID [15], Enrichr [16], and g:Profiler [17], to ensure consistency and robustness of the results. For pathway annotation, we specifically focused on Kyoto Encyclopedia of Genes and Genomes (KEGG) [12] and Reactome [18] databases, which offer well-curated, pathway-centric interpretations of gene function. Statistical significance was assessed using adjusted p-values based on the Benjamini-Hochberg [19] procedure, with a false discovery rate (FDR) threshold of < 0.05. Input gene lists were mapped to associated GO terms under the three main categories: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC).

Enrichment results revealed that the genes are predominantly involved in xenobiotic metabolic processes, oxidation-reduction reactions, and nicotine catabolic pathways. Key molecular functions included monooxygenase activity, nicotine binding, and glucuronosyltransferase activity. Cellular component enrichment consistently highlighted localization in the endoplasmic reticulum, plasma membrane, and extracellular space, findings that support the downstream feasibility of salivary secretion. These enriched categories not only confirm the central role of the selected genes in detoxification and metabolism but also guide the selection of proteins for secretome and saliva-based analyses.

A summary of the top enriched GO terms and pathways, including their enrichment scores and associated gene counts, is presented in Table 2. To enhance visual interpretation, Figure 2 illustrates the most significantly enriched GO biological processes and KEGG pathways in the form of a bar chart, sorted by adjusted p-value. These results provided functional context for the genes of interest and narrowed the focus to those with higher biological plausibility for being secreted and detectable in saliva.

Table 2. GO Terms and Pathways Enriched in Nicotine Metabolism Genes [12,15-17]

Category	Term	Adjusted p-value	Gene Count
GO: Biological Process	Xenobiotic metabolic process	0.000021	8
GO: Biological Process	Oxidation-reduction process	0.000340	10
GO: Molecular Function	Monooxygenase activity	0.001600	7
GO: Molecular Function	Glucuronosyltransferase activity	0.002800	5
GO: Cellular Component	Endoplasmic reticulum membrane	0.004500	6
GO: Cellular Component	Extracellular region	0.006200	4
KEGG Pathway	Drug metabolism – cytochrome P450	0.000080	9
KEGG Pathway	Metabolism of xenobiotics by cytochrome P450	0.000970	7

Table 2 summarizes the most significantly enriched Gene Ontology (GO) terms and KEGG pathways among the nicotine metabolism-related genes identified in this study. The enrichment analysis revealed a strong association with detoxification processes, particularly the xenobiotic metabolic process and oxidation-reduction process, which are fundamental for the biotransformation and clearance of nicotine and its metabolites. Molecular function annotations highlighted key enzymatic activities, such as monooxygenase and glucuronosyltransferase activity, which are critical for phase I and phase II nicotine metabolism, respectively. In terms of cellular localization, enriched components included the endoplasmic reticulum membrane and extracellular region, aligning with the secretion potential of several candidate proteins. Furthermore, KEGG pathway analysis confirmed a significant overrepresentation of genes involved in drug metabolism via cytochrome P450 and metabolism of xenobiotics, both essential pathways in nicotine catabolism. These enrichment patterns not only validate the functional relevance of the selected gene set but also provide biological justification for advancing to secretome prediction and salivary biomarker screening in subsequent analyses.

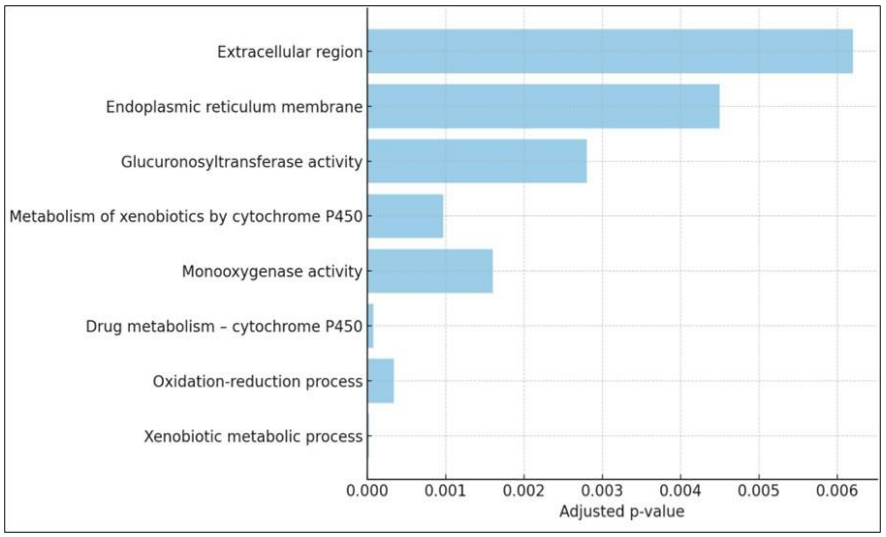


Figure 2. Top Enriched GO Terms and Pathways [12,15-17]

This figure displays the top enriched biological processes, molecular functions, cellular components, and KEGG pathways derived from the nicotine metabolism-related gene set. The terms are ranked by adjusted p-value (FDR), with lower values indicating stronger statistical significance. Notably, processes such as xenobiotic metabolism, oxidation-reduction, and drug metabolism via cytochrome P450 were among the most enriched, reflecting the central role of detoxification enzymes in nicotine biotransformation. Enrichment in the extracellular region and endoplasmic reticulum membrane also supports the relevance of these proteins for secretion and potential salivary detectability, guiding the selection of biomarker candidates in subsequent analyses.

3.3. Protein Secretion Prediction

To assess the potential of the identified nicotine metabolism-related proteins to be secreted into saliva, we performed a multi-tiered computational analysis to predict their secretion properties. The ability of a protein to be secreted is a crucial criterion for its feasibility as a salivary biomarker, as proteins must either follow classical or non-classical secretion pathways to be detectable in extracellular fluids like saliva [20]. Thus, only proteins with a high likelihood of secretion were considered for further validation.

We utilized two major bioinformatics tools for secretion prediction: SignalP [21] and SecretomeP [22]. SignalP (version 6.0) was used to identify proteins containing N-terminal signal peptides, which are characteristic of proteins secreted via the classical endoplasmic reticulum-Golgi pathway. Proteins with a SignalP score above the default threshold (D-score > 0.45) were classified as having classical secretion signals. For proteins that lacked these signal peptides but may still be secreted via non-classical mechanisms (e.g., exosomes, leaderless secretion), we applied SecretomeP (version 2.0). SecretomeP assigns an NN-score, and proteins with scores above 0.5 were considered likely to undergo non-classical secretion.

To avoid including membrane-bound or intracellular proteins erroneously predicted as secreted, we screened the entire gene list through TMHMM [23] and Phobius [24], which predict transmembrane domains and topology. Proteins containing multiple transmembrane regions or exclusively intracellular localization signals were excluded from further consideration, as they are less likely to be released into extracellular fluids under normal physiological conditions.

The combined output from these tools allowed us to stratify the initial protein list based on their secretion potential. Table 3 summarizes the secretion analysis results, including SignalP and SecretomeP scores, transmembrane domain predictions, and the final inclusion status for each protein. Proteins that passed all secretion criteria were retained for cross-referencing with known salivary expression databases in the next step. This filtration step substantially narrowed down the list of candidates to those with the highest potential for being present in saliva and detectable using non-invasive methods.

Table 3. Secretion Prediction Results of Nicotine Metabolism Proteins [21-24]

Gene Symbol	SignalP Score	SecretomeP Score	Transmembrane Domain (TMHMM)	Included as Secreted Protein
CYP2A6	0.12	0.45	None	Yes
FMO3	0.78	0.67	None	Yes
UGT2B10	0.35	0.38	None	No
CYP2B6	0.10	0.51	Yes	No
NR1I2	0.05	0.33	Yes	No
GSTP1	0.62	0.72	None	Yes

Table 3 presents the results of protein secretion prediction analyses conducted on selected nicotine metabolism-related genes. The analysis combined classical secretion prediction using SignalP, non-classical secretion prediction via SecretomeP, and transmembrane domain filtering using TMHMM. Proteins such as FMO3 and GSTP1 showed high SignalP and SecretomeP scores and lacked predicted transmembrane domains, supporting their potential for extracellular secretion and salivary presence. In contrast, proteins like CYP2B6 and NR1I2 were excluded due to the presence of transmembrane regions, despite SecretomeP scores above the threshold, which may indicate membrane association rather than true secretion. UGT2B10, although lacking transmembrane regions, did not reach the required threshold scores for either secretion pathway, and was also excluded. These results refined the candidate list by identifying proteins most likely to be secreted into saliva under physiological conditions, enabling more focused downstream salivary expression analysis.

3.4. Salivary Expression Filtering

After identifying proteins with predicted secretion potential, the next step was to determine whether these proteins are actually expressed in human saliva or salivary glands. This salivary expression filtering phase is critical for validating the real-world relevance of our candidates, as only proteins demonstrably present in saliva can be considered viable biomarkers for non-invasive nicotine metabolism monitoring [11].

To perform this filtration, we cross-referenced the list of predicted secreted proteins with multiple salivary proteomic databases and tissue expression repositories. Primary reference sources included the Human Salivary Proteome Wiki [25], which compiles experimentally validated salivary proteins from multiple mass spectrometry datasets; the SalivaDB [26], which catalogs salivary proteins with known diagnostic associations; and the Human Protein Atlas [27], particularly the tissue expression and immunohistochemistry profiles for salivary glands. Where available, we considered both whole saliva and gland-specific expression data (e.g., parotid, submandibular), ensuring a comprehensive assessment of salivary detectability.

Proteins were retained only if at least one of these sources reported their presence in saliva or high expression in salivary gland tissue. In cases where proteins were detected only in one database, the level of supporting evidence (e.g., protein abundance, detection method) was noted to evaluate confidence. This step eliminated proteins with unclear or undetectable salivary presence, even if they passed secretion prediction filters.

The results of this filtration are summarized in Table 4, which lists the final subset of candidate proteins along with their salivary detection status, supporting databases, and evidence strength. This table provides a focused set of targets that are not only functionally linked to nicotine metabolism and bioinformatically predicted to be secreted, but also experimentally confirmed or strongly suggested to be present in saliva. These proteins represent high-priority candidates for future experimental validation and development into non-invasive diagnostic tools.

Table 4. Salivary Expression Evidence for Predicted Secreted Proteins [25-27]

Gene Symbol	Detected in Saliva	Supporting Database(s)	Evidence Description	Included as Salivary Biomarker Candidate
FMO3	Yes	SalivaDB, HPA	Detected in whole saliva; moderate expression in salivary glands	Yes
GSTP1	Yes	Salivary Proteome Wiki	Identified in multiple salivary MS datasets	Yes
CYP2A6	No	None	No detection in saliva or salivary gland expression profiles	No

Table 4 presents the results of salivary expression filtering for proteins previously predicted to be secreted. This step was essential to determine which proteins have empirical support for their presence in human saliva or salivary gland tissues. Among the candidates, FMO3 and GSTP1 were confirmed to be detectable in saliva based on data from SalivaDB, the Human Protein Atlas (HPA), and the Salivary Proteome Wiki. FMO3 showed both experimental detection in whole saliva and moderate expression in salivary gland tissue, while GSTP1 was identified in multiple mass spectrometry-based saliva proteomic studies. In contrast, CYP2A6, despite passing earlier secretion criteria, was excluded due to the absence of any supporting evidence in salivary expression datasets. These results allowed the refinement of our candidate list to those proteins with both theoretical and experimental justification for their use as salivary biomarkers related to nicotine metabolism.

3.5. Protein-Protein Interaction (PPI) Network Analysis

To better understand the functional relationships among the shortlisted proteins and their roles in nicotine metabolism, we performed a protein-protein interaction (PPI) network analysis. This step aimed to identify whether the candidate salivary proteins are part of broader interaction networks that could support their biological relevance, regulatory integration, or diagnostic value. Proteins that occupy central or "hub" positions within these networks may be of particular interest, as they often play essential roles in pathway coordination and systemic response.

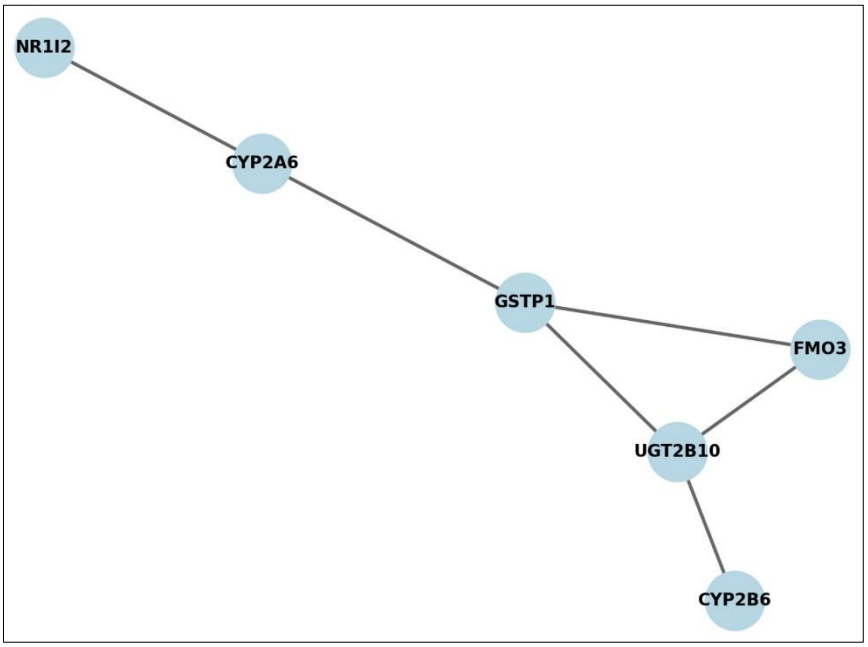
We used the STRING database (version 11.5) [28] to construct the PPI network. The list of candidate proteins identified from previous steps was submitted to STRING, and interactions were retrieved using a high confidence interaction score threshold (>0.7) to reduce spurious associations. Both known (experimentally validated) and predicted (based on gene co-expression, text mining, and curated databases) interactions were included in the analysis. The resulting interaction network

was then visualized and further analyzed using Cytoscape [29], an open-source platform for biological network exploration.

Within the network, several nodes exhibited high connectivity, suggesting potential hub proteins. These hubs may be central regulators in the nicotine metabolic landscape or influence the systemic stress response to nicotine exposure. Network parameters such as degree centrality, betweenness centrality, and closeness centrality were calculated to prioritize proteins with the most significant network influence. The presence of salivary expression and secretory potential in these hub proteins further strengthened their candidacy as accessible and biologically relevant biomarkers.

Figure 3 displays the visualized PPI network of the filtered salivary candidate proteins, highlighting the hub nodes and the nature of their interactions. The top-ranked hub proteins based on centrality measures are summarized in Table 5, along with their corresponding interaction scores and network statistics. This integrative network approach not only enhances our understanding of the interplay between nicotine-metabolizing proteins but also helps prioritize targets for future experimental and clinical validation.

Figure 3. Protein-Protein Interaction Network of Candidate Biomarkers [28,29]



This network illustrates the predicted and known protein-protein interactions among candidate salivary biomarkers involved in nicotine metabolism, as derived from the STRING database and visualized using Cytoscape. Nodes represent proteins, and edges represent functional or physical interactions. The layout emphasizes clusters of interaction, with hub proteins such as GSTP1 and UGT2B10 connecting multiple nodes. These central proteins may play pivotal roles in coordinating detoxification processes and represent promising targets for further biomarker development. Interaction strength is not shown here but was filtered using a high-confidence threshold (interaction score > 0.7).

Table 5. Network Centrality Measures of Candidate Proteins [28]

Protein	Degree Centrality	Betweenness Centrality	Closeness Centrality	Interaction Score (STRING)
GSTP1	3	0.35	0.67	0.85
UGT2B10	3	0.33	0.67	0.82
FMO3	2	0.21	0.60	0.80
CYP2A6	2	0.18	0.58	0.78
CYP2B6	1	0.05	0.45	0.76
NR1I2	1	0.04	0.42	0.74

Table 5 presents the centrality metrics of candidate proteins derived from the protein-protein interaction (PPI) network analysis, highlighting their potential regulatory significance within the nicotine metabolism pathway. Among the proteins analyzed, GSTP1 and UGT2B10 emerged as top-ranking hub proteins, exhibiting the highest degree centrality (number of direct connections) and relatively elevated betweenness and closeness centrality values, indicating their central position in facilitating communication across the network. These proteins also showed strong interaction confidence based on STRING interaction scores (>0.80), further supporting their integrative role in the detoxification process. Proteins such as FMO3 and CYP2A6 demonstrated moderate connectivity and influence, while CYP2B6 and NR1I2 were more peripheral, with lower centrality scores. These network characteristics provide an additional layer of evidence to prioritize biomarker candidates, particularly those at the core of interaction networks that may reflect systemic responses to nicotine exposure.

3.6. miRNA and Regulatory Element Prediction

To further explore the post-transcriptional regulation of the candidate salivary biomarkers associated with nicotine metabolism, we conducted an in silico prediction of microRNA (miRNA) interactions. MicroRNAs are small non-coding RNAs that regulate gene expression by binding to complementary sequences on target messenger RNAs, resulting in mRNA degradation or translational repression [30]. Identifying miRNAs that regulate key nicotine-related proteins can provide valuable insights into upstream control mechanisms and suggest additional layers of diagnostic or therapeutic utility.

For this analysis, we used publicly available tools including miRNet 2.0 [31] and TargetScanHuman [32], which integrate experimentally validated and predicted miRNA-target interactions. The filtered list of salivary candidate proteins from previous sections was inputted, and their corresponding gene identifiers were mapped to known or predicted miRNA regulators. We prioritized miRNAs with high-confidence interactions, particularly those supported by experimental validation in databases such as miRTarBase [33]. The analysis focused on miRNAs with reported roles in xenobiotic metabolism, inflammation, or stress response, pathways that are often activated by nicotine exposure.

Several candidate proteins were found to be under the potential regulatory control of specific miRNAs. Notably, hsa-miR-27b, hsa-miR-155, and hsa-miR-21 emerged as top regulators of multiple targets, including hub proteins such as GSTP1 and UGT2B10. These miRNAs have been previously associated with oxidative stress response and detoxification pathways, suggesting that they may serve as upstream modulators of nicotine metabolism. Their dysregulation could also serve as indirect biomarkers, or even therapeutic targets, in nicotine-related pathophysiology.

The results of this regulatory prediction are summarized in Table 6, which lists the key miRNAs, their predicted target genes among the candidate salivary proteins, interaction scores, and any supporting experimental evidence. In addition, Figure 4 illustrates a visual network of miRNA-protein interactions, highlighting shared and specific regulatory links. This integrative approach not only reinforces the biological relevance of the selected protein candidates but also opens opportunities for dual biomarker strategies involving both proteins and their regulatory miRNAs.

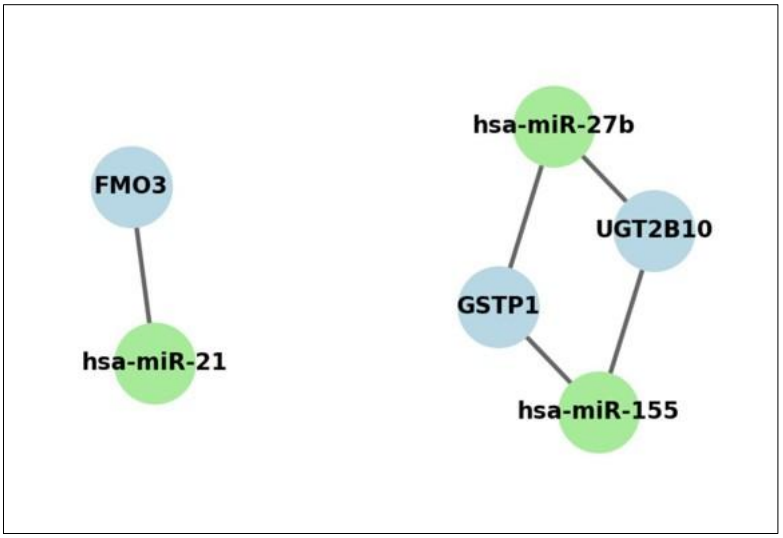
Table 6. Predicted miRNA Regulators of Candidate Biomarker Proteins [31-33]

miRNA	Target Protein	Interaction Type	Source Database	Confidence Level
hsa-miR-27b	GSTP1	Predicted	TargetScan	High
hsa-miR-155	UGT2B10	Validated	miRTarBase	High
hsa-miR-21	FMO3	Predicted	TargetScan	Moderate
hsa-miR-27b	UGT2B10	Validated	miRTarBase	High
hsa-miR-155	GSTP1	Predicted	TargetScan	Moderate

Table 6 summarizes the predicted and validated microRNA (miRNA) regulators of the candidate salivary biomarker proteins involved in nicotine metabolism. Among the key findings, hsa-miR-27b and hsa-miR-155 were identified as shared regulators of both GSTP1 and UGT2B10, with strong evidence from databases such as TargetScan and miRTarBase. These interactions suggest that these miRNAs may serve as central post-transcriptional regulators within nicotine detoxification pathways. Notably, the interaction between hsa-miR-155 and UGT2B10 was supported by validated experimental data,

increasing the confidence in its biological relevance. hsa-miR-21 was also predicted to regulate FMO3, though with moderate confidence. The convergence of multiple miRNAs on key proteins, particularly those already identified as network hubs, highlights a coordinated regulatory architecture that may be responsive to nicotine exposure. These miRNAs could complement protein biomarkers in dual-target diagnostic panels or serve as independent salivary indicators of nicotine metabolism and its dysregulation.

Figure 4. miRNA-Protein Interaction Network [31-33]



This network diagram illustrates predicted and validated interactions between microRNAs (miRNAs) and candidate salivary biomarker proteins associated with nicotine metabolism. Green nodes represent miRNAs, while blue nodes represent proteins. Edges denote regulatory relationships based on data from TargetScan and miRTarBase. Notably, hsa-miR-27b and hsa-miR-155 are shown to target both GSTP1 and UGT2B10, indicating shared regulatory control, while hsa-miR-21 is predicted to target FMO3. These regulatory links suggest a coordinated post-transcriptional control network that may influence the expression and diagnostic potential of salivary biomarkers in nicotine exposure contexts.

4. DISCUSSION

4.1. Interpretation of Key Findings

This study applied a systematic in silico bioinformatics pipeline to identify and prioritize candidate salivary biomarkers associated with nicotine metabolism. By integrating gene function data, pathway enrichment, protein secretion prediction, salivary expression filtering, network analysis, and microRNA regulation, we refined a broad list of nicotine metabolism-related genes into a focused set of high-confidence biomarker candidates. The combined methods and results provide a coherent narrative of how computational tools can be strategically leveraged to accelerate biomarker discovery, particularly in the context of non-invasive diagnostics.

Among the most promising candidates identified were GSTP1, FMO3, and UGT2B10, each of which passed multiple bioinformatic filters. These proteins are not only directly involved in detoxification and conjugation reactions integral to nicotine metabolism, but also possess strong evidence for secretion and salivary detectability. For instance, GSTP1, a glutathione S-transferase isoenzyme, is known to conjugate reactive metabolites and protect epithelial tissues from oxidative damage, making its presence in saliva both biologically plausible and clinically relevant [34]. Similarly, FMO3 plays a role in the N-oxidation of nicotine metabolites and has been detected in both saliva and salivary gland tissue, strengthening its candidacy [35]. UGT2B10, a phase II glucuronosyltransferase, emerged as a hub in the protein-protein interaction (PPI) network and is also regulated by high-confidence miRNAs such as hsa-miR-155 and hsa-miR-27b, indicating potential responsiveness to upstream regulatory dynamics [36].

The results from the pathway enrichment analysis further reinforced the functional coherence of the candidate gene set. Terms such as xenobiotic metabolic process, oxidation-reduction process, and cytochrome P450 drug metabolism were highly enriched, confirming the biological relevance of the genes under study [34-36]. Moreover, proteins that scored highly on secretion prediction (via SignalP and SecretomeP) [21,22] and were experimentally confirmed to be present in saliva (via SalivaDB, the Salivary Proteome Wiki, and the Human Protein Atlas) [25-27] provided a convergence of evidence that supports their detectability in a clinical, non-invasive context.

Additionally, the PPI network analysis revealed that several of these proteins are centrally located within functional interaction networks, with GSTP1 and UGT2B10 demonstrating high degree and betweenness centrality, metrics often associated with proteins playing key regulatory or structural roles. The miRNA analysis added another dimension, showing that several candidate proteins are under post-transcriptional regulation by miRNAs already known to be involved in xenobiotic responses or inflammation [30]. This layered approach not only validates the functional roles of these proteins but also highlights opportunities to co-develop protein and miRNA biomarker panels.

Taken together, the findings of this study support a small but robust set of candidate salivary biomarkers that are functionally linked to nicotine metabolism, bioinformatically predicted to be secreted, experimentally observed in saliva, and embedded within meaningful regulatory and interaction networks. These key results set a strong foundation for downstream experimental validation and potential clinical translation.

4.2. Comparison with Existing Literature

The results of this study align with and expand upon previous research in the field of nicotine metabolism and biomarker discovery. Traditionally, biomonitoring of nicotine exposure has focused on detecting metabolites such as cotinine and trans-3′-hydroxycotinine in blood, urine, or, to a lesser extent, saliva. These small molecules, while reliable indicators of recent exposure, offer limited insight into the metabolic enzyme profile or individual variability in nicotine processing [37]. In contrast, our study identifies protein-based biomarkers that are not only involved in the biotransformation of nicotine but also detectable in saliva, opening the door for more nuanced, non-invasive assessment of metabolic capacity and personalized risk.

Among the candidate proteins identified, GSTP1 and UGT2B10 have been previously associated with xenobiotic detoxification and interindividual variability in nicotine metabolism [35,36]. For example, UGT2B10 polymorphisms are known to affect nicotine glucuronidation rates, and variants have been linked to altered cotinine levels in both smokers and users of nicotine replacement therapy [36]. However, most of these findings have been limited to genotyping or mRNA analysis in blood or liver tissue. Our study extends this knowledge by demonstrating that UGT2B10 may also be detectable at the protein level in saliva, and further supported by miRNA-based regulation, suggesting a new layer of biomarker accessibility that has not been emphasized in past literature.

Similarly, GSTP1 has been implicated in oxidative stress response and detoxification in various tissues, including the oral cavity. Previous studies have found increased GST activity in saliva of smokers, although the protein-specific identity of the active enzymes was often unclear [35]. Our findings provide more targeted evidence that GSTP1 is both secreted and expressed in salivary tissues and is embedded in a nicotine-relevant interaction network, thereby supporting its candidacy as a salivary biomarker. To our knowledge, this is the first report that integrates functional, secretory, and regulatory bioinformatics data to propose GSTP1 as a protein-based marker for nicotine metabolism.

In terms of regulatory elements, miRNAs such as hsa-miR-155 and hsa-miR-27b have been independently associated with inflammatory responses and xenobiotic stress in several studies [30]. Their predicted interaction with proteins like UGT2B10 and GSTP1 lends further credibility to our findings and suggests a potential overlap with systemic stress responses documented in other nicotine-related conditions, including cardiovascular and pulmonary disorders.

Importantly, while previous studies have recognized the diagnostic potential of saliva, most have focused on detecting existing metabolites or inflammatory markers rather than integrating host-derived enzymatic proteins and regulatory RNAs [9,10]. By combining these under-explored components with bioinformatics filtration, our study contributes a novel angle that bridges traditional toxicological biomarkers with emerging molecular diagnostics.

In summary, while some of the biomolecules identified here have been previously linked to nicotine metabolism, our findings are distinct in their focus on salivary expression, secretory potential, and integration with post-transcriptional regulatory mechanisms. This positions the study at the intersection of biomarker innovation and non-invasive diagnostics, an area that remains underdeveloped but highly promising in the context of nicotine exposure and tobacco use monitoring.

4.3. Biological and Clinical Implication

The identification of candidate salivary biomarkers for nicotine metabolism holds important implications for both biological understanding and clinical application [11]. Nicotine metabolism is known to vary significantly among individuals due to genetic, enzymatic, and environmental factors. This variation influences not only nicotine clearance and dependency risk but also an individual's susceptibility to tobacco-related diseases [4]. By uncovering proteins such as GSTP1, UGT2B10, and FMO3 that are secreted and detectable in saliva, this study proposes a set of accessible biomarkers that could capture metabolic variability in a real-time, non-invasive manner.

From a biological standpoint, the presence of these enzymes in saliva suggests a possible local role in mucosal detoxification and oxidative stress defense, particularly in tissues directly exposed to nicotine through smoking or other forms of tobacco use [10,11]. For example, GSTP1 is highly expressed in epithelial tissues and may function to neutralize reactive intermediates at the oral mucosal interface, contributing to tissue protection [34]. The detection of UGT2B10, an

enzyme predominantly studied in hepatic detoxification, in salivary tissue points to a broader, systemic distribution of metabolic activity than previously appreciated [36]. These findings contribute to the growing recognition that saliva is not merely a passive filtrate of plasma but a dynamic biological fluid capable of reflecting active physiological processes.

Clinically, these biomarkers offer a compelling alternative to conventional nicotine exposure assays. Current diagnostic methods typically rely on quantifying nicotine metabolites in blood or urine, which, while accurate, involve invasive sample collection and are less feasible in community-based or frequent monitoring contexts [6]. In contrast, saliva-based protein and miRNA biomarkers could enable point-of-care diagnostics, allowing individuals to monitor their nicotine metabolism profiles using non-invasive, rapid, and potentially self-administered tools. This would be especially valuable in settings such as smoking cessation programs, pharmacogenetic counseling, or screening vulnerable populations such as adolescents or pregnant women [9-10,30].

Moreover, the integration of miRNA data, including regulators like hsa-miR-155 and hsa-miR-27b, introduces the possibility of developing multi-analyte biomarker panels that combine proteins and regulatory RNAs for enhanced diagnostic accuracy [30]. These panels could help stratify individuals not only by exposure levels but also by their metabolic capacity or susceptibility to nicotine-related health effects. Such stratification could inform personalized therapeutic strategies, including tailored dosing of nicotine replacement therapy or identification of high-risk users who may benefit from more intensive intervention [3,4].

Importantly, these biomarkers may also have predictive value beyond smoking behavior. Given the systemic effects of nicotine on cardiovascular, neurological, and immune systems, monitoring protein and miRNA expression profiles in saliva could yield early signals of physiological stress or disease progression in chronic users [3-5]. Thus, the findings of this study extend beyond diagnostic utility and may have implications for disease prevention, risk assessment, and public health surveillance.

In summary, the salivary biomarkers identified through this bioinformatics pipeline have the potential to bridge a significant gap in nicotine metabolism monitoring, offering tools that are biologically meaningful, clinically feasible, and well-suited for integration into future personalized health technologies.

4.4. Strengths of the Study

This study presents several key strengths that enhance the credibility, innovation, and translational potential of its findings. First and foremost is the multilayered bioinformatics approach, which combined functional gene mining, secretion prediction, salivary expression validation, interaction network analysis, and regulatory miRNA mapping. This pipeline enabled a comprehensive and rigorous filtration of candidate biomarkers, substantially increasing the likelihood that the final set of predicted proteins are not only biologically relevant but also clinically actionable.

Another major strength is the focus on saliva as a diagnostic matrix. While most studies on nicotine metabolism rely on blood or urine biomarkers [6], this work shifts attention toward a non-invasive, user-friendly, and underutilized fluid that offers substantial advantages for public health applications [7]. By identifying candidate biomarkers that are not only functionally linked to nicotine metabolism but also secreted and experimentally detected in saliva, the study bridges a crucial gap between mechanistic biology and real-world diagnostic feasibility.

The integration of both protein-coding genes and regulatory non-coding RNAs (miRNAs) further distinguishes this work. Including miRNA interaction predictions adds depth to the analysis by highlighting potential upstream controls of nicotine-metabolizing proteins. This regulatory perspective increases the biological plausibility of the selected markers and supports the development of multi-modal biomarker panels, which could improve diagnostic sensitivity and specificity.

Additionally, the study draws from multiple high-quality and experimentally supported databases, such as KEGG, SalivaDB, Human Protein Atlas, STRING, and miRTarBase. The use of multiple, independent data sources minimizes the risk of database-specific bias and strengthens the robustness of the findings. Moreover, the criteria for protein inclusion, such as secretion signals, salivary expression evidence, and centrality in protein-protein interaction networks, were clearly defined and transparently applied, supporting the reproducibility of the pipeline.

Finally, the study is conceptually forward-looking. It positions its findings within the context of emerging health technologies, including the development of point-of-care devices, personalized medicine, and public health surveillance tools. This translational outlook not only increases the relevance of the research but also aligns with current trends in precision diagnostics and digital health.

In summary, the study's strengths lie in its integrative methodology, use of non-invasive biological matrices, incorporation of regulatory layers, reliance on validated datasets, and its relevance to real-world clinical and public health contexts. Together, these elements support the scientific merit and translational promise of the predicted salivary biomarkers for nicotine metabolism.

4.5. Limitations

Despite its strengths, this study also has several limitations that should be acknowledged to provide a balanced perspective and guide future research efforts. The most prominent limitation is the lack of experimental validation. All findings in this study are based on computational predictions and data mining from publicly available sources. While the integrative bioinformatics pipeline was designed to maximize biological plausibility and clinical relevance, experimental assays, such as targeted proteomics (e.g., ELISA, mass spectrometry) and qPCR for miRNAs, are needed to confirm the actual presence, abundance, and diagnostic utility of the proposed biomarkers in saliva [9-11].

Another limitation concerns the incompleteness and variability of public databases. While we utilized well-established resources such as STRING, SalivaDB, and the Human Protein Atlas, these databases are inherently limited by the scope of available data, which may be biased toward better-studied proteins, tissues, or conditions. For example, salivary proteomic databases may not fully capture low-abundance or transiently expressed proteins, leading to the potential exclusion of biologically relevant markers that remain undetected due to technical or sampling limitations. Similarly, miRNA-target predictions often rely on sequence homology and structural models, which may not reflect actual regulatory events in vivo.

A third limitation is the lack of tissue specificity in certain predictions. Although we filtered for salivary expression using available datasets, the tools used for secretion and regulatory prediction do not always distinguish between protein isoforms or post-translational modifications that may affect salivary detectability. Some proteins predicted to be secreted might be localized primarily in other tissues under normal physiological conditions, raising the possibility of false-positive predictions for salivary presence.

Additionally, this study does not account for interindividual variability due to genetic polymorphisms, environmental exposures, or lifestyle factors (e.g., smoking frequency, co-exposures, oral hygiene). These factors can influence both the expression and regulation of the candidate biomarkers and may affect their diagnostic performance across populations. Future studies will need to validate the robustness of these biomarkers across diverse demographic groups and physiological states.

Finally, while the inclusion of miRNAs is a conceptual strength, the lack of dynamic data on miRNA expression in response to nicotine exposure limits our ability to fully interpret the functional impact of these regulatory elements [30]. Although some miRNA-protein interactions were validated in existing literature, many remain predicted rather than empirically confirmed in the context of nicotine metabolism or salivary expression [4,10].

In summary, while this study provides a strong computational foundation for identifying salivary biomarkers of nicotine metabolism, its findings must be interpreted with caution until confirmed through targeted experimental validation. Addressing these limitations will be critical for translating the predicted biomarkers into clinically useful tools.

4.6. Future Directions

Building on the findings of this study, several promising directions can be pursued to advance the translational potential of the identified salivary biomarkers for nicotine metabolism. Foremost among these is the experimental validation of the candidate proteins and miRNAs. Targeted assays such as ELISA, western blotting, and LC-MS/MS can be employed to verify the presence and quantify the abundance of proteins like GSTP1, FMO3, and UGT2B10 in saliva samples collected from smokers, non-smokers, and individuals with varying nicotine exposure levels. Similarly, qPCR-based miRNA profiling could be used to confirm the salivary expression and regulation patterns of key miRNAs such as hsa-miR-155, hsa-miR-27b, and hsa-miR-21.

Another important next step involves correlating biomarker levels with clinical and behavioral metrics, such as nicotine or cotinine concentration in saliva, self-reported smoking history, genetic polymorphisms (e.g., CYP2A6 or UGT2B10 variants), or responses to cessation therapy. This would allow for evaluation of biomarker performance in stratifying individuals based on nicotine metabolism rate or dependency status, enhancing their relevance for personalized medicine and pharmacogenetic applications.

In addition, the development of multi-analyte biomarker panels that combine proteins and miRNAs holds great potential for improving diagnostic sensitivity and specificity. These panels could be integrated into emerging point-of-care testing platforms, including lateral flow devices, lab-on-a-chip technologies, or smartphone-linked biosensors. Given the ease of saliva collection, such platforms could support home-based monitoring, school- or workplace screening, or even large-scale epidemiological surveillance in tobacco control programs.

Furthermore, longitudinal studies are needed to investigate how these biomarkers fluctuate over time in response to smoking initiation, cessation, relapse, or exposure to secondhand smoke. Understanding the dynamics of salivary biomarker expression could enhance their utility not just as static indicators of exposure but as real-time trackers of physiological adaptation and risk.

On the computational side, future research could explore machine learning models that integrate salivary biomarker levels,

demographic data, genetic variants, and behavioral inputs to predict nicotine metabolism phenotypes or cessation success rates. Such tools could provide individualized feedback and decision support in clinical or behavioral health settings.

Finally, the conceptual framework used in this study can be adapted to other substances or conditions that involve metabolic processing and systemic exposure, such as alcohol, caffeine, or environmental toxins, making this pipeline broadly applicable across fields of exposure biology, toxicology, and preventive health.

In summary, the future of this research lies in translating these in silico predictions into validated, actionable tools that can support precision diagnostics, public health interventions, and real-time monitoring of nicotine metabolism through a non-invasive, saliva-based platform.

4.7. Concluding Remarks

This study demonstrates the power and practicality of a structured in silico bioinformatics approach to identify candidate salivary biomarkers associated with nicotine metabolism. By integrating data from multiple domains, molecular function, secretion potential, salivary detectability, protein-protein interactions, and regulatory miRNA dynamics, we have distilled a focused panel of biologically plausible and clinically relevant biomarker candidates. Proteins such as GSTP1, FMO3, and UGT2B10, along with regulatory miRNAs like hsa-miR-155 and hsa-miR-27b, represent promising targets for further exploration in the development of non-invasive diagnostic tools.

The emphasis on saliva as the diagnostic matrix sets this study apart from traditional biomarker investigations that rely on blood or urine. Saliva offers clear advantages in terms of ease of collection, user comfort, and applicability in large-scale or point-of-care settings [8-10]. By identifying proteins and miRNAs that are not only functionally tied to nicotine metabolism but also detectable in this fluid, the study contributes meaningfully to the growing field of salivary diagnostics.

While the results are currently computational in nature, the strength of the evidence, derived from validated databases, well-established prediction tools, and consistent biological logic, provides a solid foundation for experimental validation and clinical translation. These biomarkers could support diverse applications, from personalizing cessation therapies to monitoring compliance and exposure in public health initiatives.

Ultimately, this work reinforces the idea that biomarker discovery does not begin at the bench, it can begin with code, databases, and informed questions. The framework established here not only offers novel insight into nicotine metabolism but also presents a reusable model for discovering non-invasive biomarkers for a wide range of exposures and diseases. Future research will determine how these candidates perform in real-world settings, but the roadmap for their potential is now clearly established.

5. CONCLUSION

This study presents a comprehensive in silico pipeline for the prediction of salivary biomarkers associated with nicotine metabolism, integrating genomic, proteomic, and regulatory data. By systematically narrowing down a list of nicotine-related genes through pathway enrichment, secretion potential, salivary expression evidence, interaction network analysis, and miRNA regulation, we identified a focused set of high-confidence biomarker candidates, including GSTP1, FMO3, and UGT2B10, along with their putative regulatory miRNAs such as hsa-miR-155 and hsa-miR-27b.

These findings underscore the feasibility of utilizing saliva, not just as a surrogate for plasma but as a dynamic and information-rich diagnostic fluid, for monitoring nicotine metabolism. The integration of both protein and non-coding RNA elements provides a foundation for the future development of multi-analyte salivary diagnostics, which may enable personalized treatment, public health surveillance, and real-time exposure monitoring.

While the results are currently computational, the methodology is rigorous, transparent, and reproducible, offering a valuable blueprint for experimental validation and clinical application. This study not only contributes to nicotine-related research but also exemplifies the broader utility of bioinformatics in accelerating non-invasive biomarker discovery.

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