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Fingerprinting the Metabolic and Lipidomic Landscapes of Pancreatic Ductal Adenocarcinoma with MALDI Imaging

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Abstract

Purpose: Metabolic reprogramming plays an integral role in progression, immune evasion, and response to treatment in many cancers, including pancreatic ductal adenocarcinoma (PDAC).

This study aimed to spatially characterize the metabolic and lipidomic alterations in PDAC and its precursor lesion, intraductal papillary mucinous neoplasm (IPMN), compared to normal/non-neoplastic pancreatic tissue.

Experimental details: Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) was employed to perform spatial metabolic and lipidomic profiling of human PDAC, IPMN, and normal/non-neoplastic pancreatic tissues. MALDI-MSI provided multiplexed metabolic images of tissue samples at 200 μ m resolution, allowing spatial characterization of the tumor metabolic landscape.

Results: Our data identified metabolic signatures characteristic of PDAC, with several of these altered in IPMN compared to normal/non-neoplastic tissue. The metabolic fingerprint

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Author contributions

SY, KG, MGG and ZMB developed the concept. SY, DRB and CT performed the MALDI-MSI and analysis of samples. MGG and MD supervised collection of the samples and clinical data. SY analyzed and interpreted the data. SY and MS performed co-registration of the MSI and histology data. SY, BK and YM performed transcriptomic analysis. SY and ZMB structured the results and SY wrote the first draft of the manuscript. All authors edited and approved the final manuscript.

Conflict of Interest

The authors declare no potential conflicts of interest.

of PDAC was characterized by increased levels of taurine, ascorbic acid, and a variety of lipid species, including most of the phosphatidylcholines and sphingomyelins compared to normal/non-neoplastic pancreas tissue, alongside elevated acetylcarnitine, butyrylcarnitine, and phosphatidylcholine (PC 34:3) relative to IPMN; significant reductions were observed in creatine, malate, lysophosphatidylcholine (LPC 16:0). In contrast, IPMN tissues demonstrated a significant reduction of xanthine, arginine, and tryptophan compared to normal/non-neoplastic pancreas tissue. These metabolic signatures were spatially heterogeneous.

Conclusion: Our findings provide novel insights into the metabolic and lipidomic underpinnings of PDAC. Metabolic alterations in PDAC were associated with proliferation, immune evasion, and treatment resistance.

Keywords

PDAC; IPMN; pancreatic tissue; MALDI-MSI; metabolomics; lipidomics

Introduction

Pancreatic ductal adenocarcinoma (PDAC) accounts for over 90% of pancreatic cancer cases. Despite advances in diagnostic techniques and treatment modalities, the overall 5-year survival rate after a PDAC diagnosis is less than 13% [1]. A combination of late-stage diagnosis, and resistance to conventional therapies, make PDAC the third-leading cause of cancer-related deaths in the United States that is projected to become the second leading cause by 2040 [2]. These grim statistics underscore the urgency of unraveling the mechanisms driving PDAC development, and identifying actionable vulnerabilities to develop more effective therapies.

PDAC is characterized by significant metabolic reprogramming, with alterations in key metabolic pathways driving tumor growth and survival [3, 4]. The most frequent mutated genes in PDAC, *KRAS*, *TP53*, *SMAD4*, and *CDKN2A*, regulate signaling pathways that impact central metabolic networks and fuel pancreatic cancer progression [5]. The PDAC microenvironment is characterized by hypovascularity, hypoxia, and nutrient deprivation [6, 7]. To adapt and survive PDAC cells rewire intermediary metabolism, including glucose, amino acid, and lipid metabolism to meet their energetic and biosynthetic demands [3], enhance nutrient acquisition through scavenging and recycling pathways [3], and establish metabolic crosstalk with other components of the tumor microenvironment (TME) [3]. This metabolic reprogramming not only supports tumor growth and survival, but also contributes to therapy resistance [4, 6].

Metabolic studies of PDAC have measured metabolic changes in biofluids such as serum, plasma, or urine samples due to their ready availability [8]. However, biofluids are non-organ specific, and in addition to PDAC-mediated changes also reflect biochemical processes occurring elsewhere in the body. Some studies have utilized PDAC tissue samples for metabolic analysis [9, 10], including high-resolution ¹H magnetic resonance spectroscopy (MRS) of tissue extracts [11, 12], mass spectrometry analysis [7, 9, 10, 13], genome-scale metabolic modeling [14], as well as molecular characterization of metabolic pathways using RNA-seq data [15]. These studies have identified evidence of significant

metabolic reprogramming such as disruptions in lipid metabolism, alterations in amino acid profiles, and the dysregulation of key metabolites that signify altered energy pathways. MRS studies of PDAC samples have also identified low lipid levels alongside elevated lactate and taurine levels [12]. Tissue analysis using HR-MAS NMR metabolomic analysis identified eight metabolites, including glycerophosphocholine, lactate, myo-inositol, methanol, taurine, methylene of lipid, β -glucose, and phosphocholine, as potential biomarkers for PDAC [11]. Mass spectrometry studies have detected upregulated levels of fatty acids and lipids, along with downregulated amino acids in tissue and serum samples of patients with PDAC [9], and increased hydroxypalmitic acid, and decreased phenylalanine, and norleucine levels in resected PDAC tissue [13]. One limitation of MRS and mass spectrometry is the lack of spatial resolution which limits the understanding of tumor heterogeneity. To address this, we employed matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) to image metabolic and lipidomic distributions within tissue sections.

MALDI-MSI leverages the principles of MALDI-MS to spatially ionize and perform mass analysis of biomolecules within tissue sections [16]. A significant advantage of MALDI-MSI is that it does not require prior extraction, purification, separation, or labeling of the samples, thus preserving the spatial integrity of the biological molecules within the tissue [16]. MALDI-MSI is particularly valuable for its ability to measure hundreds of analytes simultaneously, producing detailed mass spectra at each pixel across the tissue section. These spectra plot the mass-to-charge (m/z) ratios of ions against their relative intensities, creating ion density maps for individual molecules to map the spatial distribution and relative abundance of various biomolecular classes, including peptides, proteins, lipids, and metabolites [16]. Because the maps are from co-localized regions, relationships between the spatial distributions of these multiplexed data can be determined. Moreover, the tissue sections analyzed by MALDI-MSI can be further examined using traditional histological techniques, such as hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC), to correlate metabolic findings with histological features [16]. Previous MALDI-MSI studies of PDAC have primarily focused on mapping peptide signatures [17] and glycan profiles [18] to identify potential biomarkers. Here, we used MALDI-MSI for spatial fingerprinting of the metabolic and lipidomic profiles of primary PDAC, intraductal papillary mucinous neoplasm (IPMN), and normal/non-neoplastic pancreas tissue. Our overall hypothesis was that metabolite concentration patterns in PDAC would be significantly different from IPMN and normal/non-neoplastic tissue. MSI allowed us to evaluate the spatial distribution of the multiplexed metabolites, and to relate the distributions to histopathology.

Our analyses identified distinct metabolic and lipidomic signatures specific to PDAC, providing valuable insights into the metabolic and lipidomic landscapes that differentiate PDAC from IPMN and normal pancreatic tissue. The integration of MALDI-MSI data with histopathology was used to relate these metabolic and lipidomic landscapes to tissue morphology. Our findings provide novel insights into the metabolic and lipidomic underpinnings of PDAC, and underscore the need for further investigations to determine whether targeting these pathways has therapeutic benefits.

Materials and Methods

Patient inclusion and tissue sample collection

Flash-frozen tissue specimens were obtained from pancreatic resection specimens after pathologist review from five patients with stage IIB PDAC, five with IPMN, and five normal/non-neoplastic pancreatic tissue samples. Some of the normal/non-neoplastic tissues were derived from non-neoplastic regions adjacent to pancreatic tumors. This strategy was intentionally chosen to allow comparison of the metabolic changes in the cancer specimens with normal and non-specific tissue and metabolic changes that could arise in tumor adjacent tissue. Another reason for selecting adjacent normal tissue is that using both cancer and non-cancer tissues from the same patients allows for better control over tissue processing conditions as well as systemic patient factors. PDAC and IPMN cases were selected if patients had not undergone prior treatment, and if sufficient frozen tissue, including normal adjacent tissue was available. Flash-freezing was performed after pathologist review and sampling of the resection specimen for diagnostic purposes, after which the frozen tissues were placed in standard cryovials and stored in -70°C freezers. Although the exact timing was not recorded for these cases, pancreatic resections typically require approximately 45 minutes to process, with a few additional minutes to take additional specimen for tissue banking. Normal and neoplastic tissues were snap frozen at the same time. The amount of tissue stored for each case was similar, but tissues were not completely uniform in thickness. All the tissues had been frozen for several years before being thawed for cryosectioning for this study. The standard SOP for the biobanking applied to all samples. A standard SOP was followed for all the cryosectioning. The collection and analysis of these tissues was performed in accordance with recognized ethical guidelines and written informed consent, under a protocol approved by the Johns Hopkins Medicine Institutional Review Board that is guided by the ethical principles in the Belmont Report.

Tissue preparation

Frozen pancreatic tissue samples were mounted with Eprelia M-1 Embedding Matrix and cryosectioned at $10\text{ }\mu\text{m}$ thickness using a Leica CM1860-UV cryostat (Leica Biosystems, Wetzlar, Germany, RRID:SCR_025772). The sections were thaw-mounted onto indium tin oxide (ITO)-coated glass slides, Part no. CG-40IN-S115 (Delta Technologies LTD, Loveland, CO) which were washed with hexanes (10 minutes sonication) and ethanol (10 minutes sonication). Two adjacent sections were cut from each tissue sample: one for the MALDI-MSI experiment and another for H&E staining. Each ITO slide accommodated 7 to 8 tissue sections arranged in a randomized order. Prior to matrix application, the ITO slides were transferred to a vacuum desiccator for equilibration to room temperature and dried for at least 20 minutes. The dried slides were sprayed using an HTX M5 pneumatic sprayer (HTX Imaging, Chapel Hill, NC, RRID:SCR_023733), with 10 mg/mL 1,5-diaminonaphthalene (1,5-DAN) in 50% acetonitrile (ACN) with 0.2% trifluoroacetic acid (TFA). Four passes were used, with 0.1 mL/min flow rate, 1200 mm/min nozzle velocity, 2.5 mm track spacing, criss cross (CC) pattern, 2 L/min N_2 flow at 10 psi, with a nozzle height of 40 mm , and no dry time between passes.

MALDI-MS imaging and data analysis

MALDI-MSI experiments were carried out using a RapifleX MALDI TOF/TOF instrument (Bruker Daltonics, Billerica, MA, RRID:SCR_026525) in both positive (m/z 30-1,000 Da) and negative (m/z 30-1,000 Da) ion modes on the same tissue sections by offsetting the laser position by 100 μm within the same raster. flexControl (v 4.1) was used to control the instrument and optimize the acquisition parameters, while flexImaging (v 5.1) was used to set up the MALDI imaging experiments and select the regions of interest for MALDI imaging. Prior to data acquisition, height adjustment (target profile generation), laser focus tuning, and external mass calibration with red phosphorus were conducted to achieve a mass error <5 PPM. A 100 μm imaging laser using the M5 Smartbeam option with 200 shots per pixel at 10 kHz was selected to generate 200 μm sized pixels, and the laser fluence was optimized for each imaging experiment. All imaging data were loaded into SCiLS Lab (v2023b Pro, Bruker Daltonics, RRID:SCR_014426) using the default settings with the parameters from FlexImaging. For all pixels defined within each tissue sample, peak intensities were averaged and normalized by total ion current. After imaging experiments were conducted, the slides were vacuum-sealed and stored at -80°C until further use.

MS/MS measurements

MS/MS fragmentation analysis was utilized to confirm the preliminarily identified m/z features. For comparison with pure standard compounds, N-acetylglutamic acid, histidine, malate, creatine, N-methylnicotinamide, citrate, xanthine, taurine, ascorbate were purchased from Sigma-Aldrich (Burlington, MA). The isolation width for the parent ion was set to 1.00 Da width or narrower to fragment isolated single m/z peaks. On-tissue MS/MS spectra were acquired in tissue regions with high detected intensity of the parent ion peak, selected from the respective MALDI ion images displayed in SCiLS Lab. MS/MS spectra from collision induced dissociation (CID) with nitrogen were acquired directly from the pancreatic tissue samples using a timsTOF fleX MALDI-2 instrument (Bruker Daltonics, Billerica, MA, RRID:SCR_023615) in both positive and negative ion modes for the identification of metabolites. Spectra were collected using timsControl (v 5.1.1). Prior to data acquisition, height adjustment (target profile generation), laser focus tuning, and external mass calibration with red phosphorus were conducted to achieve a mass error <1 PPM. A 50 μm custom laser using the single M2 Smartbeam option with 1000 shots per pixel at 1 kHz was selected for profiling, and the laser fluence, collision energy (30-50 eV), and isolation width were optimized for each m/z . On-tissue MS/MS spectra were acquired in tissue regions with high detected intensity of the parent ion peak, selected from the respective SCiLS ion images. Spectra were saved and analyzed in DataAnalysis (v 6.1) and ChemDraw (v 20.0.0.41, RRID:SCR_016768) to confirm diagnostic peak fragmentation and assign an identity. Briefly, bonds with lower bond dissociation energy were targeted for breakage in ChemDraw to computationally determine fragment m/z values. Such m/z values were compared to the MS/MS spectra. If sufficient diagnostic fragments existed in the spectra, the identification was considered confirmed. For metabolites where MS/MS spectra were acquired from both tissue samples and reference standards, dot product similarity scores were calculated to assess spectral matching, with higher scores indicating greater confidence in compound identification. The dot product was calculated using the formula:

$$\text{Dot Product} = \frac{\sum(I_1 \times I_2)}{\sqrt{\sum(I_1^2)} \times \sqrt{\sum(I_2^2)}}$$

where I_1 and I_2 are the normalized intensities of matching fragment ions in the sample and reference spectra, respectively. The dot product was calculated for all fragment ions exceeding a normalized intensity threshold of 2% in both tissue and standard spectra.

H&E staining

A standard H&E protocol was employed for staining tissue sections. Sections were sequentially immersed in 95% ethanol, 70% ethanol, and distilled water for 30 seconds each, followed by staining in hematoxylin for 3 minutes. After rinsing in distilled water, sections were dehydrated in 70% ethanol and 95% ethanol for 30 seconds each, then stained in eosin for 1 minute. The sections were subsequently dehydrated in 95% ethanol and 100% ethanol for 30 seconds each and cleared in xylene for 2 minutes. Finally, cover slips were applied using xylene based mounting media (Eupredia #4112) and allowed to dry. High-resolution digital images of the H&E stained tissue sections were obtained using a Hamamatsu NanoZoomer S210 Digital slide scanner (RRID:SCR_023760).

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from frozen tissue using a Qiagen kit (Qiagen #79656) and RNeasy mini column kit (Qiagen #74106) following the manufacturer's protocol. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad #1708891). qRT-PCR was performed from cDNA using IQ SYBR Green supermix (Bio-Rad #1725121) and gene specific primers in the PCR machine (Bio-Rad, RRID:SCR_026760). The primer sequence for the variant 1 human SLC6A6 gene (NM_003043) was used. 5'-aminolevulinic synthase 1 (ALAS1, NM_000688.6) was used as a housekeeping gene. Fold change was determined using $2^{-\Delta\Delta CT}$.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 6.0, GraphPad Software, RRID:SCR_002798) and MetaboAnalyst 6.0 (www.metaboanalyst.ca, RRID:SCR_015539). The normalized peak intensities were used as an input parameter to carry out Partial Least Squares Discriminant Analysis (PLS-DA) in the MetaboAnalyst web tool. PLS-DA score plots were generated to visualize group separations, with ellipses representing 95% confidence intervals for each group. Heatmaps were generated based on the Euclidean distance measure and the Ward clustering algorithm. Correlation heatmaps were constructed using pairwise Spearman rank correlation analysis between samples, allowing visualization of metabolomic profile similarity and clustering behavior across tissue groups. To determine the statistical significance of the quantified data, an unpaired two-tailed Student's t-test was performed. p-values < 0.05 were considered significant unless otherwise stated. To evaluate significance, values from all the pixels were consolidated into a single mean value for each sample. Receiver Operating Characteristic (ROC) curve analysis was performed for the significantly dysregulated identified compounds to evaluate their diagnostic performance in

distinguishing PDAC from non-malignant pancreatic tissues. Metabolites with Area Under the Curve (AUC) > 0.85 were considered highly discriminative. Bar plots were generated for all the significant compounds (p -value < 0.05) to show differences across the pancreatic tissue samples. qRT-PCR gene expression data were analyzed using one-way ANOVA followed by Šídák's multiple comparisons test to assess statistical significance across sample groups.

Results

Metabolic fingerprinting of tissues

A total of 15 pancreatic tissue samples were analyzed, comprising 5 PDAC, 5 IPMN, and 5 normal/non-neoplastic samples. Clinicopathological details of the patient samples used in this study are summarized in Supplementary Table S1. Spatial mapping of metabolites and lipids in PDAC was achieved using MALDI-MSI using the workflow presented in Figure 1A that outlines tissue sample preparation, acquisition of MALDI-MSI data, and subsequent spatial data analyses.

Representative examples of MALDI spectra obtained from PDAC, IPMN, and normal/non-neoplastic tissue samples are presented in Figure 1B. These examples demonstrate the metabolic differences across the three groups. A data matrix consisting of 970 features in positive ion mode and 950 features in negative ion mode was generated from the analysis. 135 significantly dysregulated m/z features (p < 0.05), consisting of 42 features detected in negative ion mode and 93 detected in positive ion mode were identified.

Significantly dysregulated m/z features were initially identified using the Human Metabolome Database (HMDB, RRID:SCR_007712) and Lipid Maps (RRID:SCR_003817), providing preliminary annotations of metabolites and lipids. To confirm these preliminary annotations, MS/MS analysis was conducted, to validate the identification of these analytes. All identifications were made from on-tissue MS/MS fragmentation analysis and are based on diagnostic ions detected in this analysis which are shown in the Supporting Information in Supplementary Figure S1. For metabolites, we additionally performed comparative analysis of MS/MS fragmentation from standard compounds that were compared to on-tissue MS/MS fragmentation data (Supplementary Figure S1). For metabolites where MS/MS spectra were acquired from both tissue samples and reference standards, dot product similarity scores were calculated to assess spectral matching, with higher scores indicating greater confidence in compound identification. Identifications were considered confirmed when all fragment ions from standard compounds were detected in on-tissue MS/MS fragmentation and the dot product similarity between the on-tissue and standard spectra, calculated without the precursor (parent) ion, was ≥ 0.8 . If the dot product value without the precursor ion was < 0.8, even when diagnostic fragments were present, the compound was classified as a tentative identification. All structural identifications were carried out manually, by matching detected on-tissue MS/MS fragments with possible fragmentations occurring in chemical structures under investigation. Lipids were classified as confirmed when the precursor m/z matched the theoretical mass within ± 0.02 Da and the on-tissue MS/MS fragmentation pattern showed the expected head-group and fatty acyl chain-derived fragment ions. When the precursor m/z deviated

from the theoretical mass by more than ± 0.02 Da, but the MS/MS spectrum still showed the characteristic head-group and acyl-chain fragment ions expected for the candidate lipid and no alternative lipid species could account for the observed fragments, the identification was reported as tentative. Representative MS/MS spectra for selected compounds, demonstrating their characteristic fragmentation patterns, are presented in Figure 2. Among the validated compounds, 9 were identified in negative ion mode and 28 in positive ion mode. The MS/MS analysis, conducted to validate the identification of all identified analytes in this study, is presented in Supplementary Figure S1, showcasing the fragmentation patterns that were critical for confirming their molecular structures and ensuring accurate annotation of compounds. These validated metabolites included key intermediates involved in lipid metabolism, energy pathways, and redox regulation.

To evaluate the adequacy of the sample size, we performed a post hoc power analysis across the 135 significantly altered m/z features. Power was calculated using the observed means, standard deviations, and pooled standard deviations (with $n = 5$ per group and $\alpha = 0.05$). The results showed that power values ranged from 0.53 to 1.00, with a mean power of 0.73 and median power of 0.70. Notably, 67 of the 135 features (~50%) achieved power ≥ 0.70 , suggesting that our study was sufficiently powered to detect moderate-to-large biological effects despite the small group size.

Heat-map analysis was conducted to visualize the individual abundance profiles of the dysregulated m/z features across the PDAC, IPMN, and normal/non-neoplastic pancreas tissue samples, as shown in Figures 3A and B. The heat map uses a color-coded representation, where color temperature indicates metabolite abundance with a warmer color for higher abundance, and a colder color for lower abundance. A clear difference was evident in the abundance levels of compounds among the three groups, with several metabolites showing marked dysregulation in PDAC tissue compared to both IPMN and normal/non-neoplastic tissue, identifying the significant metabolic reprogramming that occurred in PDAC.

To characterize the metabolomic variations in normal/non-neoplastic, IPMN, and PDAC tissue, partial least squares discriminant analysis (PLS-DA) was performed. The PLS-DA results, presented in Figures 3C and D, revealed distinct clustering patterns that identified clear separation between PDAC and normal/non-neoplastic tissue and the overlap of IPMN tissue with normal/non-neoplastic and PDAC tissue in both positive (Figure 3C) and negative (Figure 3D) ion modes. The overlap of IPMN with normal/non-neoplastic and PDAC tissue reflects the transitional metabolic phenotype of IPMN, bridging the characteristics of benign and malignant states. Each dot in the score plots corresponds to an individual tissue sample, representing its unique metabolic profile. In the positive ion mode, principal component 1 (PC1) accounted for 45% of the variance, and principal component 2 (PC2) accounted for 25% of the variance. Similarly, in the negative ion mode, PC1 and PC2 accounted for 49% and 23% of the variance, respectively.

To further examine the relationships among tissue samples based on their metabolic/lipid profiles, Spearman rank correlation analysis was performed using the normalized intensities of all significantly altered metabolites. The resulting heatmap illustrated the

degree of similarity between individual samples and revealed distinct clustering patterns corresponding to tissue classification (Supplementary Figure S2). PDAC samples exhibited strong intra-group correlation and formed a distinct cluster, clearly separated from both IPMN and normal/non-neoplastic tissues, reinforcing their distinct metabolic signature.

ROC curve analysis was conducted to determine the diagnostic performance of the significantly dysregulated metabolites in PDAC. Nearly all of these compounds achieved AUC values exceeding 0.85, demonstrating strong diagnostic accuracy in distinguishing PDAC from non-malignant pancreatic tissues (Supplementary Table S2).

Spatial correlation of histological features and metabolic profiles in PDAC

H&E staining combined with MALDI-MSI provided a framework to correlate histopathological features with metabolic heterogeneity in the tissues. The histopathological analysis of pancreatic tissue sections, visualized through H&E staining, demonstrated marked differences in tissue architecture between normal/non-neoplastic, IPMN, and PDAC samples as shown in the representative example for taurine in Figure 4. Normal/non-neoplastic tissue exhibited well-preserved acinar structures, while PDAC tissue displayed disrupted architecture with dense desmoplastic stroma, characteristic of the PDAC TME. Elevated taurine levels localized within regions of dense desmoplastic stroma and invasive tumor clusters in PDAC tissue. Some normal/non-neoplastic pancreatic tissues analyzed in this study were obtained from regions adjacent to pancreatic cancer. One challenge in comparing tumor and normal/non-neoplastic pancreatic tissues is that while normal/non-neoplastic pancreata are predominantly composed of acinar tissue, PDAC exhibits a dense tumor stroma microenvironment that includes inflammatory cells, fibroblasts, and other components. Even with no tumor involvement, the histology of the pancreas is often not entirely normal, especially in older individuals where features such as PanIN, mild atrophy, fatty replacement, and small areas of acinar-to-ductal metaplasia (ADM) may be observed. Histological images for all normal/non-neoplastic pancreatic samples are shown in Supplementary Figure S3.

Spatial maps of key metabolites together with the corresponding H&E staining are presented in Figure 5 for all three groups of tissues. Spatial metabolic heterogeneity was evident in normal/non-neoplastic tissue, IPMN and PDAC as seen in the corresponding metabolic maps of taurine, acetylcarnitine, creatine, malate, ascorbic acid, and lipidomic maps of phosphatidylcholine (PC) 34:3, PC 32:0, and m/z 835.64 (tentatively identified as sphingomyelin SM 42:2;O2). The spatial heterogeneity in normal/non-neoplastic tissue was structured and closely followed tissue morphology in the H&E sections. Unstructured metabolic heterogeneity became evident in IPMN, and was most evident in PDAC. To quantitatively support these visual observations, we performed Haralick feature analysis on the ion images and determined values for the feature ‘homogeneity,’ which provides an inverse index of heterogeneity [19]. A decrease in homogeneity values from normal/non-neoplastic to PDAC (Supplementary Figure S4) quantitatively confirmed the increased heterogeneity observed visually. One advantage of MSI is the ability to investigate the relationship between the spatial distributions of metabolites in the multiplexed data. The images in Figure 5 clearly demonstrate the spatial heterogeneity of each metabolite, and

the lack of overlap of this spatial heterogeneity for the different metabolites and lipids that occupied distinct spaces in the tissues.

Metabolic and lipidomic alterations in PDAC

MALDI-MSI ion maps of selected analytes, presented in Figure 6, provide a detailed visualization of their spatial distribution within the analyzed tissue samples. These maps are accompanied by the corresponding bar plots that summarize the relative abundances of these compounds across different tissue types. MALDI-MSI ion maps and bar plots for the most significantly dysregulated ions are provided in Supplementary Figures S5 and S6, respectively.

Untargeted metabolomic and lipidomic analyses identified significant changes in the metabolite and lipid profiles across normal/non-neoplastic, IPMN, and PDAC tissue in both positive and negative ion modes. Lipid dysregulation was predominantly observed in the positive ion mode, whereas MALDI-MSI conducted in the negative ion mode identified significant dysregulation of metabolites. Dot product similarity scores were calculated both with and without the precursor ion as presented in Supplementary Table S3. Dot product values ≥ 0.8 without the precursor ion were considered as reliably identified. These were taurine, ascorbic acid, creatine, xanthine and malate. The remaining m/z values, specified as tentative identifications, included citrate (m/z 191.02), N-methylnicotinamide (m/z 137.07), histidine (m/z 154.06), N-acetylaspartate (m/z 174.03), and N-acetylglutamate (m/z 188.06). In PDAC, taurine, ascorbic acid, and tentatively assigned histidine, N-acetylaspartic acid and N-acetylglutamic acid were significantly ($p < 0.05$) upregulated, while malate, creatine and PE 36:3 were significantly ($p < 0.05$) downregulated compared to normal/non-neoplastic tissue. Additionally, xanthine levels were significantly decreased in IPMN samples compared to normal/non-neoplastic pancreatic tissue.

In the positive ion mode, we observed dysregulation of the carnitine system and lipid species across normal/non-neoplastic, IPMN, and PDAC tissue samples. PDAC tissues exhibited the most pronounced changes, with significant alterations observed in several key lipid classes, including phosphatidylcholines (PCs), sphingomyelins (SMs), and lysophosphatidylcholines (LPCs).

Lipid species that were significantly dysregulated in our study included, PC 34:3, PC 38:3, SM 36:1;O2, LPC 16:0, PC 32:0, PC 28:3;O3, SM 34:1;O2, PC 32:1, PC 28:2;O3, SM 34:2;O2, PC 30:4, PC 34:2, PC 34:1, PC 34:0, PC 36:3, PC 36:1, PC 38:4, PC 36:3;O3, PC 40:6, PC 38:3, SM 42:2;O2, PC 40:5, PC 38:2, SM 42:1;O2 and PC 40:4. In addition to lipid alterations, several metabolites were also significantly dysregulated. The classification of these analytes as confirmed or tentative identifications is provided in Supplementary Tables S4a and S4b, respectively. Of the dysregulated lipids and metabolites, PC 34:3, PC 38:3, PC 32:0, PC 28:3;O3, SM 34:1;O2, PC 28:2;O3, SM 34:2;O2, PC 30:4, PC 34:1, PC 34:0, PC 36:3, PC 36:1, PC 38:4, PC 36:3;O3, PC 40:6, PC 38:3, SM 42:2;O2, PC 40:5, PC 38:2, SM 42:1;O2, PC 40:4 and tentatively assigned N-methylnicotinamide were significantly upregulated in PDAC compared to normal/non-neoplastic tissue. Compounds such as SM 36:1;O2, PC 32:1, PC 34:2, LPC 16:0, and creatine were downregulated in PDAC compared to normal/non-neoplastic tissue. Acetylcarnitine, butyrylcarnitine, and PC

34:3/PC 32:0 were upregulated significantly in PDAC compared to IPMN. Metabolites such as arginine and tryptophan were downregulated in IPMN compared to normal/non-neoplastic tissue. These findings highlight the lipidomic and metabolomic alterations that occur with progression from normal/non-neoplastic tissue to IPMN and to PDAC.

SLC6A6 expression is upregulated in PDAC

To further understand the mechanism underlying elevated taurine in PDAC, qRT-PCR of the taurine transporter SLC6A6 was performed. SLC6A6 expression significantly increased ($p < 0.05$) by 6.74-fold in PDAC and 2.62-fold in IPMN relative to normal/non-neoplastic tissue (Supplementary Figure S7).

Discussion

MALDI-MSI allowed us to map the spatial distribution of metabolites and lipids within PDAC to visualize the metabolic landscape, and to advance our understanding of the metabolic reprogramming that occurs in PDAC. The studies were performed using surgical specimens obtained from patients with no prior treatment that eliminated treatment-related effects contributing to the differences. We observed distinct metabolic alterations in PDAC compared to normal/non-neoplastic tissue and IPMN.

The metabolic fingerprint of PDAC was characterized by increased levels of taurine, ascorbic acid, and tentatively assigned histidine, N-acetylaspartic acid, N-acetylglutamic acid, as well as several lipid species, including most of the phosphatidylcholines and sphingomyelins compared to normal/non-neoplastic tissue. Additionally, PDAC exhibited elevated levels of acetylcarnitine, butyrylcarnitine, and phosphatidylcholine (PC 32:0/PC 34:3) relative to IPMN. Conversely, significant reductions were observed in creatine, malate, PE 36:3 and lysophosphatidylcholine (LPC 16:0), in PDAC compared to normal/non-neoplastic tissues. In contrast, IPMN tissues demonstrated significant downregulation of xanthine, arginine, and tryptophan compared to normal/non-neoplastic tissue.

Several metabolites/lipids such as taurine, malate, creatine, multiple phosphatidylcholine and sphingomyelin lipids were significantly altered. These metabolites/lipids play crucial roles in supporting cancer cell proliferation, immune evasion, and resistance to stress [20-24]. Visual inspection of the images clearly identified the tissue spatial heterogeneity of the metabolites. Signal intensities were not consistently higher or lower in co-localized regions for PDAC or for IPMN. These data suggest that the metabolic fingerprint of PDAC is a composite of several elevated or decreased metabolites and lipids that do not necessarily occur within the same tumor tissue compartments but may collectively lead to malignant progression. These findings provide new insights into the metabolic mechanisms driving PDAC and identify potential therapeutic targets. Several metabolites and lipids that were significantly altered in PDAC have been previously identified in this setting [9-13, 25, 26].

The significant elevation of taurine in PDAC compared to normal/non-neoplastic and IPMN tissues aligned with previous reports of elevated taurine in PDAC [7, 12]. Taurine influences the immune microenvironment in PDAC through the SLC6A6 transporter that mediates cellular taurine uptake. qRT-PCR data revealed that SLC6A6 expression was upregulated

in PDAC compared to normal/non-neoplastic pancreatic tissue, consistent with increased taurine observed in PDAC [21]. Taurine uptake and SLC6A6 expression levels have been shown to enhance tumor aggressiveness outcomes in multiple cancers [21]. Uptake of taurine by PDAC cells may allow tumor cells to outcompete CD8⁺ T cells for this essential amino acid. As a result, CD8⁺ T cells experience taurine deficiency that increases endoplasmic reticulum (ER) stress. This stress triggers ATF4 transcription to transactivate the expression of multiple immune checkpoint genes, leading to T cell exhaustion [21].

Our MALDI-MSI analysis revealed a significant decrease of creatine in PDAC tissue compared to normal/non-neoplastic pancreatic and IPMN tissue, consistent with previous reports of reduced creatine in PDAC [9, 11]. Creatine regulates cellular energy homeostasis through creatine kinase (CK) that facilitates the rapid regeneration of ATP from ADP. Context-dependent roles have recently been reported for ubiquitous mitochondrial creatine kinase CKMT1 in cancer progression [24]. Recent studies have highlighted the role of creatine supplementation in immune cell function, particularly in T cells, where it is essential for sustaining their energy requirements during activation and effector functions [27]. CD8⁺ cytotoxic T cells rely on ATP for their activation and proliferation. The antitumor activity of creatine has been linked to its ability to modify ATP buffering in CD8⁺ T cells [27]. Additionally, creatine supplementation has been shown to enhance anti-tumor immunity by promoting ATP production in macrophages [28] and in neutrophils [29]. Consistent with these findings, creatine supplementation in mice suppressed tumor growth [30]. Furthermore, a combination of creatine supplementation with PD-1/PD-L1 blockade treatment showed synergistic tumor suppression [22].

Acetylcarnitine levels were significantly increased in PDAC tissue. The carnitine pathway is a pivotal mediator of cancer metabolic plasticity, linking essential pathways, mediators, and metabolites to meet the energy and biosynthetic demands of cancer cells [31]. The carnitine pathway facilitates the bidirectional transport of acyl moieties between the cytosol and mitochondria, which is essential for the mitochondrial β -oxidation of fatty acids to produce ATP [14, 31]. Previous research has demonstrated that in PDAC cells ATP production relies heavily on fatty acid oxidation rather than glycolysis, with enhanced β -oxidation of long-chain fatty acids in the mitochondria serving as a major energy source for PDAC cells [32]. Excess acetylcarnitine in the mitochondria is transported to the cytosol and enters the nucleus, where it provides acetyl groups for histone acetylation and lipogenesis. Elevated histone acetylation promotes tumor progression by enabling aberrant transcription of key genes involved in critical cellular functions, such as cell proliferation and cell-cycle regulation [33].

In contrast to the elevated levels of acetylcarnitine in PDAC, malate significantly decreased in PDAC compared to normal/non-neoplastic pancreatic tissue. Reduction of malate in PDAC tissue has been previously reported [9]. Malate plays a crucial role in energy metabolism as an intermediate in the tricarboxylic acid (TCA) cycle that is essential for ATP production through oxidative phosphorylation. It also participates in the malate-aspartate shuttle, which is key to maintaining redox balance in PDAC cells by facilitating the transfer of reducing equivalents across mitochondrial membranes. The observed reduction of malate may be attributed to extensive metabolic rewiring typical of PDAC cells, such as

hypoxia-induced aerobic glycolysis and glutamine dependence [34]. In most cells, glutamate dehydrogenase (GLUD1) converts glutamine-derived glutamate into α -ketoglutarate within the mitochondria to fuel the TCA cycle. However, PDAC cells rely on a noncanonical glutamine metabolism pathway to maintain redox homeostasis and tumor growth, in which glutamate derived aspartate is transported to the cytoplasm, where it is converted into oxaloacetate by glutamate-oxaloacetate transaminase 1 (GOT1) [34]. Oxaloacetate is then metabolized to malate and subsequently to pyruvate, apparently increasing the NADPH/NADP⁺ ratio and potentially maintaining the cellular redox balance [34]. A previous study indicated that cell survival in the acidic TME, driven by lactate production from increased glycolysis, relies on elevated expression of GOT1 and the non-canonical glutamine metabolism pathway [34].

We observed significantly elevated levels of ascorbic acid in PDAC compared to normal/non-neoplastic tissue. Prior studies have measured ascorbic acid concentrations with conflicting results [35, 36]. The upregulation of ascorbic acid could be an adaptive response by PDAC cells to counteract the harsh TME of cancer cells. However, the exact mechanisms driving its accumulation and function in PDAC, and its interaction with the TME, remain unclear and warrant further research.

The lipidomic data revealed profound changes in several key lipid species, particularly PCs, SMs, and LPC. Among the lipid species, sphingomyelins and phosphatidylcholine were among the most dysregulated in PDAC in our study. PC is the most abundant phospholipid in eukaryotic cell membranes, constituting 40–50% of total phospholipids [37]. We identified distinct PCs that were significantly dysregulated in PDAC compared to normal/non-neoplastic and IPMN pancreatic tissue. Most of these PCs were elevated in PDAC tissues compared to normal/non-neoplastic and IPMN pancreatic tissues. This upregulation likely reflects an increased demand for membrane biosynthesis to support the accelerated growth of PDAC cells. These findings underscore the critical role of PC metabolism in PDAC progression. Elevated levels of PCs have been reported in the blood and tissue samples of PDAC patients [10, 26]. Elevated PC metabolism supports the accelerated growth of cancer cells by providing essential components for membrane synthesis and energy production [20]. Additionally, PC-derived lipid mediators have been identified as important drivers of resistance, facilitating cancer cell survival and proliferation, and promoting immunosuppression [20].

In our study, SMs were another lipid class profoundly altered in PDAC tissues. SMs, which make up 10-20% of plasma membrane lipids, are integral components of lipid rafts—membrane microdomains involved in signal transduction and receptor organization [38]. We observed significant dysregulation of SMs in PDAC, with most showing elevated levels in PDAC tissues compared to control and IPMN tissue samples. Elevated SM levels have been reported in various cancers [39]. This increase in SM levels is likely driven by the overexpression of sphingomyelin synthase (SMS), a plasma membrane-bound enzyme involved in maintaining the SM level on the membranes. SMS 2 mRNA expression level was positively correlated with tumor-associated macrophages, the immunosuppressive microenvironment, and poor prognosis in PDAC patients [40]. Additionally, it has been reported that high expression levels of acid sphingomyelinase (ASM)—an enzyme that

catalyzes the degradation of sphingomyelin into phosphorylcholine and ceramide, strongly correlates with long-term survival of PDAC patients [41]. Elevated SM levels play a vital role in cancer initiation, progression, and immune evasion [23]. SM contributes to chemotherapy resistance by facilitating the trafficking of P-glycoprotein to plasma membrane lipid rafts and enhancing cell membrane integrity [42]. Additionally, elevated levels of sphingomyelin act as a tumor-promoting factor by reducing pro-apoptotic ceramide levels, a lipid that typically induces cell death, thus further promoting tumor survival [43].

LPCs are a class of bioactive lipids derived from the partial hydrolysis of PCs and are a main component of oxidatively damaged low-density lipoproteins (oxLDL) [44]. LPCs are involved in several physiological processes, including inflammation, endothelial activation, and atherogenesis [44]. Our study demonstrated that LPC (16:0) was significantly decreased in PDAC compared to normal/non-neoplastic tissues. Previous studies have shown a significant reduction in LPC levels in PDAC patient tissue and serum samples compared to healthy controls [10, 26]. The observed decrease in LPC (16:0) suggests an increased metabolic demand for lysophosphatidic acid (LPA) in PDAC cells, as LPC serves as a key precursor for LPA synthesis mediated by the lysophospholipase D enzyme, autotaxin (ATX) [45]. LPA is a bioactive lipid that promotes cancer cell survival, proliferation, and migration, making it a crucial factor in tumor progression [45]. ATX has been shown to be overexpressed in various cancers [46], and previous reports indicated that serum ATX activity is elevated in pancreatic cancer patients [47]. Moreover, Ras-driven tumors have been shown to scavenge extracellular lysophospholipids such as LPCs to meet their cellular demand for unsaturated fatty acids that may further account for the observed depletion of LPC in PDAC tissues [7, 48]. Overall, our lipidomic analysis highlights the profound reprogramming of lipid metabolism in PDAC, with specific alterations in key lipid species that are mechanistically linked to cancer progression, immune evasion, and therapy resistance. These findings underscore the potential of targeting lipid metabolic pathways, such as PC and SM biosynthesis, LPA signaling, and lipid metabolism, as part of a multifaceted approach to treat PDAC.

In summary, the metabolic reprogramming in primary PDAC was characterized by spatial heterogeneity. Metabolic alterations that are associated with proliferation, immune evasion and resistance to treatment, occurred in distinct compartments of PDAC tissue, supporting further analysis relating the spatial metabolic distributions to spatial transcriptomics. One of the limitations of our study was the small group of samples studied that consisted of stage IIB PDAC and the limited histological analysis. Additionally, the MALDI-MSI data were acquired at 200 μm spatial resolution that although sufficient for regional profiling, does not permit single-cell level analysis. Furthermore, the time between surgical resection and tissue freezing may contribute to metabolite drift. Prior studies have shown that cold ischemic time can affect tissue metabolomes [49, 50]; however, these alterations will likely affect both the normal/non-neoplastic and neoplastic tissues to a similar degree. Studies with expanded patient samples at different stages, and expanded immunohistological and spatial transcriptomic analysis relating the spatial metabolic heterogeneity to molecular pathways and immune and physiological microenvironments will further confirm the critical role of metabolism and its targeting in PDAC progression and treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The authors declare that all relevant data including Supplementary data used to conduct the analyses are available within the article or can be requested at any time from the corresponding author. All data are de-identified.

Code availability

The MATLAB (MathWorks, Inc., MA, USA, RRID:SCR_001622) code for generating the spatial homogeneity statistics from the MALDI images using the Haralick features (gray-level co-occurrence matrix) and the corresponding MALDI image data are available under JHU Academic Software License Agreement at: <https://doi.org/10.5281/zenodo.16956374>.

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Translational Relevance

We used matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) to obtain spatially-resolved metabolic and lipidomic fingerprinting of PDAC, intraductal papillary mucinous neoplasm (IPMN), and normal/non-neoplastic pancreas tissue. Our analyses identified distinct metabolic and lipidomic signatures specific to PDAC, providing valuable insights into the metabolic and lipidomic landscapes that differentiate PDAC from IPMN and normal/non-neoplastic pancreatic tissue. Metabolic alterations were associated with proliferation, immune evasion and resistance to treatment. Some metabolic alterations were also identified in IPMN. Our findings provide novel insights into the metabolic and lipidomic underpinnings of PDAC, and underscore the need for further investigation to determine whether targeting these pathways may have therapeutic benefits.

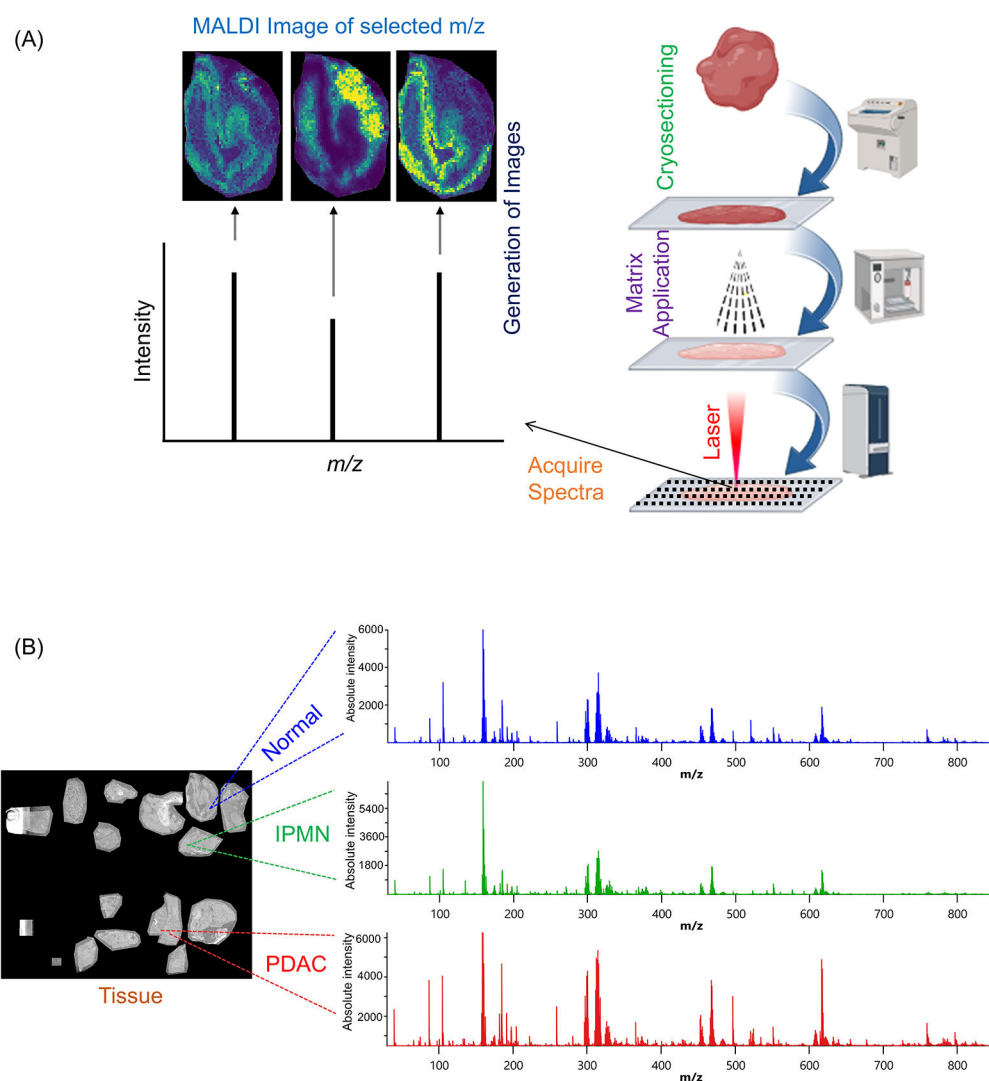


Figure 1: MALDI-MSI workflow and representative spectra from normal/non-neoplastic, IPMN and PDAC tissue. (A) Schematic representation of the MALDI-MSI workflow, including tissue section preparation, matrix application, data acquisition, and data processing for spatial metabolomics analysis. (B) Representative MALDI mass spectra obtained from normal/non-neoplastic, IPMN, and PDAC tissue samples, illustrating distinct spectral patterns and peak intensities that reflect metabolic differences across these tissue types. (Figure 1A was created in BioRender. [Yousf, S. (2026)]. [<https://BioRender.com/t5hq61o>]).

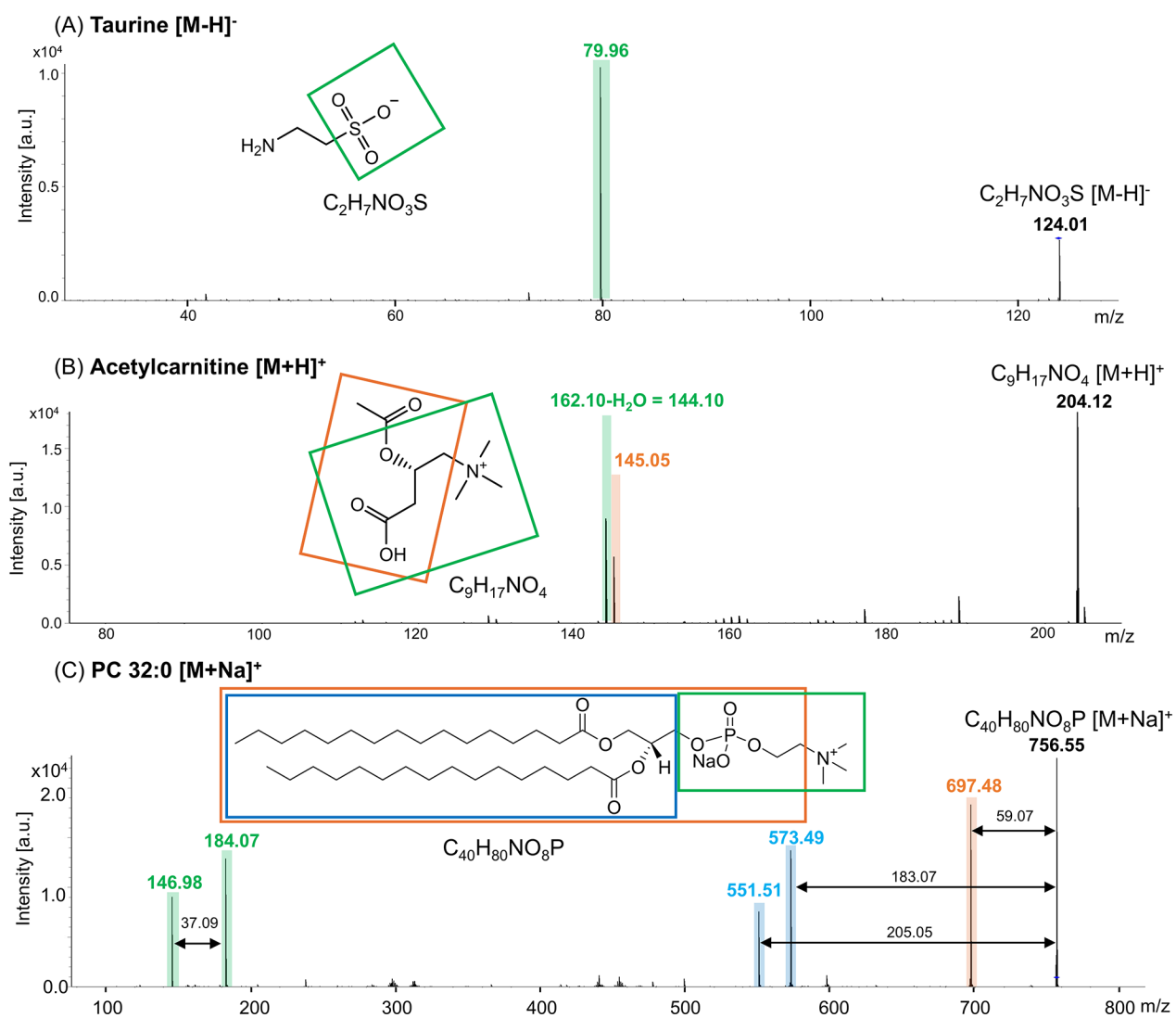
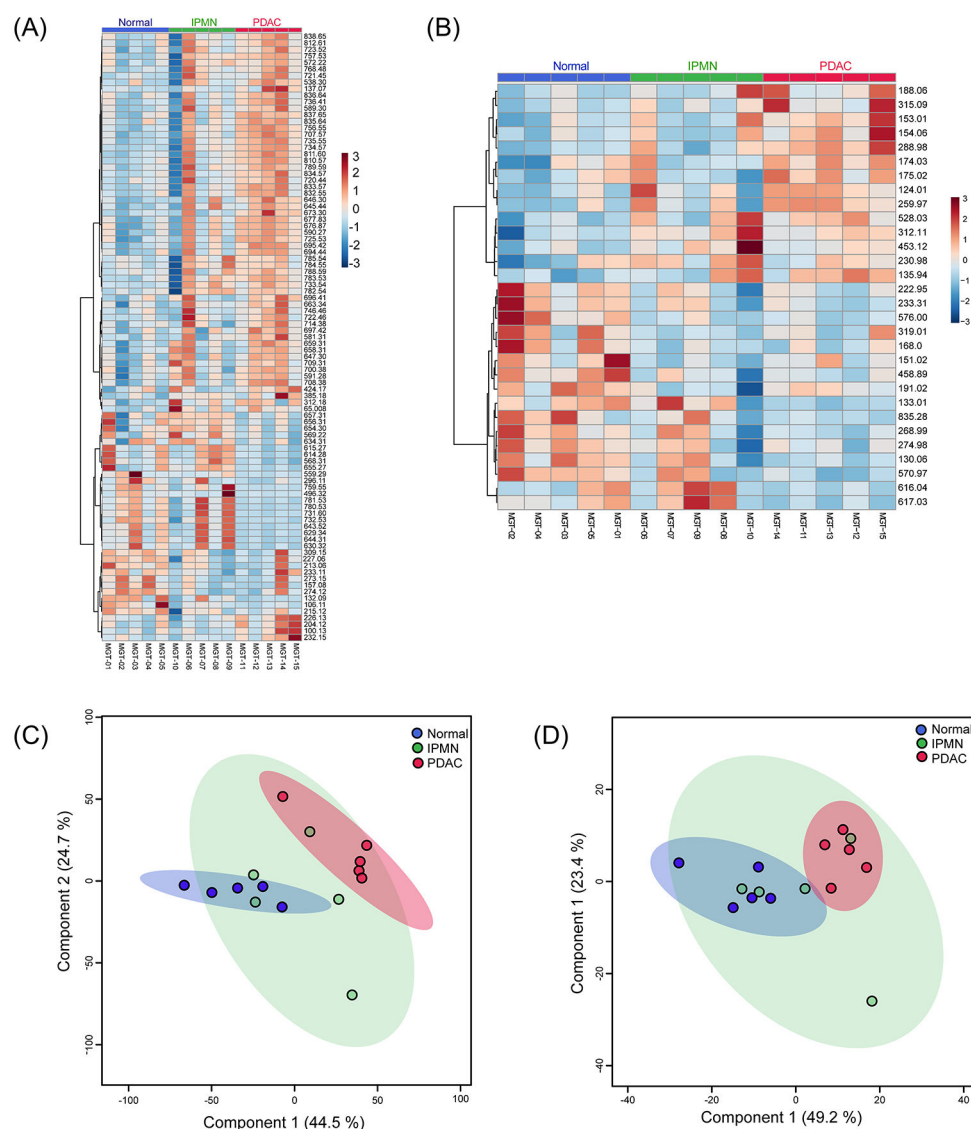


Figure 2: MS/MS fragmentation spectra for compound identification. MS/MS fragmentation spectra of (A) taurine, (B) acetylcarnitine, and (C) phosphatidylcholine (PC 32:0) obtained from tissue samples. Fragmentation patterns and key ion peaks are annotated to confirm the identities of these compounds.

**Figure 3:**

Heat maps and PLS-DA plots for positive and negative ion modes. (A, B) Heat maps displaying differences in the m/z intensity profiles of normal/non-neoplastic ($n = 5$), IPMN ($n = 5$), and PDAC ($n = 5$) tissue in positive (A) and negative (B) ion modes. (C, D) PLS-DA score plots for positive (C) and negative (D) ion modes. Two-dimensional PLS-DA representations show clustering of normal/non-neoplastic (green), IPMN (red), and PDAC (blue) pancreatic tissues. Shaded circles represent 95% confidence intervals, while individual samples are depicted as colored dots. Axes represent the first and second principal components (PC1 and PC2, respectively), with percentages of variance indicated in parentheses.

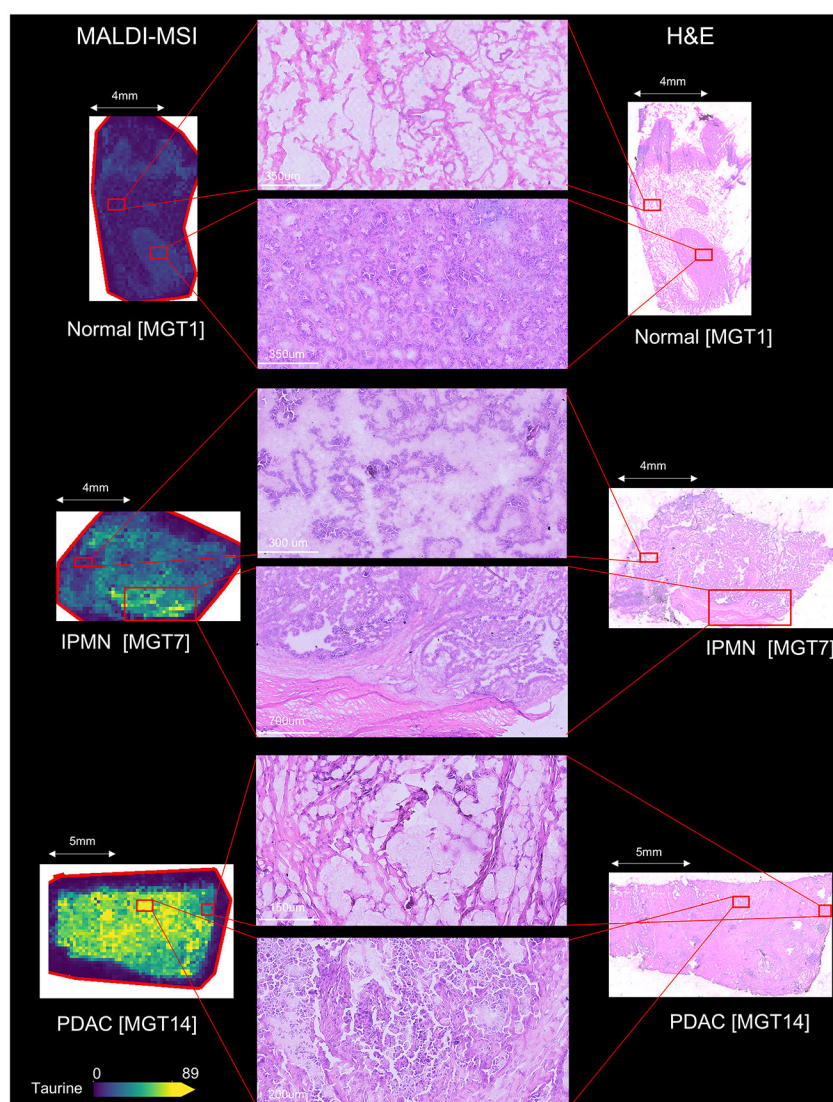


Figure 4: Representative example of histological and metabolomic profiling of pancreatic tissue. Spatial mapping of low and high taurine levels in normal/non-neoplastic, IPMN, and PDAC tissue sections using MALDI-MSI and corresponding H&E-stained tissue sections, highlighting the spatial correlation between taurine and histological features. Tissue sections were prepared at 10 µm thickness, and MALDI-MSI was obtained at a spatial resolution of 200 µm. The color scale depicts relative intensity normalized to total ion count (TIC), with blue representing the minimum signal intensity and yellow indicating maximum signal intensity.

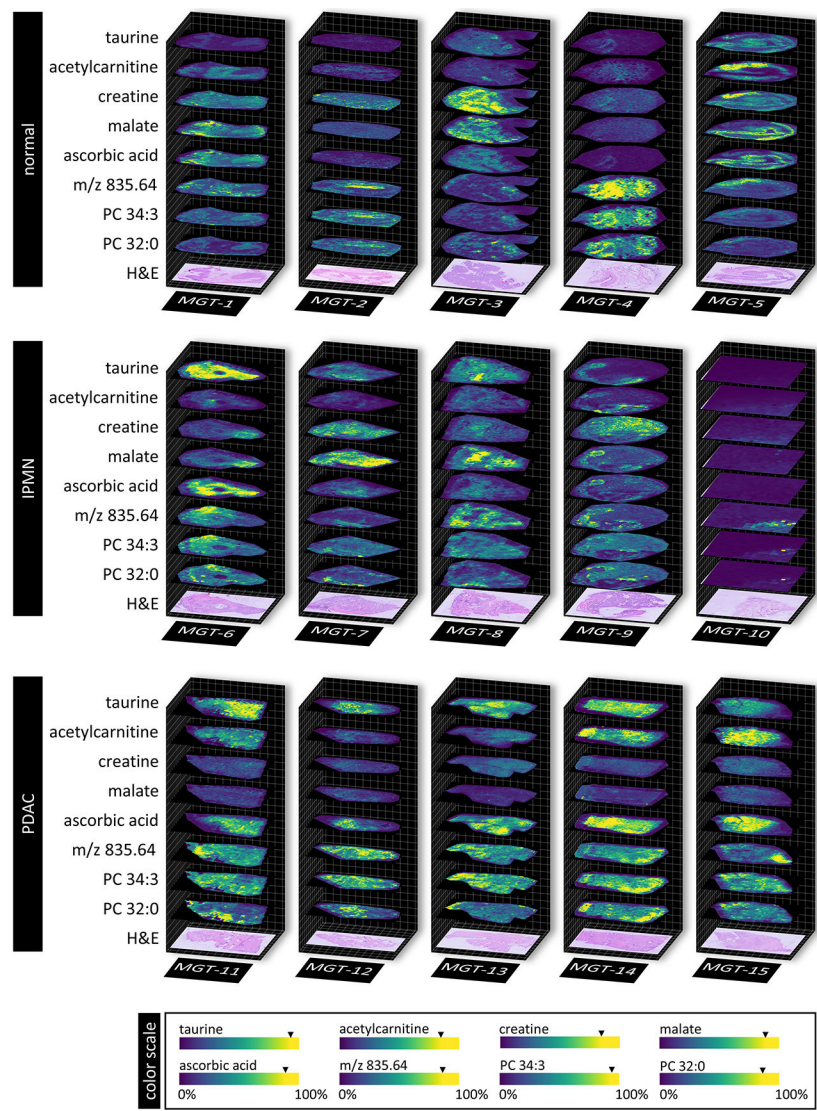


Figure 5: Spatial maps of key metabolites together with H&E staining for each tissue. Metabolic maps of taurine, acetylcarnitine, creatine, malate, ascorbic acid, m/z 835.64 (tentatively identified as sphingomyelin SM 42:2;O2), PC 34:3, PC 32:0 and the corresponding H&E staining for each normal/non-neoplastic (MGT-1 to 5), IPMN (MGT-6 to 10) and PDAC (MGT-11 to 15) tissue. Tissue sections were prepared at 10 μ m thickness, and MALDI-MSI was obtained at a spatial resolution of 200 μ m. The color scale depicts relative intensity normalized to total ion count (TIC), with blue representing the minimum signal intensity and yellow indicating maximum signal intensity. Arrowheads on the LUT mark the saturation point.

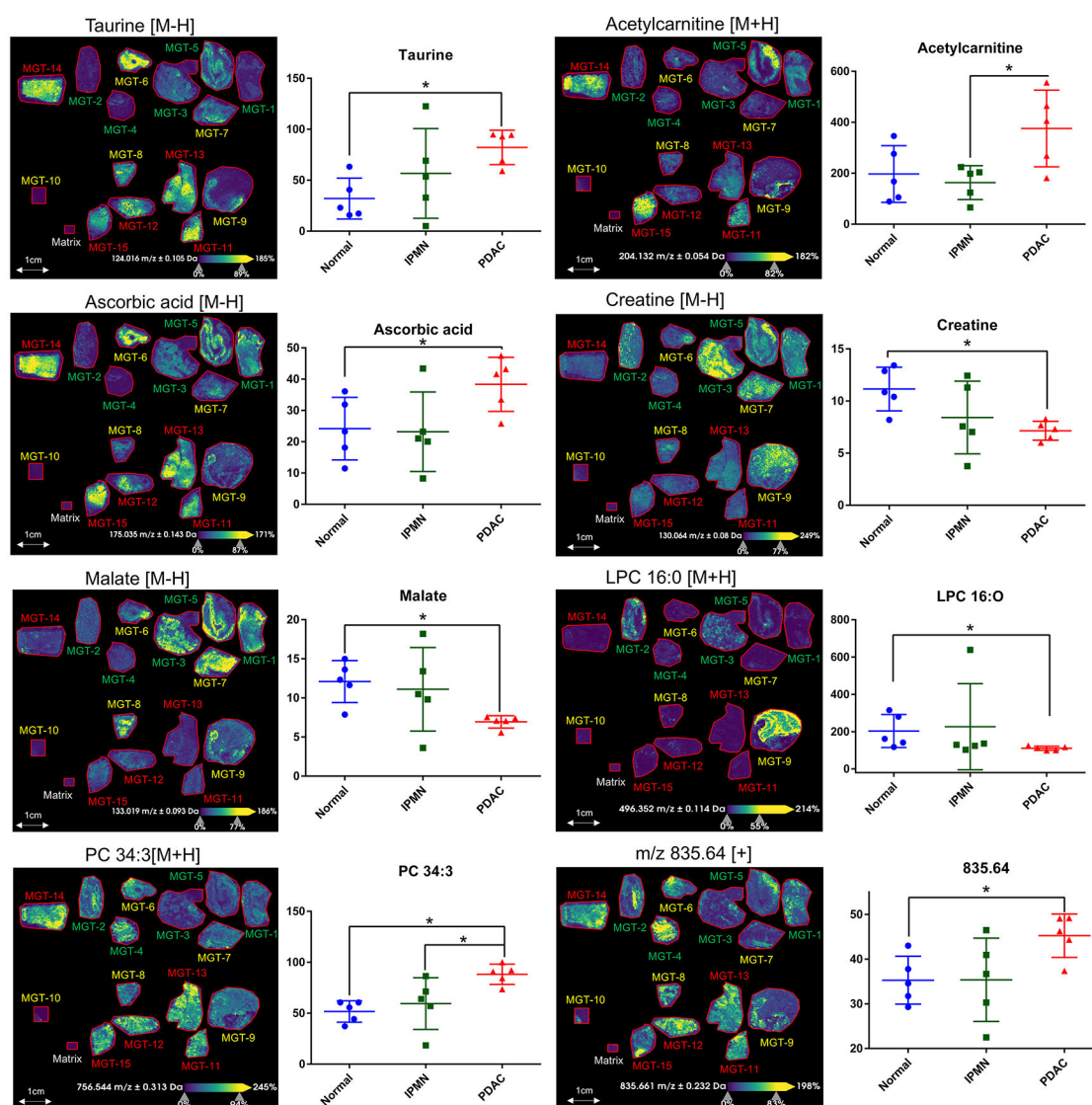


Figure 6: Spatial distribution and quantitative analysis of metabolites using MALDI-MSI. MALDI-MSI ion images displaying the spatial distribution of selected metabolites in pancreatic tissue sections from normal/non-neoplastic, IPMN, and PDAC samples, accompanied by corresponding box plots summarizing the relative intensities of these metabolites across the tissue types. Tissue sections were prepared at 10 μm thickness, and MALDI-MSI was obtained at a spatial resolution of 200 μm . The color scale depicts relative intensity normalized to total ion count (TIC), with blue representing the minimum signal intensity and yellow indicating the maximum signal intensity. Values represent the mean $\pm$ SD. Statistical significance is denoted by an asterisk (*), indicating p-values less than 0.05.