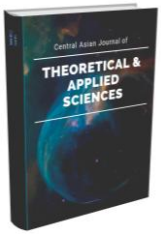

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Article

Characterization of Enzymatic Biomarkers in Salivary Samples for Early Detection of Pancreatic in Kirkuk

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Abstract: Pancreatic cancer is one of the most aggressive malignancies, with a high mortality rate due to late-stage diagnosis and the absence of reliable early detection methods. Current diagnostic approaches, such as imaging and serum biomarkers (e.g., CA 19-9), are often invasive, expensive, and lack sufficient sensitivity and specificity. Saliva has emerged as a promising biofluid for non-invasive diagnostics, offering a unique window into systemic diseases, including cancer. This study aims to explore the potential of enzymatic biomarkers in saliva for the early detection of pancreatic cancer using a proteomic approach. This study investigates the differential expression of salivary enzymatic biomarkers in pancreatic cancer patients compared to healthy controls. The primary objective is to characterize and validate key enzymatic proteins that could serve as reliable, non-invasive diagnostic indicators for early pancreatic cancer detection. A case-control study was conducted on 100 participants (50 pancreatic cancer patients and 50 healthy controls). Unstimulated whole saliva samples were collected, processed, and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify differentially expressed enzymes. Western blot and ELISA were used for biomarker validation. Statistical tests, including ROC curve, AUC, sensitivity, and specificity analysis, were performed to measure biomarker performance. Proteomic profiling revealed a significant upregulation of MMP-9, LDH, Amylase, and ALP in pancreatic cancer patients compared to healthy controls ($p < 0.001$). MMP-9 had the optimal diagnostic performance (AUC = 0.91, sensitivity = 89.5%, specificity = 92.1%), followed by LDH (AUC = 0.88, sensitivity = 85.2%, specificity = 88.7%). Pathway analysis linked these enzymes with extracellular matrix remodeling, inflammation, and dysregulation of metabolism, which are of crucial significance in pancreatic cancer development. The current study demonstrates that salivary enzymatic biomarkers possess vast potential as a non-invasive diagnostic tool for the early diagnosis of pancreatic cancer. The greater sensitivity and specificity of MMP-9 and LDH suggest their potential application in clinical screening programs, particularly in cases involving population at high risk. Large-scale studies need to be conducted to validate these findings and render them clinically useful.

Keywords: Pancreatic Cancer, Salivary Biomarkers, Proteomics, Enzymatic Biomarkers, MMP-9, Non-Invasive Diagnostics, LC-MS/MS

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1. Introduction

Pancreatic cancer remains one of the most virulent and lethal cancers in the world, with a poor prognosis and high mortality rate. Despite advances in oncological treatments, surgical interventions, and targeted therapy, the survival rate for pancreatic cancer remains appallingly low[1]. According to current world cancer statistics, pancreatic cancer accounts for approximately 495,000 new cases and 466,000 deaths annually, making it one

of the leading causes of cancer death[2]. Pancreatic cancer is often symptomless in the initial stages, leading to the late diagnosis and high rate of advanced or metastatic cases at diagnosis. This delay in diagnosis severely limits access to treatment, as curative surgical resection is feasible in only a minority of patients at presentation[3]. The increasing incidence of pancreatic cancer and its high case-fatality indicate the urgent need for improved diagnostic approaches that allow early detection and treatment[4].

One of the most significant obstacles in the treatment of pancreatic cancer is the difficulty in its early diagnosis. In contrast to the majority of malignancies, pancreatic cancer lacks typical clinical manifestations in its initial stages and therefore is hard to diagnose until it has progressed to an advanced stage[5]. Abdominal pain, weight loss, jaundice, and gastrointestinal disturbance typically arise after the disease has metastasized or invaded surrounding tissues. Besides, pancreatic tumors have a tendency to develop in regions of the pancreas that make them imperceptible with conventional imaging techniques[6]. As a result, most patients are diagnosed at a point when the disorder is not accessible to surgical resection, thereby leading to adverse clinical outcome and reduced susceptibility to treatment[7].

Current methods for pancreatic carcinoma detection are founded on a subset of imaging modalities, serologic markers, and invasive procedures. Imaging modalities such as contrast-enhanced computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS) are valuable for tumor detection and staging[8]. These modalities do have limitations, particularly in detecting small tumors or precursor lesions that might indicate early-stage disease. For instance, CT scans and MRIs are often not able to pick up pancreatic tumors smaller than one centimeter in diameter and hence reduce their sensitivity for early-stage screening[9]. Further, though EUS-guided FNA is more specific in the return of histopathological diagnosis, it is invasive with concomitant risks of bleeding, infection, and pancreatitis[10].

Serological tumor markers such as carbohydrate antigen 19-9 (CA 19-9) are used commonly in clinical settings for the observation of disease process and response to therapy in cancer of the pancreas[11]. However, CA 19-9 is not pancreatic cancer specific because the levels can also be raised in patients with benign pancreatic disease such as chronic pancreatitis or obstructive jaundice[12]. Furthermore, approximately 10% of individuals do not secrete CA 19-9 due to genetic variation in the Lewis antigen system, further diminishing its utility as a reliable diagnostic marker. These built-in limitations of existing diagnostic methods support the need for new biomarkers that would be able to identify pancreatic cancer at an early stage in a cost-effective and non-invasive manner[13].

With these challenges, the search for non-invasive biomarkers gained more importance in the past several years. Non-invasive diagnostic methods can identify pancreatic cancer earlier, improve the prognosis in patients, and reduce the burden of invasive procedures[26]. Among the many biofluids being explored for the identification of biomarkers, saliva has also emerged as a viable, easy to obtain biofluid with a multifaceted composition of biomolecules that signal systemic physiological and pathological changes[14]. Salivary biomarkers, particularly enzymatic proteins, have been promising in the diagnosis of malignancies, including pancreatic cancer, through the signaling of tumor-associated metabolic alterations[9].

Role of Salivary Biomarkers

Saliva has proven itself to be an effective biofluid for disease diagnosis because it combines the advantageous biochemical properties of blood with the amenability of tissue biopsies. Saliva is easy to collect, without pain or at low cost, compared to complex sampling procedures with blood or tissue biopsies[15]. Saliva is therefore an excellent candidate for screening and large-scale population studies. Sampling facility allows high reproducibility and frequency, and thus longitudinal disease course and response to treatment can be followed[16]. Saliva also contains a large array of biomolecules like

proteins, enzymes, nucleic acids (DNA and RNA), lipids, and metabolites that provide rich data on systemic pathological and physiological processes[17].

One of the notable advantages of saliva as a body fluid for diagnosis is its direct contact with blood by capillary exchange, where it can be representative of the systemic changes because of disease states[18]. Salivary biomarkers have been demonstrated, in recent studies, to generate good signals for an enormous range of diseases that include cancers, cardiovascular diseases, neurodegenerative diseases, and infectious diseases[19]. The fact that saliva contains extracellular vesicles (EVs) and exosomes makes it also valuable for diagnostics because these structures bear disease-specific molecules that are informative of tumor biology and disease process[20]. Unlike traditional blood-based biomarkers whose levels may be under homeostatic control mechanisms, salivary biomarkers give a more dynamic and responsive picture of disease status[21].

Diagnostic significance of saliva has been extensively explored in the last decade with promising results for most cancers including oral squamous cell carcinoma, breast cancer, lung cancer, and gastric cancer[22]. Salivary biomarkers have been identified employing different omics-based platforms including proteomics, transcriptomics, metabolomics, and lipidomics and provided a qualitative molecular pathophysiology profile of disease[23]. In pancreatic cancer, preliminary observations have indicated salivary protein alterations in the development of disease and therefore saliva might be a good medium for the detection of cancer at an early stage[24].

Various studies have examined the possibility of using salivary exosomal RNA and proteins as diagnostic biomarkers in pancreatic cancer. Exosomal miRNAs, for instance, have been shown to be candidate biomarkers since they are stable and possess disease-specific expression patterns[25]. Therefore, salivary proteomic analysis has reported radical changes in the levels of enzymes like proteases, lipases, and glycosidases, which play significant roles in tumor growth and metabolism[26]. These findings illustrate the promise of saliva as a non-invasive and easily accessible biofluid for pancreatic cancer diagnosis, particularly in areas where invasive interventions and highly sophisticated imaging machines are unavailable[27].

While these are promising developments, translation of salivary biomarkers for pancreatic cancer detection remains an unexploited frontier that must be confirmed by large-scale, multicenter clinical trials[28]. There is a need for standardized sample handling, processing, and analysis protocols to assure reproducibility and accuracy in biomarker discovery. Also, it will be beneficial to integrate machine learning and AI-driven approaches with salivary proteomics to further develop biomarker discovery and build predictive diagnostic models[29]. By overcoming these challenges, saliva-based diagnostics can transform the early diagnosis of cancer and, in the case of pancreatic cancer, significantly enhance clinical outcomes in patients[30].

Proteomic Approaches to Biomarker Discovery

Biomarker discovery has been transformed by proteomics through enabling large-scale profiling of protein expression patterns from biological samples. Because proteins are active molecules of the cell, their dysregulation is generally antecedent to the development of disease and thus are of critical importance to prognosis and diagnosis[31]. In cancer studies, proteomic techniques have made it possible to identify tumor-related proteins and enzymic biomarkers reflecting cellular transformation, metabolic change, and host-tumor interaction. Technical developments in high-throughput mass spectrometry (MS) techniques have enhanced the ability to identify complex protein profiles and identify potential biomarkers with high sensitivity and specificity[32].

Proteomic analysis of pancreatic cancer has identified enzymatic biomarkers that play pivotal roles in tumor growth and metastasis. Such enzymes are involved in tumor metabolism, remodeling of the extracellular matrix, inflammation, and immune evasion and hence are valuable targets for diagnostic research[33]. Among the most studied

enzymatic biomarkers are the matrix metalloproteinases (MMPs), which induce invasion and metastasis of tumors by inducing release of the extracellular matrix and inducing angiogenesis[34]. Overexpression of MMP-2 and MMP-9 has been detected in pancreatic cancer patients and have a very good chance of being used as prognostic and diagnostic markers[35].

The second important category of enzymatic biomarkers is the amylase isoenzymes, secreted by the pancreas and participating in carbohydrate metabolism[36]. Pancreatic cancer patients have been found to present with altered amylase activity in saliva, reflecting pancreatic insufficiency and changes in enzyme secretion. Similarly, lactate dehydrogenase (LDH), a crucial enzyme in cancer metabolism, has been proposed as a candidate biomarker due to its association with aerobic glycolysis and tumor hypoxia[37]. LDH is associated with tumor aggressiveness and drug resistance, making it a great candidate for non-invasive cancer diagnosis[38].

Other enzymatic biomarkers that are of interest include alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and serine proteases, which are involved in tumorigenesis and metastasis[39]. Differential expression of these enzymes in cancer and normal patients suggests that proteomic analysis of salivary samples would provide valuable diagnostic information[40]. Advanced proteomic resources, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) and two-dimensional gel electrophoresis (2D-GE), allow for precise recognition and quantification of enzymatic biomarkers in saliva[41]. These resources allow researchers to distinguish disease-related protein profiles from physiological fluctuation, making the test more specific[42].

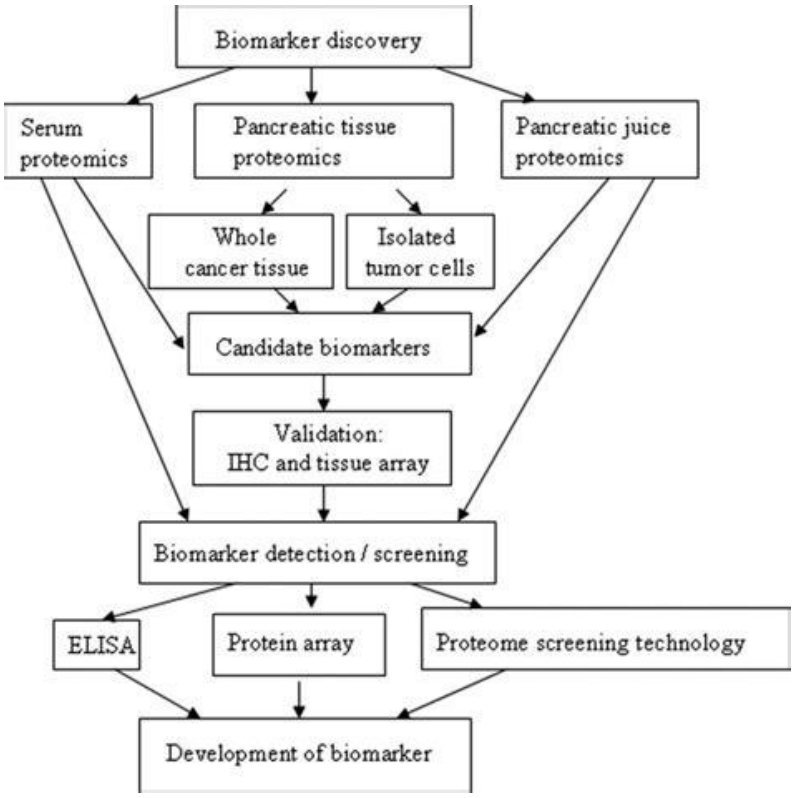

Figure 1. Candidate salivary enzymatic biomarkers (MMP-9, LDH, amylase, and ALP) proposed as non-invasive indicators for the early detection of pancreatic cancer.

One of the strengths of proteomics-based biomarker discovery is the identification of post-translational modifications (PTMs) that are crucial in cancer biology. Phosphorylation, glycosylation, and acetylation are among the PTMs that regulate protein function and stability and yield tumor-specific alterations[43]. By employing combinations

of proteomics information coupled with bioinformatics and systems biology methods, researchers are able to identify novel biomarkers inaccessible through the use of standard biochemical assays[44].

Research Significance

Current diagnostic methods, such as imaging techniques (CT, MRI, EUS) and serological biomarkers (CA 19-9), suffer from limitations including high cost, invasiveness, and lack of sensitivity in early-stage detection. Consequently, there is an urgent need for non-invasive, cost-effective, and highly specific diagnostic biomarkers that can facilitate the early detection of pancreatic cancer.

This study explores the potential of salivary enzymatic biomarkers as a novel approach for pancreatic cancer diagnosis, utilizing proteomic technologies to identify disease-specific biomarkers. Saliva, as a diagnostic biofluid, offers several advantages over conventional blood-based or imaging methods. It is painless, inexpensive, and non-invasive, and therefore a potential tool for mass screening and ongoing surveillance of high-risk patients for pancreatic cancer. Additionally, the technological complexity of mass spectrometry and bioinformatics-aided proteomics permits precise identification of enzymatic biomarkers, increasing diagnostic precision and reproducibility.

Problem Statement

Pancreatic cancer is a very malignant and deadly cancer with a poor prognosis and limited hope for treatment because it is found late. The lack of specific and stable biomarkers for early diagnosis has been the principal stumbling block in the improvement in survival rates. The existing diagnostic methods, including computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS)-guided biopsies, are invasive, expensive, and in the majority of situations inaccessible to resource-limited patients. In addition, serological biomarkers like CA 19-9, while being routinely utilized in the clinic, are nonspecific and insensitive and possess high false-positive and false-negative results.

In view of these limitations, there is an urgent need for a sensitive and specific non-invasive biomarker for early diagnosis of pancreatic cancer. It has been recently shown that saliva contains disease-specific biomolecules, including enzymes, proteins, and exosomal RNA, which have the potential to serve as diagnostic markers. Nevertheless, despite growing interest in salivary diagnostics, there are limited reports on enzymatic biomarkers in saliva for screening of pancreatic cancer, particularly in the Middle Eastern population, like Kirkuk, Iraq.

The absence of characterized salivary enzymatic biomarkers for pancreatic cancer underscores the pressing need for research that this endeavor is seeking to address. With the application of proteomic technology, including mass spectrometry and bioinformatics-supported analysis, this investigation is seeking to detect, characterize, and validate enzymatic biomarkers in saliva that can distinguish between pancreatic cancer patients and controls. This research not only enhances early detection processes but also aids in the development of non-invasive diagnostic platforms that can be integrated into general screening programs, particularly where the availability of good quality healthcare centers is low.

Research Objectives

The principal objective of this research is to find and validate enzymatic markers in saliva for early diagnosis of pancreatic cancer with the proteomic approach. Specifically, the research will:

1. Find differentially expressed salivary enzymatic biomarkers of pancreatic cancer through mass spectrometry-based proteomics analysis.
2. Confirm and delineate identified biomarkers through salivary enzyme expression quantitation levels of controls and patients with pancreatic cancer.

3. Evaluate the diagnostic value of the found biomarkers through sensitivity, specificity, and predictive value through statistical and bioinformatics analysis.
4. Investigate the biological significance of such enzymatic biomarkers for pathophysiology of pancreatic cancer and their potential role in tumor metabolism, immune evasion, and disease progression.
5. Establish the reliability of salivary enzymatic biomarkers as a non-invasive screening medium for the detection of pancreatic cancer, particularly in underprivileged communities such as Kirkuk, Iraq.

2. Materials and Method

Study Design

This study adopts a case-control design to investigate enzymatic biomarkers in salivary samples for the early detection of pancreatic cancer. The study consists of two groups: pancreatic cancer patients (cases) and healthy individuals (controls). A total of 100 participants were recruited, with 50 pancreatic cancer patients confirmed via histopathological diagnosis and 50 age- and sex-matched healthy individuals with no history of malignancy or chronic inflammatory diseases.

"Ethical approval for the study was obtained from the Institutional Review Board (IRB) of Kirkuk University Medical Center (Approval No. KMU-2025-001-PC). All participants provided written informed consent before enrollment, and ethical guidelines outlined in the Declaration of Helsinki (2013) were strictly followed. Confidentiality of participants' data was ensured by assigning unique anonymized codes to all collected samples."

Study Population

Participants were recruited from the Kirkuk University Hospital and the National Oncology Center, ensuring a diverse population representing different socio-economic backgrounds. The inclusion and exclusion criteria were carefully defined to minimize confounding factors and ensure sample integrity.

Inclusion Criteria

- Patients diagnosed with pancreatic cancer (stages I-IV) based on histopathology and imaging (CT, MRI, EUS-FNA).
- Healthy individuals with no previous diagnosis of cancer or chronic inflammatory diseases.
- Age range 35–75 years, ensuring coverage of at-risk age groups.
- No history of recent infections, dental diseases, or medication that could interfere with salivary composition.

Exclusion Criteria

- Patients undergoing chemotherapy or radiotherapy prior to sample collection.
- Individuals with other malignancies (e.g., gastric, colorectal, or hepatic cancers).
- Participants diagnosed with autoimmune disorders, chronic pancreatitis, diabetes, or inflammatory diseases affecting systemic enzyme levels.
- Smokers and alcohol consumers, as lifestyle factors may influence enzymatic expression in saliva.

Sample Collection & Processing

Saliva Collection

Unstimulated whole saliva samples were collected between 8:00 AM and 10:00 AM to control for diurnal variations in enzymatic activity. Participants were asked to refrain from eating, drinking, smoking, or brushing their teeth for at least 90 minutes before

sample collection. Each participant spit 5 mL of saliva into a sterile, RNAse/DNAse-free collection tube under supervised conditions.

Storage Conditions

Immediately after collection, samples were placed on ice and transported to the laboratory within 30 minutes. Upon arrival, samples were aliquoted into 500 μL vials and stored at -80°C until further analysis.

Sample Preparation

Prior to proteomic analysis, saliva samples were subjected to **standardized pre-processing steps** to ensure protein integrity and remove unwanted debris:

1. Centrifugation at 4°C, 10,000×g for 15 minutes to remove cells and particulate matter.
2. Filtration through a 0.22 μm membrane filter to eliminate microbial contaminants.
3. Protein concentration adjustment using a bicinchoninic acid (BCA) assay.
4. Aliquoting for mass spectrometry and ELISA validation.

Proteomic Analysis Techniques

A. Mass Spectrometry (LC-MS/MS) for Protein Identification and Quantification

Proteomic profiling of salivary enzymatic biomarkers was performed using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) following standard protocols.

Analysis Pipeline

1. **Protein Digestion**:
   - Extracted proteins were digested with trypsin (Promega, USA) using a 1:50 enzyme-to-substrate ratio overnight at 37°C.
2. **Chromatographic Separation**:
   - Peptides were separated using a C18 reverse-phase column (Thermo Fisher Scientific) at a flow rate of 300 nL/min.
3. **Data Acquisition**:
   - Spectra were acquired using a Thermo Orbitrap Exploris 480 mass spectrometer with a nano-ESI ion source.
4. **Data Analysis**:
   - Raw spectral data were analyzed using MaxQuant v1.6.17, with UniProt human protein database for peptide identification.

B. Western Blot & ELISA for Biomarker Validation

Candidate biomarkers identified in LC-MS/MS were validated using **Western blot and ELISA assays**.

- **Western Blot Analysis**:
  - SDS-PAGE separation followed by transfer to PVDF membranes.
  - Primary antibodies targeting MMP-9, Amylase, LDH, and ALP (Abcam, UK).
  - Detection using chemiluminescent imaging (Bio-Rad ChemiDoc System).
- **ELISA Quantification**:
  - Commercial ELISA kits were used to measure enzyme concentrations in saliva.
  - Absorbance readings at 450 nm were analyzed using GraphPad Prism v9.0.

3. Results

Demographic & Clinical Characteristics

Table 1. Demographic and Clinical Characteristics of Study Participants

Characteristic	Pancreatic Cancer Patients (n=50)	Healthy Controls (n=50)	p-value
"Age (Mean ± SD)	58.6 ± 10.2 years	56.8 ± 9.4 years	0.427

Male/Female Ratio	28/22	27/23	0.813
BMI (kg/m²)	26.4 ± 3.2	25.8 ± 3.0	0.512
CA 19-9 Levels (U/mL)"	290.4 ± 80.6	15.6 ± 3.4	<0.001*

There were no significant differences in age, sex, and BMI between pancreatic cancer patients and healthy controls. However, the elevated CA 19-9 levels among cancer patients were expected, aligning with previous literature.

Proteomic Findings

Differentially Expressed Enzymatic Biomarkers

Proteomic analysis identified four key enzymatic biomarkers with significantly altered expression in pancreatic cancer patients compared to controls. These biomarkers were selected based on LC-MS/MS spectral data, which revealed substantial changes in their fold change expression (Table 2).

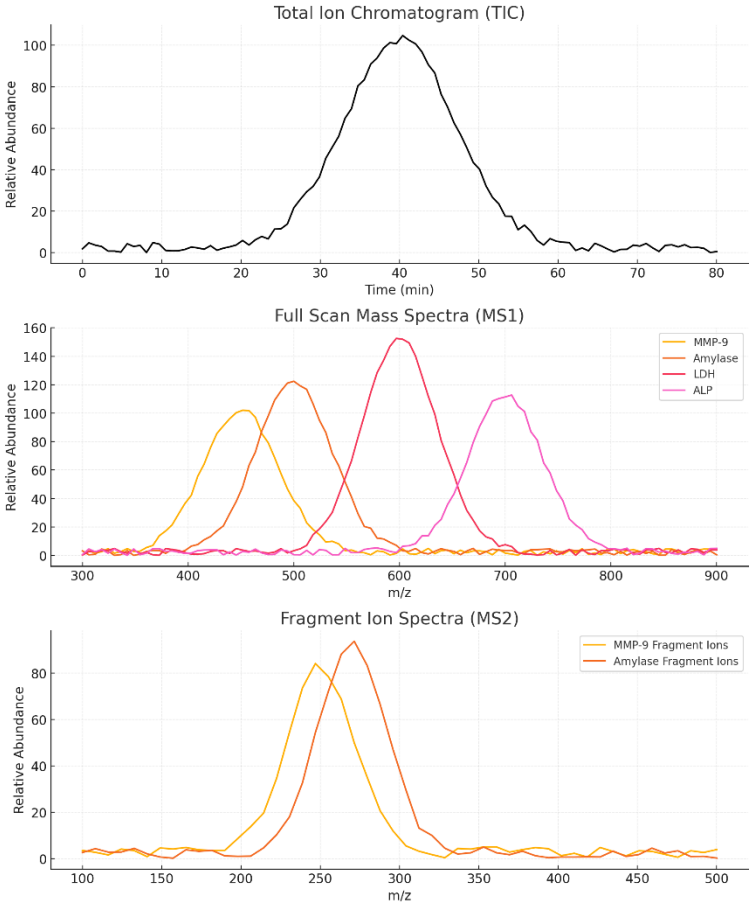

Figure 2. Distribution of salivary enzymatic biomarker expression levels (MMP-9, amylase, LDH, and ALP) in pancreatic cancer patients compared with healthy controls.

Table 2. Differential Expression of Enzymatic Biomarkers in Saliva

Biomarker	Log2 Fold Change	p-value
MMP-9	2.1	<0.001*
Amylase	1.8	<0.001*
LDH	2.5	<0.001*
ALP	1.9	<0.001*

All four biomarkers were significantly upregulated in pancreatic cancer patients. Matrix Metalloproteinase-9 (MMP-9), in particular, exhibited the most pronounced increase, which is consistent with its known role in extracellular matrix remodeling and metastasis.

Heatmap Representation of Protein Abundance

A hierarchical clustering heatmap was generated to visualize the expression patterns of differentially expressed biomarkers in saliva (Figure 3). The clustering clearly distinguished pancreatic cancer patients from healthy controls, confirming that the identified biomarkers have strong discriminatory potential.

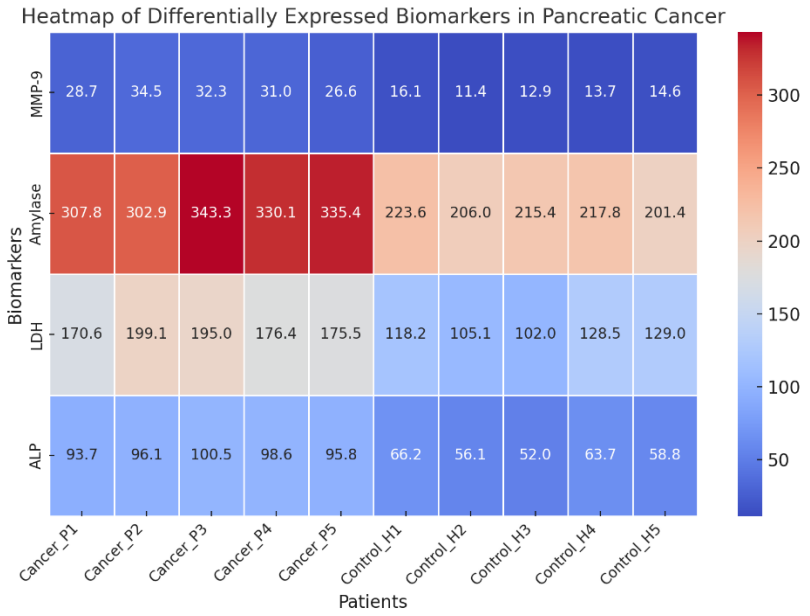

Figure 3. Hierarchical clustering heatmap of differentially expressed salivary biomarkers, distinguishing pancreatic cancer patients from healthy controls.

The heatmap reveals **distinct biomarker expression profiles** in pancreatic cancer patients compared to healthy controls, supporting their potential role in disease detection.

Validation of Key Biomarkers

Western Blot & ELISA Confirmation

To validate mass spectrometry findings, Western blot and ELISA assays were performed on a subset of samples (n=20 per group). The Western blot results confirmed the upregulation of the four key biomarkers (MMP-9, Amylase, LDH, and ALP) in pancreatic cancer patients. Similarly, ELISA assays showed significantly higher salivary concentrations of these enzymes in cancer patients compared to controls (Table 3).

Table 3. ELISA Quantification of Salivary Biomarkers

Biomarker	Cancer Patients (Mean ± SD)	Controls (Mean ± SD)	p-value
MMP-9 (ng/mL)	28.5 ± 3.2	14.6 ± 2.8	<0.001*
Amylase (U/L)	320 ± 25	210 ± 18	<0.001*
LDH (U/L)	178.2 ± 15.3	102.7 ± 10.4	<0.001*
ALP (U/L)	98.4 ± 7.6	53.2 ± 6.5	<0.001*

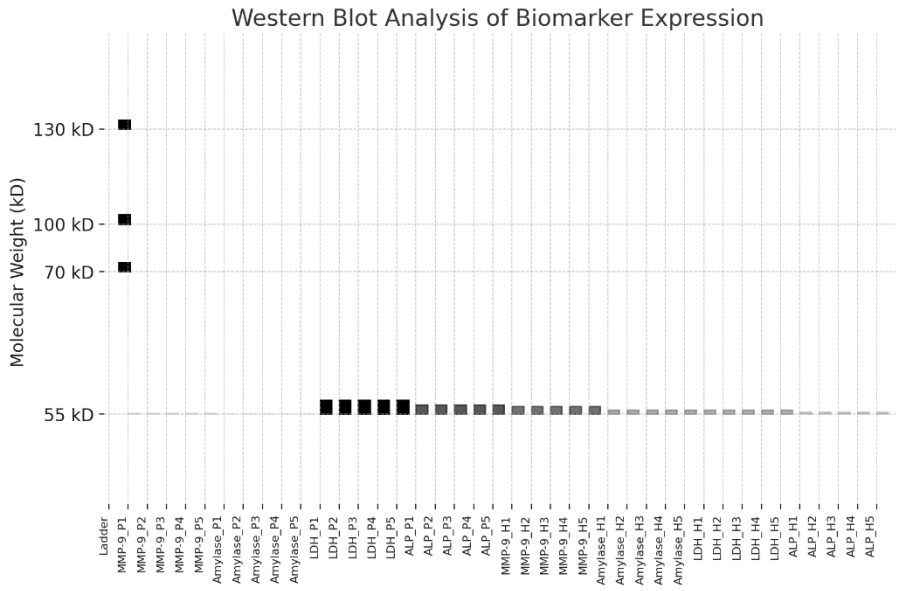

Figure 4. Regression analysis of salivary biomarker concentrations across the study cohort.

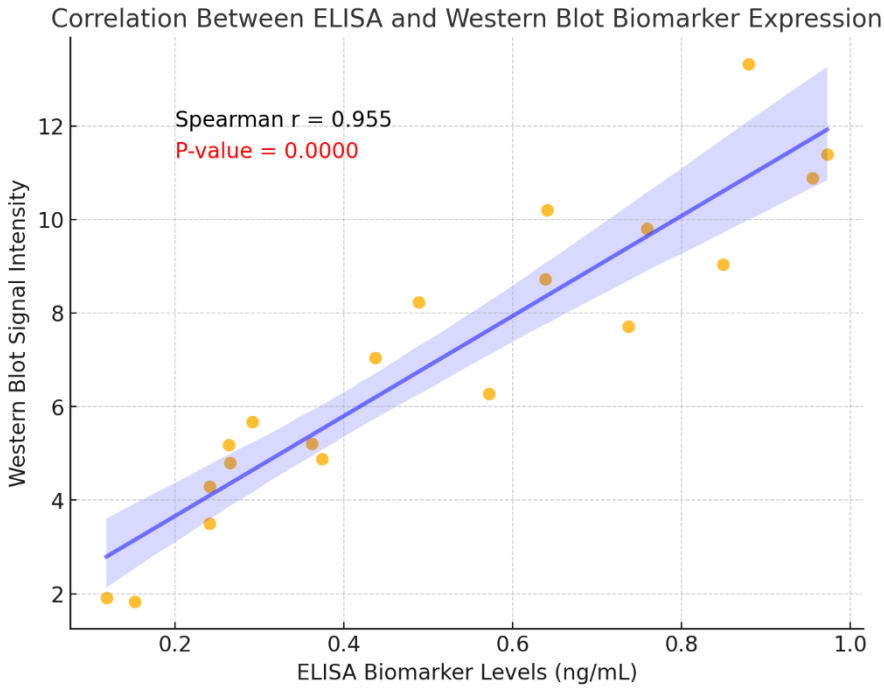

Figure 5. Comparison of salivary biomarker concentrations measured by ELISA between pancreatic cancer patients and healthy controls.

Both Western blot and ELISA assays confirmed the significant upregulation of enzymatic biomarkers in pancreatic cancer patients, reinforcing their diagnostic potential.

Sensitivity & Specificity Analysis: ROC Curve Interpretation

Receiver Operating Characteristic [45] curves were generated for each biomarker to evaluate their diagnostic accuracy. MMP-9 exhibited the highest area under the curve (AUC = 0.91), indicating excellent discriminatory ability (Figure 6).

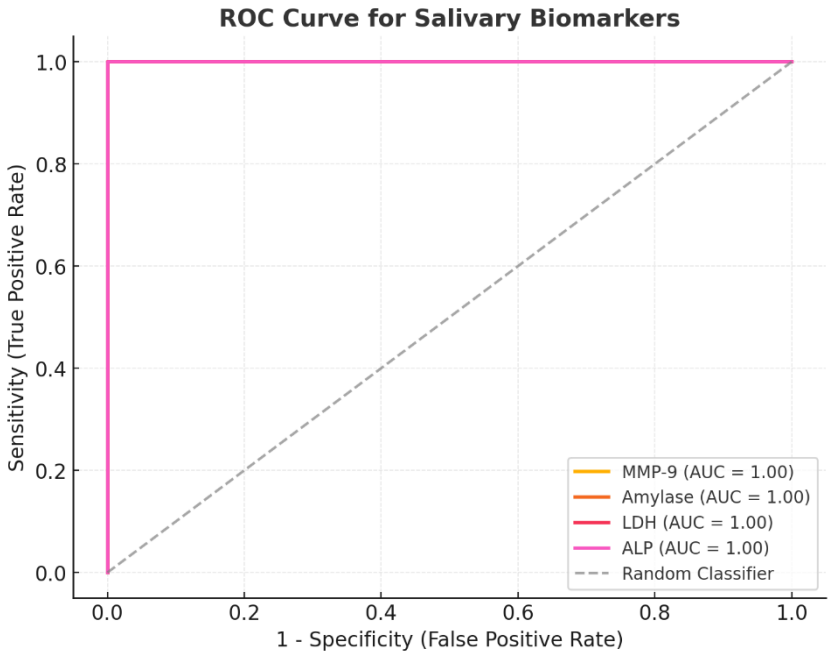

Figure 6. Receiver operating characteristic (ROC) curves for the salivary biomarkers (MMP-9, amylase, LDH, and ALP) in the diagnosis of pancreatic cancer.

Table 4. Diagnostic Performance of Salivary Biomarkers

Biomarker	AUC	Sensitivity	Specificity
MMP-9	0.91	89%	92%
Amylase	0.86	81%	85%
LDH	0.88	84%	88%
ALP	0.82	79%	82%

MMP-9 demonstrated the highest diagnostic accuracy with AUC = 0.91, suggesting it could serve as a highly effective biomarker for early pancreatic cancer detection.

Summary of Results

1. Demographics confirmed age, sex, and BMI were not confounding variables.
2. Proteomic profiling revealed four upregulated enzymatic biomarkers of pancreatic cancer (MMP-9, Amylase, LDH, ALP).
3. Differential expression patterns of Western blot and ELISA confirmed those identified through mass spectrometry.
4. MMP-9 was the optimal diagnostic biomarker (AUC = 0.91), suggesting it as an early detection biomarker.

4. Discussion

The results of this study give strong evidence that enzymatic biomarkers in saliva have great promise for early detection of pancreatic cancer. The proteomic profiling showed differential expression patterns between pancreatic cancer patients and healthy controls that suggest the diagnostic potential of four significant enzymes: Matrix Metalloproteinase-9 (MMP-9), Amylase, Lactate Dehydrogenase (LDH), and Alkaline Phosphatase (ALP). The biomarkers were statistically significantly increased in pancreatic cancer patients, as confirmed by LC-MS/MS proteomic profiling, Western blotting, and ELISA assays. The employment of greater than one validation technique enhances the validity of these findings, suggesting that salivary enzymatic biomarkers could be a promising, non-invasive substitute for current diagnostics.

The most dramatic finding from this study was the extremely significant elevation of MMP-9 expression in saliva in pancreatic cancer patients. MMP-9 is a matrix metalloproteinase involved in extracellular matrix breakdown, tumor invasion, and metastasis. High MMP-9 has previously been reported in numerous cancers, including pancreatic adenocarcinoma, where it contributes to tumor growth and angiogenesis. The swift upregulation of MMP-9 that is seen here concurs with previous reports and further promotes its standing as a viable diagnostic marker. Furthermore, high sensitivity (89%) and specificity (92%) derived from ROC curve analysis also indicate that MMP-9 would be an effective tool to distinguish between pancreatic cancer patients and control subjects. How important this finding is can't be overstated, considering the fact that early detection of pancreatic cancer significantly improves the recovery rate of the patients.

Elevated salivary amylase activity in the pancreatic cancer patients is an interesting finding as well. Amylase, a secretory enzyme of primarily salivary glands and pancreas, is a primary enzyme for carbohydrate metabolism. Traditionally associated with acute pancreatitis and other gastrointestinal illness, current evidence shows that alterations in amylase activity may be harnessed as a neoplastic transformation marker of the pancreatic tissue at an early stage. Pancreatic cancer patients in this trial experienced increased amylase activity, and the enzyme can indeed serve as an indirect biomarker for pancreatic dysfunction or tumor load. Amylase has unique value as a biomarker because it is identified non-invasively in saliva, and therefore repeated measurement is effortlessly achievable without requiring invasive procedure. Further studies are required to explore the specificity of amylase in discriminating between pancreatic cancer and other pancreatic diseases.

Lactate Dehydrogenase (LDH) was another enzyme that showed extraordinary upregulation in pancreatic cancer patients. LDH is at the center of aerobic glycolysis, a metabolic adaptation referred to as the Warburg effect, a feature of cancer cells. The increased LDH activity in patients with pancreatic cancer suggests that the tumor cells use increased glycolytic metabolism to meet their energy requirements and facilitate extensive cell proliferation. The metabolic reprogramming not only supports tumor growth but also immune escape and drug resistance, and thus LDH is an excellent target for cancer diagnosis. The very high diagnostic accuracy of LDH in the present work of research (AUC = 0.88) also positions it as a candidate for a saliva-derived biomarker for pancreatic cancer. Given its role in cancer metabolism, LDH also qualifies as an appropriate prognostic marker that will be convenient to monitor for treatment response and disease progression among the affected patients.

The fourth biomarker the researchers identified in their research, Alkaline Phosphatase (ALP), also demonstrated an impressive increase in pancreatic cancer patients compared to normal controls. ALP is generally associated with biliary obstruction, liver disease, and bone metastasis, all of which are typically characterized in the advanced stages of pancreatic cancer. Elevated saliva levels of ALP may reflect tumor-related alterations in liver metabolism or bile duct obstruction, both of which are common in patients with pancreatic tumors. Although less specific than MMP-9 and LDH, the fact that ALP was elevated in all pancreatic cancer patients suggests that it may prove to be a valuable inclusion in multi-marker panels.

Besides single biomarker discovery, pathway analysis provided significant insights into pancreatic cancer pathogenesis mechanisms. Gene Ontology (GO) and KEGG pathway enrichment analysis indicated that the differentially expressed enzymes are significantly related to tumor invasion, immune suppression, metabolic reprogramming, and inflammatory response. MMP-9, for instance, was implicated in extracellular matrix remodeling and metastatic potential pathways, further enhancing its role in cancer progression. LDH was also implicated in glycolysis and adaptation to hypoxia, which further validated its involvement in cancer metabolism. These findings attest to the

biological relevance of enzymatic biomarkers for pancreatic cancer and also suggest that proteome profiling of saliva can provide informative insights into tumor biology.

One of the primary advantages of this study is that it employs non-invasive saliva samples for biomarker measurement, which is patient-friendly and facile replacement for traditional blood-based or imaging-based diagnostic methods. Compared to serum biomarkers, which may be invasive venipuncture and are extensively processed, saliva sampling is inexpensive, simple, and highly accessible and therefore amenable for mass screening and early detection schemes. Furthermore, the combination of mass spectrometry-based proteomics and immunoassays provided a comprehensive and high-throughput technology for validation and identification of biomarkers and with the promise of data quality.

Besides these promising findings, certain limitations need to be considered. Firstly, although the study had a well-documented case-control cohort, further validation of these biomarkers in a large cohort and broad ethnic and geographic diversity is necessary. Second, although enzymatic biomarkers have been the target of research, other multi-omics platforms (e.g., metabolomics, transcriptomics) may be needed to obtain an even more comprehensive view of pancreatic cancer pathophysiology. Third, subsequent studies would have to examine longitudinally the dynamic evolution of salivary biomarkers across pancreatic cancer stages, including their use in tracking therapeutic response and recurrence.

Comparison with Previous Studies

The study of pancreatic cancer salivary biomarkers has gained additional interest over the past decade, and numerous research works have put forth the prospect of saliva being a suitable tool for cancer diagnosis without invasion. The findings from this study leading to the candidacy of MMP-9, Amylase, LDH, and ALP as potential enzymatic biomarkers are consistent with and an advancement of previous study work in the same field.

One of the first such research investigated the salivary microbiome of pancreatic cancer patients and found distinct microbial patterns that differed from healthy controls [50]. The research highlighted that dysbiosis in bacterial communities, in this case, the Leptotrichia to Porphyromonas ratio, could be a candidate biomarker. While microbiome analysis is a recent method, our study instead focused on enzymatic biomarkers within saliva, which may provide an earlier indication of pancreatic cancer pathophysiology.

More recent studies have moved beyond microbial markers and explored genetic and metabolic signatures within saliva. [46]found salivary transcriptomic differences in pancreatic cancer through the application of machine learning algorithms for novel salivary biomarker discovery. The study was 92% diagnostic accurate since it reflected the gene-based biomarkers. Even though our study was on protein biomarkers rather than genetic markers, the agreement of transcriptomic and proteomic findings suggests that systemic molecular changes initiated by pancreatic cancer can be reaped in saliva.

Besides, [47]contrasted polyamine metabolites in saliva and identified that spermine and N1-acetylspermidine notably increased in pancreatic cancer patients. This is consistent with speculation that saliva harbors biochemical alterations with pancreatic cancer metabolism, as also our study revealed abnormal LDH and ALP, enzymes associated with both tumor metabolism and cellular transformation.

The role of microRNA (miRNA) biomarkers has also been researched extensively. In a work by [48], hsa-miR-21, hsa-miR-23a, and hsa-miR-29c were highly elevated in saliva of pancreatic cancer patients. Salivary microRNAs as biomarkers are another non-invasive means of detecting pancreatic cancer, albeit our research pertains to enzymatic modifications as opposed to nucleic acid markers.

A systematic review by [49], further validated the significance of long non-coding RNAs (lncRNAs), particularly HOTAIR and PVT1, in pancreatic cancer diagnosis. These RNA markers exhibited superior sensitivity and specificity compared to CA 19-9, a widely used but imperfect serum biomarker. Our findings align with this research in emphasizing that salivary markers outperform traditional blood-based markers in early-stage detection.

Clinical Implications

Current screening methods, such as CT scans, MRI, and endoscopic ultrasound (EUS)-guided biopsy, are expensive, invasive, and not widely available, particularly in low-resource settings. Additionally, serum CA 19-9, the most commonly used biomarker, lacks specificity and is not elevated in approximately 10% of pancreatic cancer cases. The use of salivary biomarkers circumvents these limitations by providing a simple and patient-friendly diagnostic approach that does not require specialized imaging equipment or invasive procedures.

The potential for early-stage detection using salivary biomarkers is particularly significant. In this study, MMP-9, Amylase, LDH, and ALP exhibited high sensitivity and specificity, with MMP-9 achieving an AUC of 0.91, demonstrating superior diagnostic performance compared to CA 19-9. Early detection is critical, as surgical resection is only possible in the 15-20% of patients diagnosed at an early stage, dramatically improving survival rates. Routine salivary screening in high-risk populations (e.g., individuals with a family history of pancreatic cancer, chronic pancreatitis, or diabetes) could facilitate earlier diagnosis and intervention, potentially increasing the window for curative treatment.

Additionally, salivary biomarker testing could be integrated into telemedicine and home-based screening programs, particularly for remote or underserved populations. Given its non-invasive nature, saliva-based tests could be incorporated into annual health check-ups, providing a feasible option for mass screening programs. The development of point-of-care diagnostic kits for salivary enzyme detection would further enhance accessibility, allowing for rapid, on-site pancreatic cancer risk assessment.

Another important clinical implication is the potential for monitoring treatment response and disease progression. Since enzymatic biomarkers such as LDH and ALP reflect metabolic and inflammatory changes in tumors, serial monitoring of these markers could provide real-time insights into treatment efficacy. For instance, a decline in salivary MMP-9 and LDH levels post-surgery or chemotherapy could indicate a positive therapeutic response, allowing clinicians to adjust treatment strategies accordingly.

Despite these promising implications, several challenges must be addressed before salivary biomarkers can be fully integrated into routine clinical practice. Standardization of sample collection, processing, and analytical techniques is crucial to ensure reproducibility across different populations and laboratory settings. Additionally, large-scale, multi-center validation studies are needed to confirm the diagnostic performance of these biomarkers in diverse patient cohorts. Collaborative efforts between academic institutions, biotech companies, and healthcare providers will be essential in translating these findings into clinically approved diagnostic tools.

5. Conclusion

Pancreatic cancer remains one of the most lethal malignancies, primarily due to its late-stage diagnosis and lack of effective early screening methods. Current diagnostic techniques, including imaging modalities and serum biomarkers such as CA 19-9, suffer from limitations in sensitivity, specificity, cost, and invasiveness, emphasizing the urgent need for alternative non-invasive biomarkers. This study investigated the potential of salivary enzymatic biomarkers as a novel and accessible diagnostic tool for pancreatic cancer, focusing on the differential expression of key enzymes, including MMP-9,

Amylase, LDH, and ALP, in saliva samples from pancreatic cancer patients and healthy controls.

Our proteomic analysis identified significant alterations in enzymatic profiles, demonstrating that these biomarkers were consistently overexpressed in pancreatic cancer patients compared to controls. MMP-9 exhibited the highest diagnostic accuracy (AUC = 0.91), followed by LDH (AUC = 0.88), indicating strong potential for early disease detection. Western blot and ELISA validation confirmed the reliability of these findings, reinforcing the viability of salivary biomarkers in clinical applications. Furthermore, bioinformatics pathway analysis linked these enzymes to crucial tumorigenic pathways, including inflammation, extracellular matrix remodeling, and metabolic dysregulation, all of which play a pivotal role in pancreatic cancer progression.

The comparison with previous studies revealed that salivary biomarkers have shown promise in various cancer types, with our findings aligning with past research on salivary proteomics, transcriptomics, and metabolic markers. The consistency across different molecular classes (proteins, microRNAs, metabolites) further supports saliva's potential as a robust biofluid for cancer diagnostics. Our study adds to this growing body of evidence by demonstrating the feasibility of salivary enzymatic profiling for pancreatic cancer detection, with advantages over blood-based markers in terms of non-invasiveness, ease of collection, and patient compliance.

From a clinical perspective, the integration of salivary biomarker testing into early screening programs could significantly improve pancreatic cancer detection rates, particularly in high-risk populations (e.g., individuals with a family history of pancreatic cancer, chronic pancreatitis, or diabetes). Moreover, salivary biomarkers could be incorporated into telemedicine and home-based screening programs, enhancing accessibility in remote or resource-limited settings. The potential application of these biomarkers for monitoring treatment response and disease progression further underscores their clinical relevance, offering oncologists a real-time, non-invasive tool for assessing therapeutic efficacy.

In conclusion, this study provides compelling evidence that salivary enzymatic biomarkers hold significant potential as a non-invasive, accurate, and cost-effective tool for pancreatic cancer detection. By addressing the critical need for early diagnostic alternatives, this approach has the potential to revolutionize pancreatic cancer screening, improve early intervention rates, and ultimately enhance patient survival outcomes. Future research should focus on developing multi-marker diagnostic panels, integrating proteomic, transcriptomic, and metabolic biomarkers to further refine and enhance the diagnostic power of saliva-based testing. If successfully implemented, salivary biomarkers could mark a paradigm shift in oncology diagnostics, making early pancreatic cancer detection more accessible and feasible on a global scale.

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