University of Alberta

Urinary Metabolomic Signature of Pancreatic Ductal Adenocarcinoma
by

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Abstract

Pancreatic cancer is a leading cause of cancer-related death, due to its aggressive biology, lack of tools for early diagnosis and screening, advanced presentation and resistance to adjuvant therapy. Metabolomics, the newest of the “omics” sciences, may offer the potential for non-invasive screening of early tumor associated perturbations in cellular metabolism. We applied metabolomic techniques as a potential discriminating tool in the diagnosis of early stage and locally advanced pancreatic cancer. Urinary nuclear magnetic resonance spectroscopic analysis of pancreatic ductal adenocarcinoma was associated with a distinct metabolomics signature, was detectable in both early stage and locally advanced disease when compared with healthy, age and gender matched controls and was extinguished following complete, R0 surgical resection. These preliminary results suggest that metabolomics approaches may facilitate discovery of novel biomarkers capable of early disease detection.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

PDAC	Pancreatic Ductal Adenocarcinoma
EUS	Endoscopic Ultrasound
CA	Carbohydrate Antigen
NFPTR	National Familial Pancreatic Tumor Registry
PET	Positron Emission Tomography
PanIN	Pancreatic Intraductal Neoplasia
MCN	Mucinous Neoplasm
IPMN	Intraductal Papillary Mucinous Neoplasm
NMR	Nuclear Magnetic Resonance
MS	Mass Spectrometry
GC	Gas Chromatography
LC	Liquid Chromatography
PR	Pattern Recognition
PCA	Principal Component Analysis
PLS-DA	Partial Least-Squares Discriminant Analysis
MRS	Magnetic Resonance Spectroscopy
FNA	Fine Needle Aspiration
MRSI	Magnetic Resonance Spectroscopic Imaging
GIST	Gastrointestinal Stromal Cell Tumor
CRC	Colorectal Cancer Patients
UPLC	Ultra-high Performance Liquid Chromatography
TCA	Tricarboxylic Acid Cycle

OSC	Orthogonal Signal Correction
VAST	Variable Stability Scaling
SNP	Single Nucleotide Polymorphism
2-DE	Two-Dimensional Electrophoresis
MALDI TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometer
GFR	Glomerular Filtration Rate
ROC	Receiver Operating Characteristics
AUROC	Area Under the ROC
NOESY	Nuclear Overhauser Effect Spectroscopy
VIP	Variable Importance on Projection
IL	Interleukin
TNF	Tumor Necrosis Factor
IFN	Interferon
FDR	False Discovery Rate

Chapter 1: Introduction

1.1 Summary and Statement of the Problem

Pancreatic ductal adenocarcinoma (PDAC) is associated with dismal prognosis, and surgical resection is rarely curative. These poor outcomes are in part due to the late presentation and biological aggressiveness of this tumor, coupled with resistance to standard and innovative chemotherapeutic approaches. While a subset of patients may be cured by major pancreatic resection, most are not, and despite dramatic progress in other surgically treatable cancers such as colorectal malignancies, overall outcomes for PDAC patients have changed little in recent decades. It should be noted however that surgical outcomes with the Whipple resection for pancreatic head cancers have improved substantially, and the previous 50% mortality of 30 years ago currently is of the order of 1-5% in high volume centers [1, 2].

These outcomes are unlikely to change substantially until effective tools become available for early detection of pancreatic cancers in high-risk populations, and until the biological basis of the tenacity of this cancer is better understood. Because PDAC is such a devastating and pervasive malignancy, any tool with the potential for early diagnosis could have a major impact on outcome.

Metabolomic science offers one new potential approach that could provide a means for early detection based on identification of a discrete metabolomic

signature associated with PDAC. Furthermore, armed with a better understanding of the underlying biochemical pathways disrupted in PDAC, metabolomics has the potential to bring a number of advancements not only to early cancer detection and diagnostics, but also may lead to development of new targeted therapies tailored to molecular processes underlying the disease. As such, metabolomics has the potential to dramatically alter the field of surgical oncology in the realm of diagnosis, treatment and understanding of tumor biology. Furthermore, an understanding of metabolomic pathways active in PDAC that differ from normal cellular metabolism may open up new pathways for interventional treatment.

1.2.1 Pancreatic Cancer - An Overview of Diagnosis, Screening and Molecular Pathogenesis

PDAC is a biologically complex malignancy with dismal prognosis. This has largely been attributed to difficulty with diagnosis and late stage of presentation, as well as a lack of responsiveness to radio- and chemotherapy. Surgical resection provides the only potential for cure, however as a result of delayed presentation combined with the biological aggressiveness of PDAC and lack of potent adjuvant therapies, only 15-20% have surgically resectable disease [3]. The vast majority of patients are diagnosed after their disease has spread locally

or metastasized. Even those who undergo surgical resection, however have a dismal prognosis, with 5-year survival rates as low as 10-30% [3] **(Table 1.1).**

Five-year overall survival for resected pancreatic adenocarcinoma from the National Cancer Data Base[1]

Surgical patients			Observed survival					Median survival, months
Stage	Number of patients	Percent	1-year, percent	2-year, percent	3-year, percent	4-year, percent	5-year, percent	
IA	1886	8.8	71.3	50.2	40.7	34.7	31.4	24.1
IB	2364	11.0	67.3	45.4	35.3	29.6	27.2	20.6
IIA	3846	17.9	60.7	34.9	23.8	18.4	15.7	15.4
IIB	7828	36.4	52.7	23.8	14.4	10.2	7.7	12.7
III	2850	13.2	44.5	19.3	11.0	8.1	6.8	10.6
IV	2738	12.7	19.2	8.4	5.3	3.7	2.8	4.5
Total	**21,512**							**12.6**

Table 1.1 Survival Outcomes Following Resection in Pancreatic Ductal Adenocarcinoma
(*Reproduced with permission from John Wiley and Sons Bilimoria et al.,Validation of the 6th edition AJCC pancreatic cancer staging system. Cancer 2007; 110(4): 738-744)*

These survival rates have remained largely unchanged for decades, despite parallel advances in surgical and adjuvant therapies for other cancers such as colorectal malignancies. Clearly improvements are urgently needed in the area of diagnostics and screening, therapeutics and global biological understanding of this heterogeneous and complex malignancy.

1.2.2 Diagnostic Challenges in Pancreatic Cancer

Early detection and precise preoperative diagnosis of PDAC remains an elusive area in desperate need of refined diagnostics and improved understanding of tumorigenesis. Prognosis is dismal, in part due to delayed diagnosis and the

aggressive nature of disease [4]. Given that the vast majority of patients with PDAC are not diagnosed until after their disease is incurable, the search for accurate, non-invasive and sensitive diagnostic tests will be a critical component if curative outcomes are to be transformed.

Complete surgical resection remains the only potentially curative treatment. However, in nearly 80% of patients, disease is not detected until an advanced stage when curative-intent surgery is not longer an option [3]. Clinical features of pancreatic cancer presenting in the pancreatic body or tail are often vague and non-specific (abdominal pain, weight loss). Acute onset of painless jaundice is the one herald sign of relatively small pancreatic head and uncinate tumors. Nonetheless, even in this setting nearly half of patients have locally advanced, unresectable disease when pain occurs in the setting of jaundice, and jaundice is often a feature of more advanced disease [5, 6]. Jaundice can on occasion be a non-specific finding as it may be associated with benign disease such as cholelithiasis, pancreatitis or benign biliary strictures [5]. Currently available imaging techniques lack the sensitivity necessary for detecting early-stage disease and radiographic findings are not specific [5]. Reported accuracies of endoscopic ultrasound (EUS) are highly variable and are likely largely dependent on local expertise. The reported sensitivity of EUS is generally high (85%), however negative predictive values are low (64%) [5]. While useful in diagnostic confirmation of patients presenting with symptomatic pancreatic tumors, EUS is not a palatable approach that can be widely used for screening of patients at

higher risk of pancreatic cancer. Furthermore, negative biopsies provide no benefit in clinical decision making [5, 7, 8]. Accurate diagnosis even at later stages can be challenging given that benign pancreatic disease can often mimic pancreatic cancer. Early and accurate detection of disease is crucial in helping to minimize unnecessary morbidity and mortality associated with surgery for benign disease, as well as improve survival in the case of true malignancy. In fact, between 10-25% of patients who undergo radical surgery (pancreaticoduodenectomy or radical pancreatectomy) for pancreatic lesions are found to have benign disease on final pathology [9-13]. Likewise, necessary surgical intervention is often delayed inappropriately in situations of diagnostic uncertainty.

1.2.3 Molecular Markers in Pancreatic Cancer

In an attempt to improve on early and accurate diagnosis of pancreatic cancer, a wide array of molecular markers have been described, the majority of which are still at the preclinical phase. The most widely investigated markers are outlined in **Table 1.2**. To date, however no marker has adequate sensitivity and specificity to provide adequate clinical diagnostic resolution [14]. The carbohydrate antigen CA19-9 remains the only widely used and clinically recognized tumor marker for pancreatic cancer. Reported sensitivities and specificities of CA19-9 are relatively high (80-90%), however they are closely linked with tumor size [15-18].

While useful in assessing prognosis and as an indicator of recurrence following potentially curative surgery, the role of CA19-9 as a diagnostic tool in detecting early stage disease amenable to surgical resection is limited and largely not established [19-23].

Table 1. Summary of new molecular markers for pancreatic cancer.

Source	Marker	Sensitivity	Specificity	Author
Serum	MUC1	71%	96%	Gold [3]
Serum	CEACAM1	85%	98%	Simeone [4]
Serum	MIC1	90%	62%	Koopmann [5]
Serum	Alpha4GnT	76%	83%	Ishizone [6]
Serum	CK-19 mRNA	64%	100%	Hoffmann [7]
Serum	K-*ras*	0	0	Marchese [8]
Pancreatic juice	Methylation pattern	82%	100%	Matsubayashi [9]

CEACAM1: carcinoembryonic antigen-related cell adhesion molecule 1; CK-19: cytokeratin-19

Table 1.2 Investigational Molecular Markers for Pancreatic Ductal Adenocarcinoma
(*Reproduced with permission from Lee et al., Screening for Early Pancreatic Ductal Adenocarcinoma: An Urgent Call. Journal of the Pancreas 2009; 10(2): 104-108)*

Investigation for reproducible, accurate molecular markers of PDAC has involved primarily serum based studies, but has also addressed molecular markers expressed in pancreatic fluid, duct brushings, duodenal aspirates and pancreatic tissue obtained by EUS, core biopsy, drainage or from surgically resected specimens. The later approaches are not ideal for routine clinical use given their invasive nature and to date, none have proven to be clinically relevant. Broad categories of putative molecular markers include serum carbohydrate antigens (CA19-9, CA125), detection of specific genetic mutations (including activated

oncogenes, K-*ras*, and inactivated tumor suppressor genes, p16, p53, DPC4 and BRCA2), altered telomerase activity, growth factor and receptor overexpression, aberrant expression of mucins, abnormally methylated DNA, and abnormal protein expression [14].

Both genomic and proteomic-based research have been exploited in an attempt to identify more reproducible, sensitive and specific biomarkers capable of timely and accurate disease detection. However, despite early optimistic reports these have largely failed to demonstrate clinical efficacy [24, 25]. In an effort to advance biomarker discovery, metabolomics-based research in the area of cancer diagnostics has grown dramatically in the past decade [26-35]. Metabolomics offers a unique approach to early cancer diagnosis through detection of early metabolic signals of cellular perturbation, which occur prior to the surfacing of gross phenotypic change [36]. Only a handful of preliminary studies however, have examined the role of metabolomics in screening for early tumor-associated perturbations in cellular metabolism specific to pancreatic cancer. Virtually all of the research thus far has been serum-based, limited in sample size, and inclusive of advanced stage disease [5, 25, 37-39]. While it is still not yet established which biofluid is optimal for cancer-related metabolomics-based studies, urinary analyses have a number of clear advantages. Sampling is non-invasive, requiring minimal sample processing and issues of degraded spectral resolution (encountered with serum) are avoided [40, 41]. In contrast to previous studies, we chose to focus on early stage disease, felt to be most

relevant in the context of developing an early detection and screening tool. Given the shortcomings of current diagnostic tools and the paucity of metabolomics-specific research, further, in-depth investigation into the role of metabolomics and PDAC diagnostics is clearly needed. In this thesis, we therefore set out to investigate the potential of urinary metabolomic screening of early stage or locally advanced PDAC.

1.2.4 Role of Screening in Pancreatic Cancer

A fundamental precept of cancer screening is that early detection will improve survival, free of lead-time bias. When malignancies are detected late in the course of disease, effectiveness of therapies is compromised and curative intent surgery may no longer be an option. An ideal screening test should be highly sensitive and specific, cost effective, widely available and considered palatable by the at risk population being screened. Currently, early detection combined with surgery offers the only chance of survival in treatment of PDAC. However, unlike advancements made in screening and early detection of a number of other cancers such as breast and colorectal cancers, reliable, sensitive, primary screening modalities for pancreatic cancer in the general population do not yet exist [42]. Due to the low prevalence of disease and the low accuracy and invasive nature of presently available screening and diagnostic tests, population-based primary screening for pancreatic cancer in the asymptomatic population is

neither advisable nor cost effective [43, 44]. However, it is becoming increasingly recognized that a higher-risk patient subgroup exists for PDAC where selected, targeted screening may indeed be both appropriate and advisable.

1.2.5 Familial and Hereditary Pancreatic Cancer Syndromes

While little is known about the etiology of PDAC, there are several clearly established risk factors. The strongest risk factor for the development of PDAC is a family history of the disease. Kindred pancreatic cancer families and familial predisposition have been recognized since the first case series was described in 1967 [45, 46]. This notion was strengthened further by observational studies, which were followed by genetic analysis of families and the eventual development of nationwide family-based pancreatic cancer registries [47]. It is estimated that somewhere between 4-17% of pancreatic cancers are either familial or syndromic [47]. As outlined in **Table 1.3** below, there are six widely recognized hereditary cancer syndromes which predispose patients to pancreatic cancer [46]. Arising from germline mutations of a number of genes, each has its own associated lifetime risk of development of pancreatic cancer. Genetic testing is now available for the majority of these hereditary cancer syndromes [48].

Table 1
Selected conditions and cancer syndromes associated with increased pancreatic cancer risk.

Syndrome (abbreviation)	Location	Gene	Gene type	Inheritance	Estimated relative risk	Frequency in sporadic cases
Hereditary pancreatitis (HP)	7q35	PRSS1	Cationic trysinogen	Autosomoal dominant, 80% penetrance	20–75	Unknown
Cystic fibrosis (CF)	7q31.2	CFTR	Chloride ion channel	Autosomal recessive	~5	Unknown
Peutz–Jeghers syndrome (PJS)	19p13.3	STK11/LKB1	Tumour suppressor, serine threonine kinase	Autosomoal dominant	132	4%
Familial atypical multiple mole melanoma (FAMMM)	9p21	p16 INK4a/ MTS1	Tumour suppressor	Autosomoal dominant	13–22	98%
Hereditary breast Ovarian Cancer (HBOC)	17q21-24 and 13q12-13	BRCA1 and BRCA2	Tumour suppressor, linked to RAD51	Autosomoal dominant	2.3–3.6 for BRCA1 3–10 for BRCA2	7% for BRCA2 N/A for BRCA1
Familial adenomatous polyposis (FAP)	5q21	APC	Tumour suppressor	Autosomoal dominant	~5	40%
Hereditary nonpolyposis colorectal cancer (HNPCC)	2p22-21 and 3p21.3	MSH2 and MLH1•	Mismatch repair	Autosomoal dominant	Unknown	4–11%
Family X: site specific pancreatic cancer	4q32-34	Palladin	Cytoskeleton structure	Autosomal dominant	Unknown	Unknown

Table1.3 Selected Conditions and Cancer Syndromes Associated with Increased Risk of Pancreatic Cancer
(*Reprinted from Best Practice & Research Clinical Gastroenterology (23), Greer et al. Hereditary Pancreatic Cancer: A Clinical Perspective. p159-170; 2009 with permission from Elsevier)*

Familial pancreatic cancers are defined as cancers arising in kindreds with two or more family members who have been diagnosed with pancreatic cancer and who are first degree relatives [48]. Inheritance patterns are autosomal dominant with a high degree of penetrance. While still largely unknown, it is thought that familial pancreatic cancer has more than one genetic cause, however these mutations are remain elusive [45, 49]. Unlike the hereditary pancreatic cancer syndromes, familial pancreatic cancer is not associated with the development of any other cancers. The overall lifetime risk of developing pancreatic cancer is dependent on the gene inherited, as well as other environmental factors (i.e. smoking) and therefore exact estimates of risk remain unknown but estimates range from 5-100% (13, 15).

1.2.6 Natural History of High Risk Pancreatic Cancer Syndromes

There remains a limited understanding of the natural history of familial pancreatic cancer and lifetime risk estimates vary in the literature. In families with one family member diagnosed with pancreatic cancer, it is estimated that the risk of development of cancer in all first-degree relatives is 6.3 fold greater than the baseline, population risk of 1:10,000. In families with two relatives affected, the risk of development of pancreatic cancer in first-degree relatives has been reported to increase substantially with estimates ranging from 10 - 18 fold increase in risk, and an estimated 57-fold increase in risk exists in kindreds who have 3 affected relatives [48]. Sporadic pancreatic cancers occur rarely before the age of 45, and the incidence rises steeply thereafter. One series has suggested that the mean age of diagnosis in patients with familial pancreatic cancer is 44, with ages ranging between 29-63 [48]. Therefore many cases of familial pancreatic cancer demonstrate an early age of onset of disease, similar to other familial cancers [50].

Pancreatic cancer family registries have been recorded since the 1990's. The largest registry to date worldwide is the National Familial Pancreatic Tumor Registry (NFPTR), which as of July 1, 2008 has a total of 2,877 families enrolled. This registry, first established at Johns Hopkins University, enrolls patients nationally in the United States and has been used as a research tool in the continued investigation of familial pancreatic cancer [51]. Development of cancer

registries such as the NFPTR provide an essential resource to allow researchers to examine the pathogenesis, natural history, biomarkers, underlying gene alterations and new diagnostic and therapeutic strategies, in managing high-risk pancreatic cancer patients [50].

1.2.7 Current Screening Strategies

Currently, early detection combined with surgery offers the only chance of survival. However, unlike advancements made in the screening and early detection in a number of other cancers such as breast and colorectal, reliable, sensitive screening tests for pancreatic cancer do not yet exist [42]. As a consequence, the vast majority of patients present with advanced stage disease when curative surgery is no longer possible. Significant challenges remain in screening for early pancreatic cancer in these high-risk groups and current screening guidelines vary from institution to institution, based largely on expert opinion. There are no consensus recommendations with regards to the modality or frequency of surveillance screening [52]. Most centers use a combination of annual pancreas protocol CT imaging and endoscopic ultrasound (EUS) evaluation, followed by fine needle biopsy or endoscopic retrograde cholangiopancreatography if concerning features are noted [52]. Other variations include tumor markers, such as CA 19-9, abdominal MRI scanning, MR cholangiopancreatography or positron emission tomography (PET) combined

with cross-sectional CT imaging. Each of the hereditary cancer syndromes associated with pancreatic cancer has its own set of surveillance guidelines, with variations in age of initial surveillance as well as frequency and screening modality [52]. Despite aggressive surveillance programs, currently available screening modalities are too insensitive to detect early-stage disease, when a cure is still possible. New, highly sensitive screening tests are needed, given the clear shortcomings of current surveillance tools and the fact that the majority of patients are not diagnosed until after their disease is incurable. Discovery of minimally invasive, accurate and cost-effective screening-modalities through the combined use of novel biomarkers and imaging studies is therefore critical for effective screening of these at-risk populations.

1.2.8 Molecular Pathogenesis of Pancreatic Cancer

A greater understanding of the biology of pancreatic cancer could lead to improved, targeted therapeutics in the treatment of this aggressive and devastating malignancy [53]. While many aspects surrounding the molecular pathogenesis of pancreatic cancer remain incompletely understood, PDAC arises from both inherited and acquired mutations in a number of different cancer-associated genes [54, 55].

Advances in the understanding of these genetic mutations have helped in establishing the progression model of pancreatic tumorigenesis. Pancreatic intraductal neoplasia (PanIN) is one of three known precursor lesions of PDAC which has been most fully characterized [4]. While exact molecular events resulting in progression of PanIN lesions to invasive carcinoma remain unknown, an increasing number of genetic mutations appear to be associated with increasing degrees of dysplasia (**Figure 1**.**1**). Thought to originate from ductal epithelium, PDAC evolves from premalignant lesions to invasive cancer [4]. One of the earliest events in pancreatic tumorigenesis appears to be mutational activation of the oncogene K-ras, first noted in low-grade lesions (PanIN 1). Activating mutations of this oncogene result in a spectrum of molecular events leading to cellular proliferation, survival and invasion. The precise role of K-ras effector pathways in PDAC carcinogenesis remain largely unknown, however there is some evidence pointing towards to the role of autocrine epidermal growth-factor receptor signaling [53, 56, 57]. Tumor suppressor gene mutations become increasingly apparent (p16/CDKN2a, SMAD4 and TP53) in higher-grade lesions (PanIN-2 and 3 respectively). This cancer-progression model resulting from successive accumulation of genetic mutations has been demonstrated in numerous animal studies [58-60].

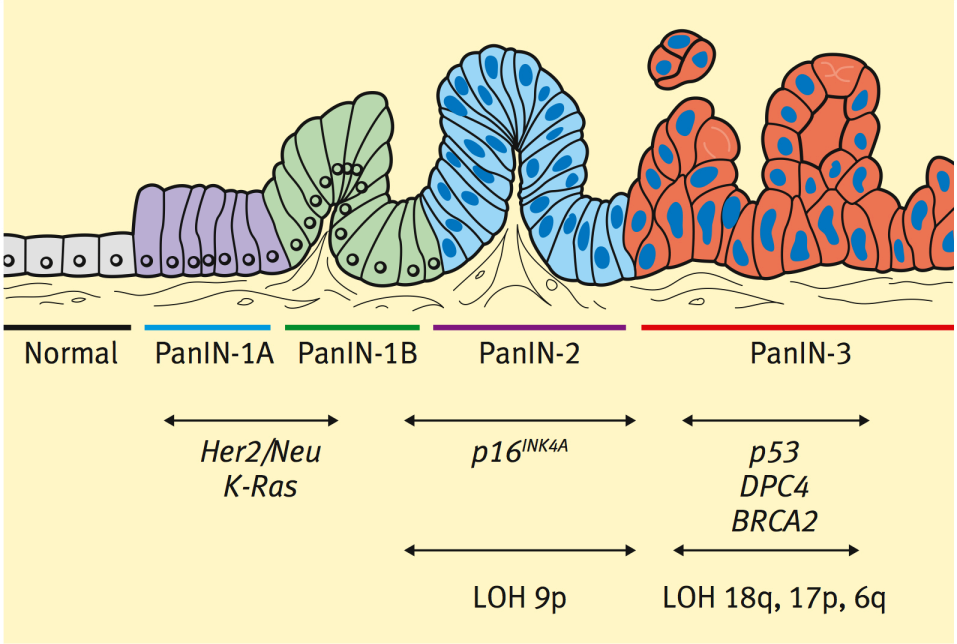

Figure 1.1 Pancreatic Cancer Progression Model
(*Reprinted from The American Journal of Pathology permission 156(6), Hruban et al. Genetic Progression in the Pancreatic Ducts. p1821-1825; 2000 with permission from Elsevier)*

Mucinous neoplasm (MCN) and intraductal mucinous neoplasm (IPMN) represent the other two recognized precursor lesions of pancreatic cancer, however, these are less well characterized. Identification and comprehensive molecular characterization of non-invasive precursor lesions in pancreatic cancer could provide an important target for screening, early diagnosis, and therapeutic interventions [61]. Furthermore, in-depth development of a stepwise molecular progression model capable of comprehensively integrating the role of genetic, proteomic and downstream metabolic alterations in the molecular pathogenesis of PDAC will be critical in developing novel biomarkers and targeted therapeutic strategies [61, 62].

The majority of genetic mutations present in pancreatic cancer can be categorized into 3 broad categories; i) activation of oncogenes (i.e. K-ras) ii) inactivation of tumor suppressor genes (i.e. TP53, p16/CDKN2A, and SMAD4) and iii) inactivation of genome maintenance genes responsible for DNA repair (i.e. hMLH1 and MSH2) [62]. A recent study found an average of 63 genetic alterations per tumor, representing abnormalities of 12 core signaling pathways (**Figure 1.2**) (see below). There are a number of other genetic mutations not identified here also thought to be involved in PDAC progression. These include, BRCA2 (breast, ovarian, and pancreatic cancer), PALB2 (breast and pancreatic cancer), STK11 (Peutz-Jeghers syndrome and pancreatic cancer) and PRSS1 (familial pancreatitis and pancreatic cancer) [63].

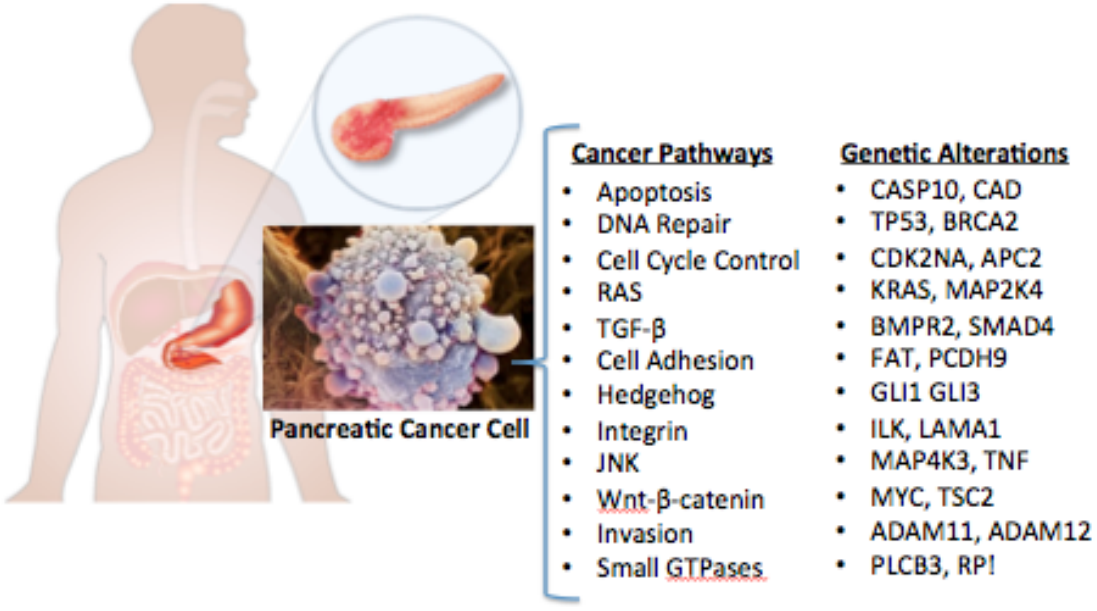

Figure 1.2 Summary of Cancer Pathways and Associated Genetic Perturbations in Pancreatic Ductal Adenocarcinoma
(*Reproduced with permission from Hidalgo et al., Pancreatic Cancer. New England Journal of Medicine 2010; 362: 1605-1617)*

Multiple combinations of varying genetic mutations are present however, resulting in large degree of tumor heterogeneity [4, 64]. Pancreatic cancer cells do not exist in isolation, but are in close communication with surrounding dense stroma. The peritumoral stroma and stromal to tumoral cross-talk plays a key role in tumorigenesis and is critically involved in tumor genesis and progression [53, 65, 66]. Making up 1-5% of the tumor cell population are pancreatic cancer cells with stem-cell properties. These cells are capable of unregulated regeneration of more-differentiated cells and are resistant to chemo and radiotherapy [4, 67, 68]. Both the supporting stroma and stem-cell population of cells have their own altered cancer-related cellular pathways, which are still largely poorly understood but could however represent additional therapeutic targets.

1.2.9 Clinical Applications of Tumor Biology

A further understanding of tumor biology may also lead to discovery of clinically useful prognostic biomarkers that could assist in stratifying pancreatic cancer patients by identifying those with occult metastatic disease prior to aggressive surgical treatment. Microscopically negative margins do not necessarily confer long-term survival status. Reported 5-yr overall survival estimates following curative surgical resection vary widely and range between 3.5-15% [1, 69-73]. Regardless, these outcomes may indicate the presence of occult metastatic

disease at the time of surgery [74]. Furthermore, on occasion patients with positive margins or the presence of lymph node metastases may show a delayed tendency towards metastasis, with improved survival compared to those in whom complete surgical resection was achieved, indicating a large degree of tumor heterogeneity [74]. PDAC appear to be represented by distinct subtypes of disease progression. While some tumors metastasize early, a subset exhibit a tendency for continued local growth, with limited evidence of metastatic potential [75, 76]. These findings of disease heterogeneity suggest that underlying fundamental molecular differences may be responsible for differences in patterns of tumor spread [77]. Better predictors of individual tumor behavior are needed. Development of molecular markers capable of identifying these subtypes preoperatively, could provide a means to stratify patients for different treatment regimens [77]. A biomarker that could more accurately predict biological behavior preoperatively could help avoid potentially futile but aggressive, radical and potentially morbid surgical treatments on occasions where there is likely to be no substantial survival benefit [74]. Conversely, preoperative stratification of patients based on tumor biology could broaden the role of radical surgical resection of locally advanced disease previously thought to provide no survival benefit. Numerous candidate molecular markers have been explored as potential biomarkers of prognosis in PDAC, however to date none has provided clinical utility [14, 24].

Integration of metabolomics discoveries with genomics and proteomics research, could provide a new and valuable avenue for discovery of clinically useful tools for preoperative molecular prognostication of PDAC patients. While genomics research has led to the identification of specific gene mutations involved in PDAC progression, a clear understanding of the specific downstream biochemical and molecular events resulting from these mutations still remains largely unknown [53]. Cancer genetic studies have provided the conceptual framework for future in-depth analysis of downstream molecular effects of specific genetic mutations. By comprehensively assessing overall metabolic profiles of biological samples, metabolomics may have a role in establishing the missing link between gene/protein expression profiles and the final cellular phenotype of PDAC.

1.3 Summary

Early detection and precise preoperative diagnosis PDAC remains an elusive area in desperate need of refined diagnostics and improved understanding of tumorigenesis. Prognosis remains dismal primarily as a result of delayed diagnosis and aggressive biological behavior. Given that the vast majority of patients with PDAC are not diagnosed until after their disease is incurable, the search for accurate, non-invasive and sensitive diagnostic tests is critical. Metabolomics, the newest of the "omics" sciences, may alter the landscape of surgical oncology through the discovery of a novel translational tool capable of

bringing the molecular world of cancer care to the bedside. By comprehensively assessing overall metabolic profiles of biological samples, metabolomics offers the potential for non-invasive screening of early tumor associated perturbations in cellular metabolism. This program of research aims to uncover the discriminating metabolomic profile associated with PDAC and examine the effects of surgical resection in modulating this metabolomic signature. Elucidation of a non-invasive, metabolomics-based multi-molecular biomarker associated with pancreatic cancer could allow for population based screening of at-risk populations, facilitate early intervention at a curable stage as well as potentially aid in the discovery of distinct molecular targets for future interventional therapies.

1.4 Objectives

The objectives of this program of research were to:

1) Provide an in-depth overview of metabolomic applications in the areas of early cancer detection, personalized therapeutics and tumorigenesis;
2) Establish a clearly defined metabolomic signature for early stage and locally advanced pancreatic adenocarcinoma compared with healthy controls;
3) Investigate the impact of complete surgical resection (R0) in extinguishing this cancer-associated metabolomic signature;
4) Briefly examine the relationship between metabolomics profile and tumor metabolic kinetics.

1.5 Program of Research

This thesis therefore sets out in **Chapter 1** to provide an up-to-date overview of pancreatic cancer in the area of diagnosis, screening and pancreatic biology**.**

This introductory overview is followed by two papers, which contribute, to the overall study goals. The first study **(Chapter 2)** provides a detailed overview (published in the *Journal of Surgical Oncology*, Davis *et al* 2011) of applied metabolomic science, placing metabolomics in context of genomics, proteomics and other –omic sciences, and its potential clinical oncological applications in the

realm of diagnostics, personalized cancer care and further delineation of tumorigenesis.

The second study (**Chapter 3)** provides a detailed comparison of urinary metabolomics profiles of a cohort of patients with early and locally advanced stage PDAC, compared with normal healthy control subjects. A series of important and substantial differences are identified in the PDAC cohort that are distinct from the controls, and these innovative findings represent a potentially applicable screening tool for further validation. Additionally, effects of complete surgical resection on the metabolomic signature associated with PDAC are examined (currently under review *Annals of Surgical Oncology*, Davis *et al.*).

Chapter 4 is the concluding chapter of this Masters Thesis, and places the findings in PDAC in context while further discussing future proposed studies for definitive validation and potential application of these findings. Different metabolomic pathways active in PDAC are discussed in detail, together with potential explanations, including a direct association with PDAC biological processes, and the alternative confounding possible explanations related to the discrete metabolomic effector pathways that could be active in patients with aggressive cancer and underlying cachexia processes.

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REVIEW

Metabolomics and Surgical Oncology: Potential Role for Small Molecule Biomarkers

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Metabolomics, the newest of the "omics" sciences, has brought much excitement to the field of oncology as a potential new translational tool capable of bringing the molecular world of cancer care to the bedside. While still early in its development, metabolomics could alter the scope and role of surgery in the multidisciplinary treatment of cancer. This review examines potential roles of metabolomics in areas of early cancer detection, personalized therapeutics and tumorigenesis
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Chapter 2: Metabolomics and Surgical Oncology: Potential Role for Small Molecule Biomarkers

2.1 Abstract

Metabolomics, the newest of the "omics" sciences, has brought much excitement to the field of oncology as a potential new translational tool capable of bringing the molecular world of cancer care to the bedside. While still early in its development, metabolomics could alter the scope and role of surgery in the multidisciplinary treatment of cancer. This review examines potential roles of metabolomics in areas of early cancer detection, personalized therapeutics and tumorigenesis.

2.2 Introduction

The new “omics” technologies have opened up exciting opportunities for screening, identifying novel biomarkers that may help in defining underlying mechanisms of tumorigenesis and development of 'cancer models'. These technologies also have the potential to help identify new targets for intervention at various stages of malignancy. With completion of the human genome map, omic sciences such as genomics, transcriptomics and proteomics, have focused on establishing links between gene/protein expression profiles and final cellular phenotype in normal and diseased states, such as cancer, by providing vast arrays of data relating to changes in gene profiles, RNA transcription, and DNA expression. While new technological advances in these areas have led to the discovery of a multitude of therapeutic targets and tumor biomarkers, a complete, comprehensive picture of cellular networking is still lacking and both genomics and proteomics remain labor-intensive and expensive. The new field of metabolomics reduces these cellular changes to a more sensitive and interpretable level, and provides an opportunity for non-invasive screening of early tumor- associated perturbations in cellular metabolism.

Metabolomics (often used interchangeably with metabonomics) describes the “*quantitative measurement of time-related multiparametric metabolic responses of multicellular systems to pathophysiological stimuli or genetic modification*” [1]. Biomarkers of interest therefore consist of metabolites, small molecules which

are intermediates and products of metabolism, including molecules associated with energy storage and utilization; precursors to proteins and carbohydrates; regulators of gene expression; and signaling molecules [2, 3]. Although generally complementary to other omic sciences, metabolomics has several unique advantages, which could help overcome limitations of its predecessors [4]. While genomics and proteomics focus on upstream gene and protein products, metabolomics focuses on downstream outputs of global cellular networking. As a result of their downstream nature, changes in the metabolome may be amplified in comparison with changes in the transcriptome and proteome [5]. Thus, the metabolome represents a functional portrait of cells or the organism, reflecting the true cellular phenotype **(Figure 2.1).**

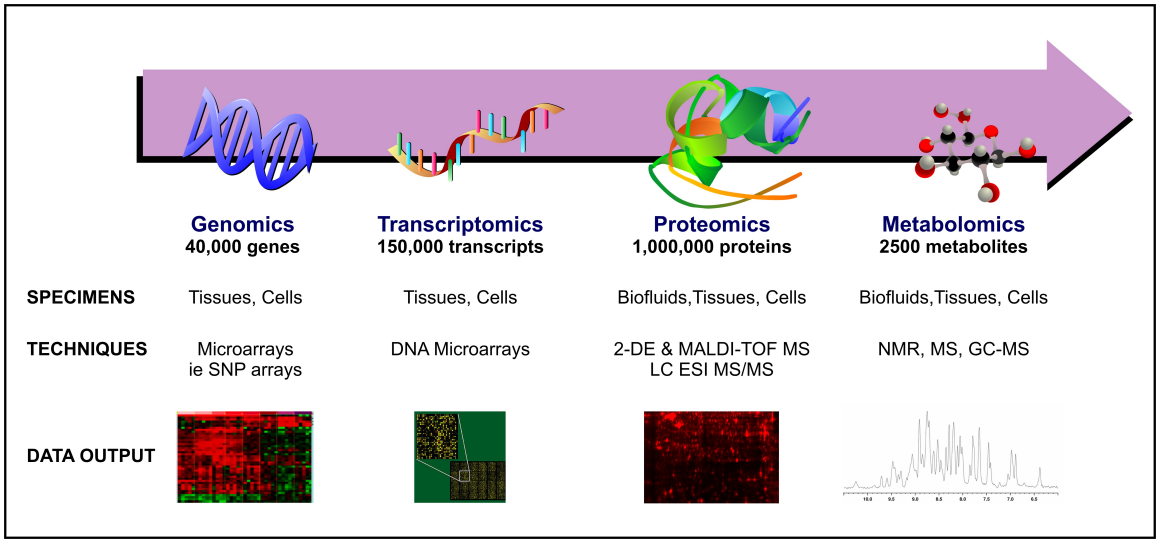

Figure 2.1. The Omics Sciences: Side-by-Side Comparison

Unlike genomics and proteomics, metabolomics permits the study of endpoint metabolites which represent the ultimate, downstream response of biological systems to genetic or environmental change [6]. In addition, because the metabolome is quite discrete and because metabolites appear in patterns (as determined by defined biochemical pathways), analysis of the metabolome provides an opportunity to gain further insight on functional changes of the organism associated with a disease state. It is estimated that the number of metabolites in any given biological system are in the range of 2000 to 3000 [7]. In contrast, the number of transcripts and proteins in any biological system is in the range of 40,000 to 100,000 products [7, 8]. Reduced complexity in metabolomic data greatly simplifies analysis and increases the likelihood of detecting meaningful changes that reflect alterations in biology [4]. Moreover, changes in metabolism result in alterations of the abundance of *groups* of metabolites. Therefore, identifying patterns of change in metabolites would provide insight on functional changes that occur due to any given condition. It is therefore conceivable that individual disease states will produce a specific metabolomic profile that reflects the underlying biology of each disease state.

While analysis of cellular metabolites is not a new field, burgeoning technological advancements have allowed for identification and quantification of a substantially broader number of individual metabolites. The concept of studying metabolites identified in tissue extracts and bodily fluids as a reflection of overall health status predates the era of large scale genome sequencing and comprehensive profiling

of the proteome [9]. However, relevant clinical applications have lagged slightly behind. Initial clinically relevant applications were established in areas of toxicology, functional nutrigenomics and in detecting inherited metabolic disorders, and have helped in bridging the gap between bench and bedside [10, 11]. Oncologic applications were soon to follow as knowledge of the complex heterogeneity amongst different cancers grew and technology continued to advance. Using nuclear magnetic resonance (NMR) -based approaches, differing spectral regions between cancer patients and healthy matched controls have been identified [12]. While encouraging, clinical applications of metabolomics-based research remain in their infancy, with many barriers to overcome before gaining widespread clinical use.

The metabolome is dynamic by nature, as it is reflective of the continuous fluxes of metabolic and cellular signaling pathways and is responsive to both host and environmental factors **(Figure 2.2)** [13]. Through its ability to capture a myriad of subtle shifts in multiple and complex metabolic pathways of a given biological system, metabolomics holds promise as a tool capable of linking integrated metabolism to human health. Evaluation of the metabolome may therefore offer a novel and sensitive approach to simultaneously evaluating multiple pathways and their downstream biological consequences, prior to any visible morphologic changes [14]. Here, we review potential applications of metabolomics in the field of surgical oncology in the realm of biomarker discovery, targeted therapeutics and interrogation of tumor biology.

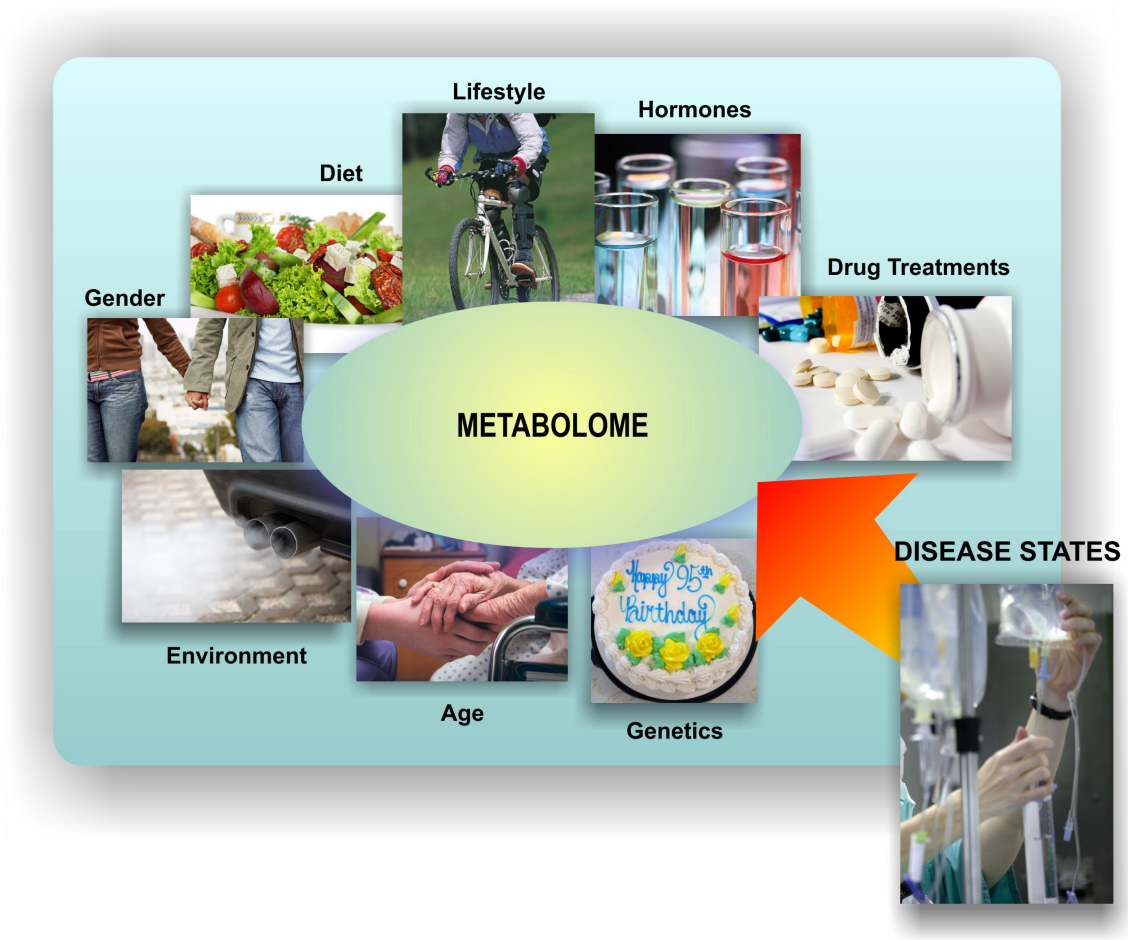

Figure 2.2 Factors Influencing the Metabolome

2.3.1 Overview of Metabolomics: Principles, Techniques and Analysis

2.3.2 Basic Principles and Samples

Several factors distinguish earlier metabolic profiling work from today's metabolomic research. There have been many advancements in analytical technologies and the number of analytical platforms available for metabolomics research has expanded dramatically. Also, advances in computing and modern software programs have allowed for processing and handling of previously prohibitive amounts of raw data and have permitted the use of multivariate statistical evaluation of metabolomic data [9]. Together, these advancements have made metabolomic profiling a key player in the field of biomarker discovery [9].

The overarching goal of any metabolomic experiment is to perform a quantitative assessment of all endogenous metabolites in a given cellular system. Metabolomic analyses have been described in a wide variety of tissues and complex biologic fluids. Each approach has its advantages and disadvantages. Analysis of tissue metabolites may provide insight into the tumor microenvironment and effects of metabolic perturbations on tumor biology. On

the other hand, tissue metabolomics has important limitations when used for diagnostic purposes. Like other biopsy-based diagnostic tests, results are incumbent on successful sampling of the lesion. Moreover, biopsies are invasive and have the potential to induce bleeding, infection or seeding of tumor cells. Fluids such as cerebrospinal fluid, bile, expressed prostatic secretions and bronchoalveolar lavage fluid have been studied, but use of these fluids for diagnostic purposes is limited to specific situations, such as in patients with lesions in direct contact with these biofluids.

The vast majority of clinical research has been carried out on urine and serum specimens [6], perhaps because these fluids have the greatest potential to provide diagnostic information on the general health of the organism. Metabolites in blood or urine reflect products of the disease state and of the host response to disease. Only very small volumes are required, and when stored at -40°C, both blood and urine samples can be kept for extended periods without alteration on subsequent analysis [15]. Samples can be procured by relatively noninvasive means at multiple time points, permitting a temporal-based analysis. It is conceivable that more immediate changes in metabolites are seen in blood in acute physiologic events. Moreover, serum metabolite profiles have demonstrated less diurnal variation as well as decreased inter-and intra-subject variability in comparison with urine. On the other hand, preparation and processing of serum or plasma is much more complex, and even small changes in technique have been shown to alter the recovery of certain metabolites [16,

17]. Moreover, lipid and protein content of serum specimens may contribute to degraded spectral resolution in the analysis phase, a phenomenon which is avoided when using urine samples [18]. The metabolomic profile of serum/plasma and urine has been investigated for diagnostic purposes for a variety of malignancies including bladder, renal, liver, colon, breast, prostate and ovarian cancers [19-29]. Initial observations in these studies support the feasibility of using either blood or urine specimens for clinical studies of the metabolomic state of the patient as well as for biomarker studies.

2.3.3 Analytical Platforms

In contrast to other omics technologies, it is not possible to make a comprehensive analysis of the metabolome using a single analytical method. Multiple spectroscopic methods exist which are capable of generating metabolomic data sets, each with their unique advantages and disadvantages. The choice of analytical platform depends on the analysis goals as well as the biological specimen to be analyzed. The two major technological platforms used for most metabolomic applications are mass spectrometry (MS) and ^{1}H-NMR spectroscopy [9, 30]. Coupling spectroscopic and MS-based analytical techniques to gas chromatography (GC) or liquid chromatography (LC) steps may improve the resolution, sensitivity and selectivity of these technologies further [31]. Each analytical method has advantages and disadvantages, most

notably differences in sensitivity, reproducibility and equipment costs. Comprehensive metabolomic analysis of any biofluid requires use of several analytical techniques.

NMR spectroscopy is an analytical technique that exploits magnetic properties of atomic nuclei. When strong magnetic fields and bursts of radiofrequency pulses are applied to atomic nuclei, absorbed energy causes them to transition from low-to-high energy states. When the perturbing radiofrequency pulse is removed, they return to their original lowest energy state and during this process emit a predictable spectrum of radiation. This is detected by a radiofrequency receiver and is represented as a unique pattern of peaks, specific to each molecule [28, 32]. The area of each resonance peak is representative of the relative concentrations of each nuclei resonating at a particular frequency [13].

^{1}H-NMR spectroscopy is currently the most commonly utilized analytical platform for studying the metabolome for a number of reasons. Aside from its unparalleled analytical reproducibility, nearing greater that 98%, it remains one of the only technologies capable of analyzing metabolites in their liquid form as well as being capable of analyzing intact tissues. There are minimal requirements for sample preparation, so the sample is preserved in its native form. Unlike MS, NMR has the ability to quantitate compounds in mixtures, as well as to identify unknown metabolites [6, 30], both of which are critical elements of biomarker discovery when using biofluids. ^{1}H-NMR's throughput capacity has also continued to improve with the introduction of technological advancements such as robotic

assisted sample preparation and transfer techniques, permitting analysis of up to 200-300 samples per day [6]. On the other hand, there are also limitations related to NMR spectroscopy. Perhaps the most significant limitation is that low abundance metabolites are not detectable [33].

MS involves identifying metabolites through generation and separation of ions on the basis of a mass-to-charge ratio [34]. MS must be used with a separation technique, as MS alone cannot distinguish isobaric metabolites. Combination with a separation technique also improves analytical resolution, as it reduces the complexity of the mass spectra and provides additional (identifying) information on physicochemical properties of metabolites. The two most commonly used separation techniques are LC and GC. GC-MS is useful for analysis of volatile metabolites or for metabolites that are rendered volatile by chemical derivatization. LC-MS is useful for analysis of non-volatile metabolites.

MS in combination with a separation technique has a higher overall sensitivity than NMR spectroscopy and it has the capability to detect a wider range of metabolites, providing a more comprehensive picture of the metabolome. There are also significant disadvantages. These techniques require a sample preparation step, which can cause loss of certain metabolites and which results in destruction of the sample. Moreover, quantification, which is crucial for recognizing potentially useful biomarkers, remains a weakness of MS [30].

2.3.4 Data Acquisition and Interpretation

The shear size and complexity of biochemical data generated from NMR spectra of tissues and biofluids remains a limiting factor in comprehending the wealth of information. Specialized mathematical, statistical and bioinformatic tools are necessary for adequate processing, analysis and storage of metabolomics data [6]. With several thousands of resolved lines apparent in the NMR spectra of biofluids, an efficient and categorical means of analysis is necessary. Data reduction techniques as well as chemometric and bioinformatic methods are crucial in helping to derive meaningful information from such an immense number of complex variables. The most efficient way to investigate this complex, multiparametric data is through the use of pattern recognition (PR) methods of analysis and in particular, multivariate statistical techniques. By reducing the dimensionality of complex data sets, these methods facilitate visualization of inherent patterns within each data set [6].

Multivariate statistical techniques generally fall into two categories: supervised and unsupervised. One of the first and most widely applied unsupervised tools is principal component analysis (PCA) [6]. This approach can be used to determine whether any intrinsic clustering exists within a complex pool of data without *a priori* knowledge of sample class. This statistical method of data reduction enables rapid identification of inherent data clustering, assessment of disease-related patterns, and identification of outliers with minimal loss of accuracy [35].

Used in isolation, however, PCA provides minimal information on variables responsible for separation, thereby adding little to biomarker discovery and further elucidation of tumorigenesis. Supervised methods include partial least squares-discriminant analysis (PLS-DA) or ANOVA where class information is supplied, and variables or metabolites giving rise to the class distinction are more easily discovered. Other chemometric tools exist; however discussion of these is beyond the scope of this review.

Whether the aim of analysis is identifying a characteristic fingerprint of disease or biomarker discovery, several stages of analysis are involved. The first stage involves PR and results in group clustering, thereby permitting discrimination of healthy controls from those with disease, for example. The next stage involves identifying specific molecules responsible for differences in spectral patterns, specifically those that lead to the group clustering. Elucidating specific set(s) of metabolite(s) responsible for clustering patterns is accomplished through the use of published data and database searches [36-38], linking observed spectra with those of known metabolites [11, 39]. There are several databases available, including the Human Metabolome Database and the number is on the rise [39, 40].

The final stage of biomarker discovery involves statistical validation of the biomarker in question. Using conventional statistical approaches, such as Student's t-test, ANOVA or nonparametric equivalents, the strength of

association between the putative biomarker and the particular clinical characteristic of interest is determined. This association may be of diagnostic, prognostic or therapeutic significance, and includes such associations as response to a particular treatment or tumor grade [11]. **Figure 2.3** outlines stages of analysis beginning with sample collection through to the validation process.

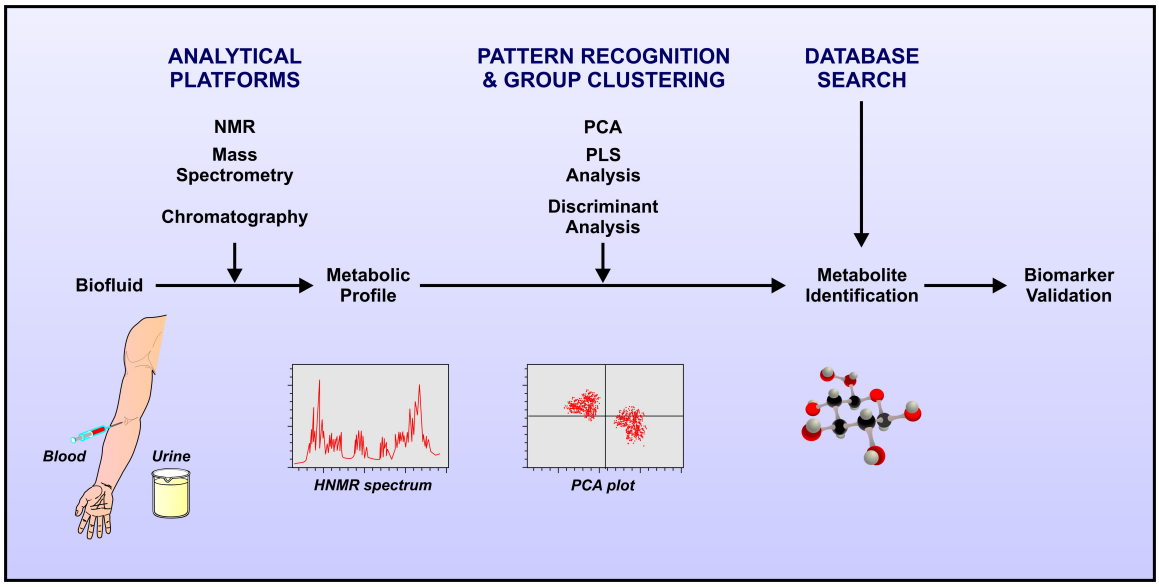

Figure 2.3 Stages of Analysis

2.4.1 Clinical Applications in Oncology: From Bench to Bedside

2.4.2 Potential Role in Early Cancer Detection and Improved Diagnostics

The fundamental precept of cancer screening is that early detection will improve survival. When malignancies are detected late in the course of disease, effectiveness of therapy may be compromised and curative intent surgery may no longer be an option. Screening tests are most relevant in populations in which a disease is prevalent and in situations where an effective therapy is available early in the disease course. Most effective screening tests are highly sensitive and specific, cost effective, widely available, and accepted by patients. Metabolomics holds promise as a non-invasive, high-throughput and cost-effective means of analysis of metabolic biomarkers capable of detecting early-stage malignancy, but also conceivably capable of identifying residual disease following surgical resection, monitoring treatment efficacy and assisting in the development of novel, targeted therapeutics. As a result, metabolomic research in biomarker discovery has grown dramatically in recent years. **Figure 2.4** depicts broad areas of research focusing on biomarker discovery aimed at early cancer detection. The majority of clinical research in metabolomics is pilot and exploratory in nature and has not yet been subject to the rigors of further validation in phase 3 and 4 studies. However, these early studies have clearly demonstrated that spectral-region differences are identifiable which discriminate healthy controls from patients with cancer in a variety of malignancies [12].

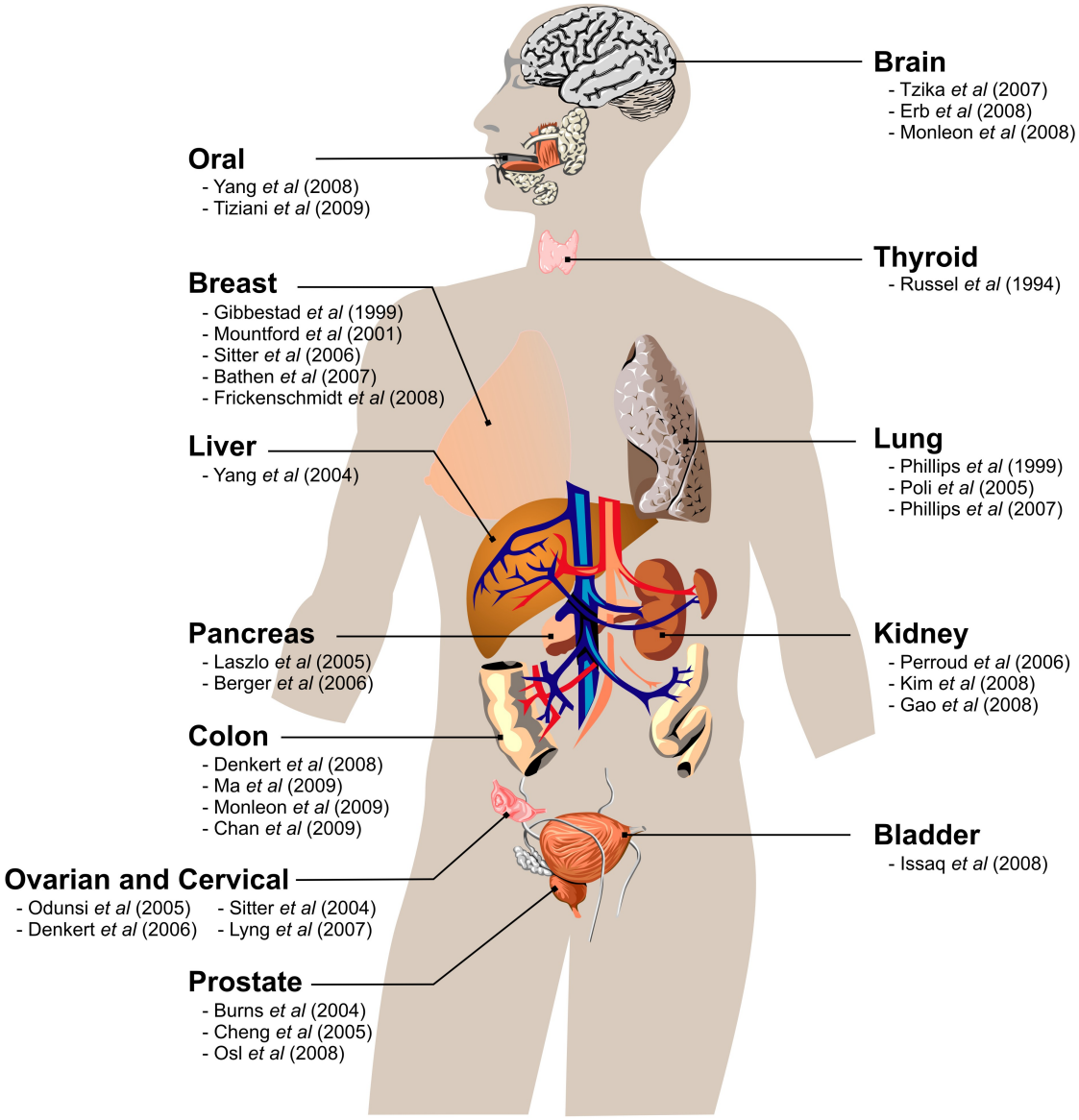

Figure 2.4 Metabolomics and Biomarker Research: What Has Been Done?

Much of the research aimed at identifying biomarkers capable of early diagnosis has focused on breast [26, 41-43], ovarian [2, 28], colon [23, 44-46] and prostate cancers [27, 47, 48]. Highlighting breast cancer research, NMR studies have identified over 30 different metabolites reliably elevated in breast cancer tissue, including glycine and choline containing compounds such as phosphocholine [49]. By no means meant to be a comprehensive list, **Table 2.1** (see below) serves to provide a sampling of some of the key metabolic disturbances associated with a number of malignancies uncovered through metabolomic-based applications. It is important to note that the majority of malignancies investigated thus far are represented by a spectrum of metabolic change rather than variations in a single metabolite, and are therefore represented by a metabolomic profile or signature of disease. A variety of related analytical platforms have been utilized to successfully discriminate malignant from healthy breast tissue, with a primary focus on the dissimilar pattern of choline-containing metabolites [26, 41-43]. The current conventional 'triple assessment' technique used in the investigation of a breast mass includes clinical evaluation, mammography and fine-needle aspiration biopsy, and has a reported sensitivity of 77-94% and specificity of 92-95% [43]. Using magnetic resonance spectroscopy (MRS) and data generated from fine-needle aspiration biopsies of benign (n=57) and malignant (n=57) breast tissue, Mountford *et al.* were able to distinguish benign from malignant breast lesions with a similarly high degree of sensitivity and specificity (93% and 92% respectively) based on relative intensities of choline-containing compounds.

Key Cancer Types	Sample of Key of Metabolite Differences (Healthy Controls/Benign Disease vs Malignancy)
Breast	Phosphocholine ↑ Choline ↑ — **Choline Metabolism** Taurine ↑ — **Amino Acid Metabolism** Glucose ↓ — **Energy Metabolism** 5-hydroxymethyl-2-deoxyuridine ↑ 8-hydroxy-2-deoxyuridine ↑ — **Modified Nucleosides: Oxidative DNA Damage**
Ovarian	3-hydroxy-butyrate ↑ Lactic acid ↑ — **Propanoate Metabolism** Glycerolphosphate alpha ↑ Phosphoric acid ↑ Inositol-2-phosphate ↑ — **Glycerolipid Metabolism** Uracil ↑ — **Pyrimidine Metabolism** Glutamic acid ↑ Proline ↑ Glycine ↑ — **Amino Acid Metabolism** Nonadecanoic ↓ Stearic acid ↓ — **Free Fatty Acid**
Colon	Beta-alanine ↑ Taurine ↑ Methionine ↑ — **Amino Acid Metabolism** Uracil ↑ — **Pyrimidine Metabolism** Oleic Acid ↑ — **Free Fatty Acid** Inositol ↑ — **Inositol Phosphate Metabolism** Choline containing compounds ↑ — **Choline Metabolism** Succinate ↓ Isocitrate ↓ — **TCA cycle** p-hydroxyphenylacetate ↑ Phenylacetylglutamine ↑ — **Gut Flora Metabolism**

Table 2.1. Breast, Ovarian and Colon Cancers: Key Metabolites Responsible for Observed Differences in Spectral Patterns

MRS was also capable of predicting lymph node status based on cellular material obtained from a fine-needle aspirate (FNA) of the primary tumor with a sensitivity of 97% and specificity of 96% [43]. They were also able to predict nodal involvement with a high degree of accuracy (sensitivity 97%, specificity 96%) based on cellular material derived from the aspirate of the primary tumor alone. Using a different analytical platform, high resolution magic-angle spinning magnetic resonance spectroscopy (HR-MAS NMR), Sitter *et al.* were able to distinguish tumor samples (n=85) from non-involved, healthy breast tissue (n=18) with a high degree of accuracy when the intensity of choline, phosphocholine and glycerophosphocholine were compared between normal and malignant breast tissue (sensitivity 83% and specificity 100%) [42]. Bathen *et al.* used the same technology to identify a metabolic phenotype that was also capable of predicting histologic grade, hormone receptor status, and axillary lymph node spread in breast cancer patients with a high degree of accuracy on the basis of metabolite information alone [41]. These results emphasize the abundance of information that can be obtained from the MR spectrum of an FNA-biopsy of a breast lesion alone [43]. While these studies and others demonstrate the ability of metabolomic-based technologies to identify a metabolic signature associated with breast cancer, and more precisely, a metabolic tool also capable of differentiating other morphologic and histological features, further research is warranted in order to fully validate these optimistic but early results [26, 43]. Bathen *et al.* were later unable to reproduce the high degree of diagnostic

accuracy reported in previous studies when model verification by blind sample prediction was performed, pointing to the need for increased standardization and transparency of both analysis and verification techniques [41]. While limited by sample size, Kvistad *et al.* have also put into question the reliability of previous reports having demonstrated elevated levels of choline-containing compounds in the breast tissue of breast-feeding women (n=5) using ^{1}H-MRS, and have proposed that the physiologic increase in metabolic activity in breast tissue during lactation has falsely paralleled the pathologic increase in cellular proliferation and turnover exhibited in malignancy [50]. Clearly, while initial results have been encouraging, continued research and validation of early results in larger, more heterogeneous patient cohorts is warranted.

Metabolomics-based surgical applications could also conceivably arise in managing malignancies that pose a diagnostic challenge. For a number of tumors, current diagnostic technologies are often unable to correctly discriminate between benign and malignant disease. Such diagnostic uncertainty arises frequently in managing both cystic and solid pancreatic neoplasms. Studies indicate that accurate preoperative diagnosis of cystic neoplasms is achieved in less than 30% of cases, resulting in potentially morbid operations being performed for benign disease or conversely, inadequate operations for potentially malignant disease [51]. Promising early results with breast, ovarian, colon and prostate cancer suggest that metabolomics-based tumor signatures may provide

a means of correctly distinguishing benign from malignant disease in situations where current technologies fall short.

Combining metabolite information with imaging techniques may also help to strengthen current diagnostic technologies. Proton magnetic resonance spectroscopic imaging (MRSI) is a metabolite imaging technique capable of visualizing and determining spatial relationships of metabolites *in vivo*, thereby providing a link between metabolite expression and anatomic distribution. Initially developed for clinically assessing brain tumors, it is now being used in the examination of anatomic and metabolic processes of prostate and breast cancers. As an adjunct to magnetic resonance imaging (MRI), studies have examined the role of MRSI in managing breast cancers that elude detection by conventional means such as mammography [52-54]. Use of MRI alone often necessitates subsequent biopsy for a confirmative diagnosis, resulting in many unnecessary biopsies for benign disease. In a prospective study by Bartella *et al.,* MSRI was used to differentiate benign (n=20) from malignant tissue (n=12) with a 100% sensitivity and 85% specificity, and could have spared 68% of patients from further investigation with biopsies without compromising the diagnosis of breast cancer [52]. While there were no false-negative results, study size was limiting and patients with any form of breast hematoma or clips associated with the lesion were excluded in order to avoid any inhomogeneities of the magnetic field. Additionally, only lesions greater than 1cm were included in

order to optimize NMR spectra and minimize data imaging time, criteria which could certainly be limiting in the clinical setting.

While biomarkers have been widely utilized in many areas of cancer care, advances in metabolomics in conjunction with the other omic sciences have the potential to offer a spectrum of novel, informative multivariate biomarkers capable of fully characterizing the malignant phenotype. These multivariate biomarkers, or disease signatures, are representative of metabolic patterns which characterize a state of cancer [11]. The true significance of metabolomic-based biomarker research aimed at early cancer detection may result from identifying early signals of cellular perturbation which occur prior to the surfacing of gross phenotypic change [14].

While there have been many early promising results, many challenges do remain. The heterogeneity of analytical platforms and the complexity and at times restrictive nature of the statistical process necessitate both standardization and further validation of these techniques with a larger number of samples given the true variability of pathologies and patient demographics encountered in clinical practice. Standardization of sample handling, processing and analysis are also necessary in order to ensure both intra and inter-laboratory reproducibility, a problem as evidenced by the wide range of reported diagnostic accuracies when different analytical platforms and statistical techniques are utilized. While encouraging, these early findings warrant validation in larger studies and in

cohorts with diagnostic uncertainty before metabolomics-based technologies are ready for widespread use in the clinical setting.

2.4.3 Potential Role in Personalized Cancer Care

Advances in molecular diagnostics have the potential to provide a more targeted approach to cancer care. In contrast to the genome or proteome, the metabolome responds to stimuli nearly instantaneously thereby permitting assessment of tumor response to environmental perturbations such as drug treatment or surgical resection, on a nearly real-time basis. The highly responsive nature of the metabolome can be exploited to monitor treatment efficacy in response to either pharmacologic or surgical intervention. Identification of specific markers indicative of therapeutic efficacy as well as other pharmacodynamic endpoints such as early drug-toxicity, could improve both the efficacy of treatment while helping avoid unnecessary toxicity and morbidity. While the majority of research in these areas is preliminary, both animal model research and human studies have had promising results.

In addition to advances in diagnostics, novel techniques linking metabolomics with imaging modalities have shown promising results in the realm of individualized therapeutics. PET, a form of metabolomic imaging done *in vivo* by measuring radiolabelled glucose, choline or thymidine as metabolic endpoints [11], is a well known example of metabolomic imaging which has been utilized

extensively in the field of oncology with respect to diagnosis, staging and monitoring treatment of a variety cancers. As an example, much success has been demonstrated with the use of [^{18}F] fluorodeoxyglucose-PET in predicting response to therapy with imatinib in treating recurrent gastrointestinal stromal tumors (GIST) much earlier than conventional CT [55, 56].

Studies using MRSI have demonstrated that malignant breast lesions contain significantly increased amounts of choline containing compounds [50, 57-59]. Preliminary research has suggested that optimization of neoadjuvant chemotherapy in patients with locally advanced breast lesions can be achieved by monitoring variations in total choline concentrations recorded within 24 h of treatment with the first dose of chemotherapy [60]. Changes in total choline concentrations from baseline correlated significantly with changes in tumor size thereby providing a method of detecting immediate response to a specific chemotherapeutic regimen [61]. In comparison, MRI assessment is unable to reliably detect response to chemotherapy until nearly 6 weeks of treatment [62].

Currently, the use of metabolic-markers of treatment response employing metabolomics-based applications remains limited. To date, there have been several pilot studies of limited sample size examining effects of surgery on the preoperative metabolomic signature of colon cancer [23, 63, 64]. Ma *et al.* were able to detect patterns of metabolic disturbances in preoperative colorectal cancer (CRC) patients which appeared to resolve postoperatively [23]. Coupling ultra-high performance liquid chromatography (UPLC)/MS technology with PLS-

DA analysis of urine specimens, metabolic patterns of healthy controls (n=80) and preoperative CRC patients (n=24) differed significantly. While the structural identification of the low molecular weight compounds accounting for these differences was not ascertained in this study, compounds which were significantly elevated in the preoperative group decreased significantly postoperatively. Qiu and colleagues had similar results using OPLS-DA analysis of GC-MS urinary metabolite spectra. While several of the metabolic differences observed between the pre and postoperative CRC patients were thought to be secondary to factors such as changes in nutritional supplementation (up-regulated amino acid metabolism), muscle protein breakdown (increased 3-methyl-histidine), and the preoperative bowel prep (down-regulated gut flora metabolites), cancer-specific metabolic changes such as normalized TCA metabolism (decreased TCA intermediates indicative of down-regulated energy metabolism) and recovered tryptophan metabolism towards a healthy state were also evident (see **Table 2.1** above*)*. Studies have been limited in size, potentially biasing the statistical analysis and the structural identity of low molecular weight compounds responsible for the clear separations of preoperative, postoperative and healthy counterparts need to be both identified and further validated.

Such metabolomic-based techniques offer a means of non-invasive preoperative phenotyping, providing information critical in subsequently guiding both surgical and medical treatments. Subsequent discoveries, such as development of biomarkers capable of identifying micrometastatic disease undetectable by

conventional diagnostic modalities, could help prevent some of the morbidity associated with aggressive operations done for truly unresectable disease while also allowing for more radical surgical options if disease is detected before becoming widespread. Furthermore, biomarkers capable of detecting residual disease following surgical resection could provide a reliable means of guiding the need for further adjuvant therapy, thereby individualizing treatment based on metabolic information. Early research suggests a vast potential for metabolomics-based applications to contribute to personalized cancer care through non- or minimally-invasive monitoring of metabolic-disturbances associated with a variety of malignancies and their unique responses to surgery and other adjuvant therapies. It is only a matter of time before further research exploring the effects of surgical interventions and adjuvant therapies on the metabolomic signature of cancers becomes more widely available, and whether these changes to the biochemical profile can reliably guide and personalize therapy.

2.4.4 Tumor Biology: Shedding Light on the Unknown

Many characteristic changes which tumor cells exhibit can be traced back to alterations in a number of key biochemical pathways involved in cell growth and proliferation. Multiple metabolic pathways that demonstrate characteristic differences between tumor cells and physiologically normal cells have been

identified. A non-exhaustive list of those metabolic pathways commonly altered in malignancy includes the glycolysis and pentose phosphate pathways, nucleotide and protein biosynthesis pathways, lipid and phospholipid turnover pathways, the citric acid cycle and the redox stress pathways [65].

Knowledge of these metabolic pathways and their mutations has drastically transformed management of surgical malignancies such as GIST. Prior to the advent of targeted molecular therapies, surgery was the only available treatment option and in approximately 50% of patients [66], complete resection was not possible. Median survival rates have increased approximately 6-fold following introduction of small molecule tyrosine kinase inhibitors, such as imatinib [66, 67]. Success of targeted therapy in GIST tumors highlights the need for continued research and understanding of tumor biology and metabolic pathways altered in malignancy. Because the metabolome is an amplified reflection of upstream changes in the genome and proteome, its characterization represents an integrated approach to the simultaneous evaluation of multiple pathways and their biological consequences [14].

It is also possible that one tumor species may affect these metabolic pathways slightly differently across individuals and molecular differences may account for the spectrum of clinical outcomes seen among patients with similar TNM stage. Current cancer classification systems do not take these molecular differences into account. Rather than relying on tumor morphology alone, complementary

metabolic information may help elucidate a more inclusive tumor portrait, while providing a more accurate estimate of disease prognosis [13]. As demonstrated by the research of Denkert *et al.,* metabolomic techniques were able to detect some of the metabolic changes associated with ovarian cancer while also detecting varying metabolic patterns associated with invasive ovarian carcinomas and ovarian borderline tumors [2]. Similar to functional genomics, metabolomics has a promising role in helping to classify different tumor types based on molecular differences. Discoveries such as these, may lead to a new a classification of cancers based on differences in metabolic tumor portraits which may in turn lead to a more accurate estimate of disease prognosis [13].

2.5 Challenges and Future Directions

Despite these encouraging results, there are still many challenges that must be overcome before metabolomic tools can be utilized on a routine basis for clinical purposes in both medical and surgical oncology. Metabolite analysis remains a key barrier to further advancements in the field. The numbers, diversity and dynamic concentration ranges of metabolites under study pose a significant challenge to researchers. Comprehensive management of massive amounts of data generated from high throughput screening, and simplification of complex data-outputs into more interpretable and user-friendly formats are just a few of

the areas to be addressed [13]. This discipline remains dependent upon continued sophistication in technologic, computational and analytical approaches [1].

Both inter- and intra-individual metabolite variability also remain a complicating feature of metabolomics research. Internal and external factors, such as instrumental, environmental and physiologic factors (i.e. diet, hormonal milieu, stress, diurnal cycles) contribute to the overall expression profile, and could potentially confound the results of any metabolomic study. Several techniques have been developed for use in the preprocessing stage of analysis to minimize the influences of these extraneous confounders [68]. Orthogonal signal correction (OSC) or variable stability scaling (VAST) are both examples of techniques capable of removing the confounding effect of biologic variation, which may mask effects of disease-associated biochemical change [68, 69]. Further discussion of data filtering and scaling is beyond the scope of this review. The same issues of standardization that have plagued the fields of genomics and proteomics also remain a threat to metabolomics. Attempts to address this issue have been initiated by the Metabolomics Society through creation of a Chemical Analysis Working Group to develop standards for metabolomics analysis [70]. Adherence to developed standards through inter-laboratory data comparison will help with quality control and will help speed discovery of clinically important metabolite profiles.

Metabolomics is an emerging discipline with the potential to link molecular tools with clinically relevant applications in the field of surgical oncology as well as other areas of medicine, and has brought promising advancements in the areas of improved diagnostics and individualized therapy. Knowledge gained from metabolomic research may permit development of treatments tailored to the molecular processes underlying the disease rather than treating the phenotypic expression of the disease's consequences [71]. This renewed level of information which focuses on detecting cellular changes long before phenotypic expression, has the potential to change the scope, role and goal of treatment. Improved diagnostic tools may help in avoiding morbid operations that have a high likelihood of futility but conversely may widen the scope of surgical intervention by allowing detection of disease long before loco-regional or metastatic spread. Early detection of premalignant conditions or metabolic alterations indicative of malignant processes could further expand surgical indications, and in essence, result in the need for prophylactic surgeries. Real-time, guided therapies based on metabolic endpoints rather than symptomatology or imaging alone will allow for personalized treatments rather than one-size-fits all surgical and chemotherapeutic regimes.

The future success and promise of metabolomic research will be dependent on continued collaborative and multidisciplinary research with integration of the other omic sciences in order to provide a truly holistic view of the malignant phenotype and the changes associated with malignant transformation.

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Pancreatic Ductal Adenocarcinoma is Associated with a Distinct Urinary Metabolomic Signature

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Currently Under Review

Chapter 3: Pancreatic Ductal Adenocarcinoma is Associated with a Distinct Urinary Metabolomic Signature

3.1 Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with poor prognosis in part due to the lack of early detection and screening methods. Metabolomics provides a means for non- invasive screening of early tumor associated perturbations in cellular metabolism.

Methods: Urine samples of PDAC patients (n=32) and healthy, age and gender-matched controls (n=32) were examined using ^{1}H-NMR spectroscopy. Paired pre and postoperative urine samples (n=20) were also examined. Targeted profiling of spectra permitted quantification of 66 metabolites. Unsupervised (principal component analysis, PCA) and supervised (orthogonal partial-least squares discriminant analysis, OPLS-DA) multivariate pattern recognition techniques were applied to discriminate between sample spectra using SIMCA-P^{+} (version12, Umetrics, Sweden).

Results: Clear distinction between PDAC and controls were noted when using OPLS-DA. Significant differences in metabolite concentrations between cancers and controls (P<0.001) were noted. Model parameters for both goodness of fit, and predictive capability were high (R^2 = 0.85; Q^2 = 0.59, respectively). Internal

validation methods were used to confirm model validity. Sensitivity and specificity of the multivariate OPLS-DA model was summarized using a receiver operating characteristics (ROC) curve, with an area under the curve (AUROC) = 0.988, indicating strong predictive power. Preliminary analysis suggests that the cancer-associated metabolomic signature was extinguished following RO resection.

Conclusions: Urinary metabolomics detected distinct differences in the metabolic profiles of pancreatic cancer patients compared with healthy controls. These preliminary results suggest that metabolomic approaches may facilitate discovery of novel biomarkers capable of early disease detection.

3.2 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is associated with dismal outcomes, and despite radical surgical resection and adjuvant chemotherapeutic strategies, these outcomes have not changed substantially in decades. Incidence and mortality rates almost parallel each other, with a median survival of 12 months [1]. The only potentially curative treatment is surgical resection, but even with negative margin (R0) resection, recurrence rates are high, with 5-year survival rates varying between 2.8% and 31.4% based on disease stage [1]. Furthermore, 75% - 80% present with advanced, unresectable disease [2]. Failure to detect early cancers and a lack of understanding of the unique biological aggressiveness of PDAC are major contributors to poor outcomes. Current radiographic studies do not have sufficient spatial resolution to detect early stage disease, and accurate diagnosis even at later stages can be challenging given that benign pancreatic disease can sometimes mimic pancreatic cancer [3].

Application of urinary metabolomics to PDAC offers an opportunity to define unique tumor-related signatures. Identification of such signatures could open up new avenues for non-invasive screening of high-risk populations; up to 17% of PDACs are associated with familial or hereditary cancer syndromes, including BRCA2 mutations, thereby representing one sub-population suitable for targeted screening [4]. Furthermore, a unique PDAC-specific metabolomic signature could potentially uncover novel pathways for therapeutic intervention, and novel

targeted adjuvant therapies where surgical resection fails to eradicate the disease. Knowledge gained from metabolomics research may permit development of treatments tailored to molecular processes underlying the disease rather than treating the phenotypic expression of the disease's consequences [5].

To date several studies have looked at metabolomic profiles in PDAC [3, 6-12]. However, the majority are based on serum profiles and have been universally carried out in late, stage IV disease. The current study sets out to compare metabolomic profiles in urine of patients with early stage or locally advanced PDAC, with appropriate age and gender-matched, healthy controls. We hypothesize that global metabolite analysis of urine samples using nuclear magnetic resonance (NMR) -based approaches and multivariate statistical techniques will reveal a characteristic metabolomic signature associated with PDAC.

3.3.1 Methods

3.3.2 Study Design and Sample Collection

This study was approved by the Alberta Cancer Research Ethics Board and the Human Research Ethics Board of the University of Alberta. Written and informed consent was obtained from all participants prior to study enrollment. Midstream urine samples were collected preoperatively from patients with PDAC (n=32) in the Edmonton region. All cases were correlated with histologic findings and follow-up data was available in all cases to ensure accurate classification of disease. Controls (n=32) were healthy, age and gender-matched male and female volunteers with no declared history of malignancy. Breastfeeding or pregnant women were excluded from study enrollment, as were patients with uncontrolled bacteria, viral or fungal infection. Additionally, subjects with compromised renal function reflected by impaired creatinine clearance (based on estimated glomerular filtration rate (GFR)) were excluded to prevent confounding effects of impaired metabolite excretion. Paired pre and postoperative (median 3.7 months) urine samples were also compared in patients with resectable disease undergoing complete R0 resection (n=20).

Urine samples were stored at -80°C until NMR data acquisition. Prior to data acquisition, samples were thawed and prepared by adding 75 µl of a chemical shift standard (Chenomx Inc., Edmonton Canada containing 5.046 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate-d_6 [DSS-d_6] and 0.2% NaN_3 in 99.8% w/v

D_20) to 675 µl of urine. The pH was adjusted using small additions of NaOH or HCl to obtain a final pH of 6.75 +/- 0.05 in order to reduce pH variation among samples. A 700 µl aliquot of prepared sample was then transferred to a 5mm NMR tube (Wilmad, Nuena, NJ) immediately prior to NMR acquisition.

3.3.3 ^{1}H-NMR Spectroscopic Acquisition and Targeted Profiling

^{1}H-NMR spectra were acquired according to previously published and accepted methods [13, 14]. Briefly, one-dimensional ^{1}H-NMR spectra of urine samples were optimized, and excitation pulse calibrated based on single pulse nutation [15-17]. Spectra were acquired using the first increment of a standard NOESY pulse sequence [18]. Experiments were executed on a 2 channel 600 MHz VNMRS spectrometer (Agilent Inc., Palo Alto, CA) equipped with a 5mm-HX dual tune probe. Spectra were acquired at 25°C with an observation width of 12 PPM, 100-ms mixing time, 4 second acquisition time, 4 steady state scans, and 32 transients. Water suppression was achieved utilizing an 80-90 Hz gammaB_1 ^{1}H continuous wave saturation pulse applied on the optimized water resonance during the 0.9s presaturation period and throughout the 100-ms mixing time. All spectra were zero-filled to 131k data points followed by apodization with a line-broadening weighting function of 0.5 Hz.

Using Chenomx NMR Suite 7.0 software (Chenomx Inc. Edmonton, Canada), metabolites were identified and quantified using a targeted profiling approach [19, 20]. This method compares the integral of a known reference signal, DSS,

with signals derived from a documented database of 297 compounds in order to determine concentrations relative to the reference signal [13]. All samples were analyzed blindly in a random fashion. A minimum of two analysts independently analyzed the spectra and only those compounds whose identity and concentrations were agreed upon were included. A set of 66 metabolites was identified and quantified. Additionally, creatinine concentrations of 12 randomly selected urine samples were verified using non-NMR, laboratory based colorimetric techniques using a commercially available kit (Arbour Assays, DetectX Urinary Creatinine Kit, Cat K002-H5), and 95% correlation was achieved.

3.4.1 Data Analysis

3.4.2 Data Preprocessing and Statistical Analysis

Prior to further analysis, drug metabolites and drug vehicle constituents were excluded. Because of a low yield of detection among samples (only 1-2 values differing from the median concentration), gluconate, glycerol, ornithine, uracil and 1,6-anhydro-β-D-glucose were also excluded. The remaining 58 metabolites were included in all subsequent model generations.

Metabolite concentrations were log-transformed to account for non-normal distribution of metabolite data, mean-centered to improve interpretability of the models generated and scaled univariately to ensure all metabolites, both high

range and low range, were given equal weight in analysis. Patient characteristics were compared using Welch's two-sample *t*-test for continuous variables and exact methods for categorical variables. Metabolite differences between PDAC and healthy controls were compared using Mann-Whitney non-parametric statistical analysis. Statistical significance was set at $p < 0.05$. GraphPad Prism Version 5.0c was used for all descriptive statistics (GraphPad Software, San Diego, CA).

Unsupervised (principal component analysis, PCA) and supervised (orthogonal partial least-squares discriminant analysis, OPLS-DA) multivariate pattern recognition techniques were applied to pre-processed metabolite concentration data to discriminate between sample spectra of PDAC patients and healthy controls using SIMCA-P^+ (version 12, Umetrics, Umeå, Sweden). By reducing the dimensionality of a set of measured variables, PCA provides a crude dataset overview and is used for initial exploratory analysis. For class discrimination, OPLS-DA with an integrated orthogonal signal correction (OSC) filter was applied. Partitioning of predictor variables improves both model transparency and interpretability [21, 22].

Cross validation and permutation testing were applied for internal validation [23-25]. A receiver operating characteristics (ROC) curve was generated to define the predictive accuracy of the OPLS-DA model from cross-validated predicted Y-values (SIMCA-P^+ software, Y-predcv, predictive Y). Area-under-the ROC curve

(AUROC) was calculated using STATA/SE 10.1 (TX, USA). The variable importance on projection (VIP)-parameter was generated for a weighted, quantitative measure of discriminatory power of the metabolites. Represented by a unit-less number, the higher the value, the greater the discriminatory power of the metabolite. VIP scores >1 generally represent those metabolites carrying the most class discriminating information [23].

3.5.1 Results

3.5.2 Patient Characteristics

Table 3.1 (see below) provides a detailed outline of pertinent patient and tumor characteristics. Patients with PDAC (n=32) were age and gender-matched with healthy controls (n=32, p=0.80 for age and p=1 for gender). PDAC was confirmed histologically in all cases. Sixty-three percent of cases were located in the pancreatic head and the majority were moderately differentiated, stage IIb disease (69%). Fifty nine percent of cases were amenable to surgical resection. The majority of patients with PDAC exhibited significant weight loss (≥ 5% over 6-12 months).

Number of Subjects (n)	PDAC (32)	Controls (32)	*p*
Age [median (range)]	69.5 (48-83)	69.5 (47-84)	0.802
Gender (male/female)	18/14	18/14	1
TNM Stage			
Ia/b	2/32 (6.3%)	-	-
IIa	3/32 (9.4%)	-	-
IIb	22/32 (68.8%)	-	-
III	5/32 (15.6%)	-	-
Histologic Type			-
Invasive Ductal Adenocarcinoma	31/32 (96.9%)	-	-
Invasive Adenosquamous Carcinoma	1/32 (3.1%)	-	-
Histologic Grade			-
1	3/32 (9.4%)	-	-
2	14/32 (43.6%)	-	-
3	6/32 (18.8%)	-	-
Unavailable	9/32 (28.1%)	-	-
Location			-
Head	21/32 (62.5%)	-	-
Head/Uncinate	7/32 (21.9%)	-	-
Body/Tail	4/32 (12.5%)	-	-
Resectable	19/32 (59.3%)	-	-
Unresectable	13/32 (40.6%)	-	-
Weight Loss (≥5%)			-
Yes	21/32 (65.6%)	-	-
No	2/32 (6.3%)	-	-
Unavailable	9/32 (28.1%)	-	-

Table 3.1. Clinical Features of Study Subjects and Tumor Characteristics

3.5.3 Metabolomic Profile Differences Between Pancreatic Cancer and Healthy Controls

Metabolite concentration data was then analyzed using both unsupervised (PCA) and supervised (OPLS-DA) multivariate pattern recognition methods. Group clustering based on disease status (cancer vs. healthy) was observed at the exploratory, unsupervised phase **(Figure 3.1A)** (see below). Supervised methods were then applied using OPLS-DA and resulted in clear discrimination between PDAC and healthy controls **(Figure 3.1B).** OPLS-DA model parameters for explained variation (R^2) and cross-validated predictive ability (Q^2) were robust (R2= 0.85, Q2 = 0.59).

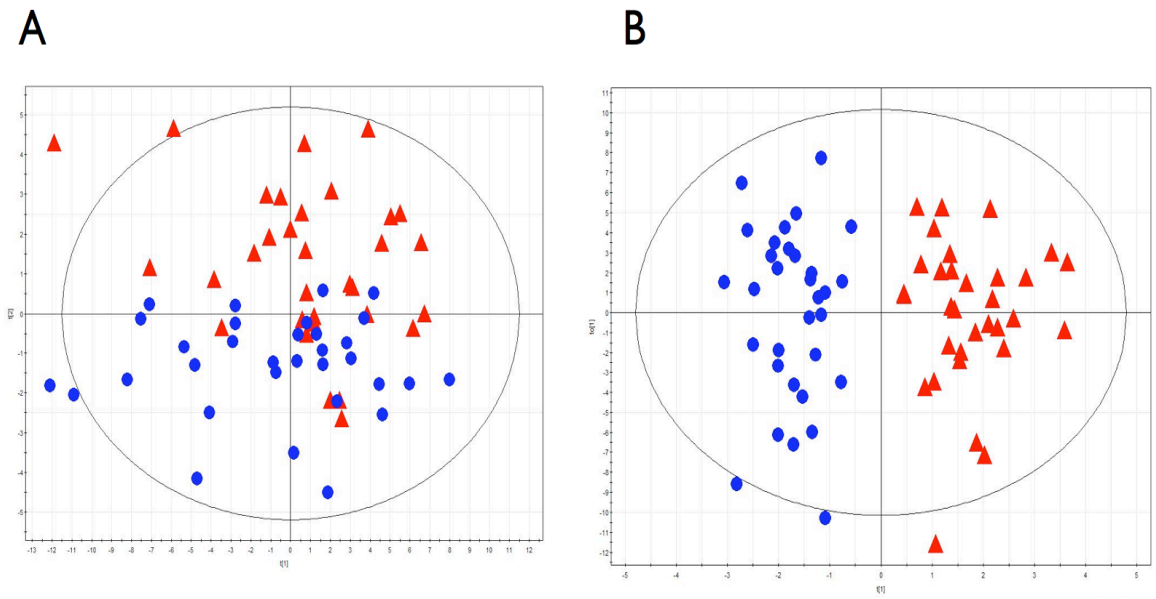

Figure 3.1. PCA and OPLS-DA Score Plots of Urinary Metabolite Profiles Derived from PDAC and Healthy Controls

PDAC samples are represented by red triangles and black circles depict controls. Both are 2 component models based on 59 measured metabolites. A) Unsupervised, PCA score plot. B) Supervised OPLS-DA score plot.

Unique metabolite expression patterns were observed when comparing PDAC patients and controls. Twenty-two metabolites showed significantly different levels of expression (p < 0.0001-0.026). While all perturbations in metabolites contribute to the OPLS-DA model, the direction of change for key metabolites according to the VIP-parameter is reflected in **Table 3.2** (see below). It is apparent that simultaneous perturbations in multiple metabolic pathways are responsible for the observed class separation.

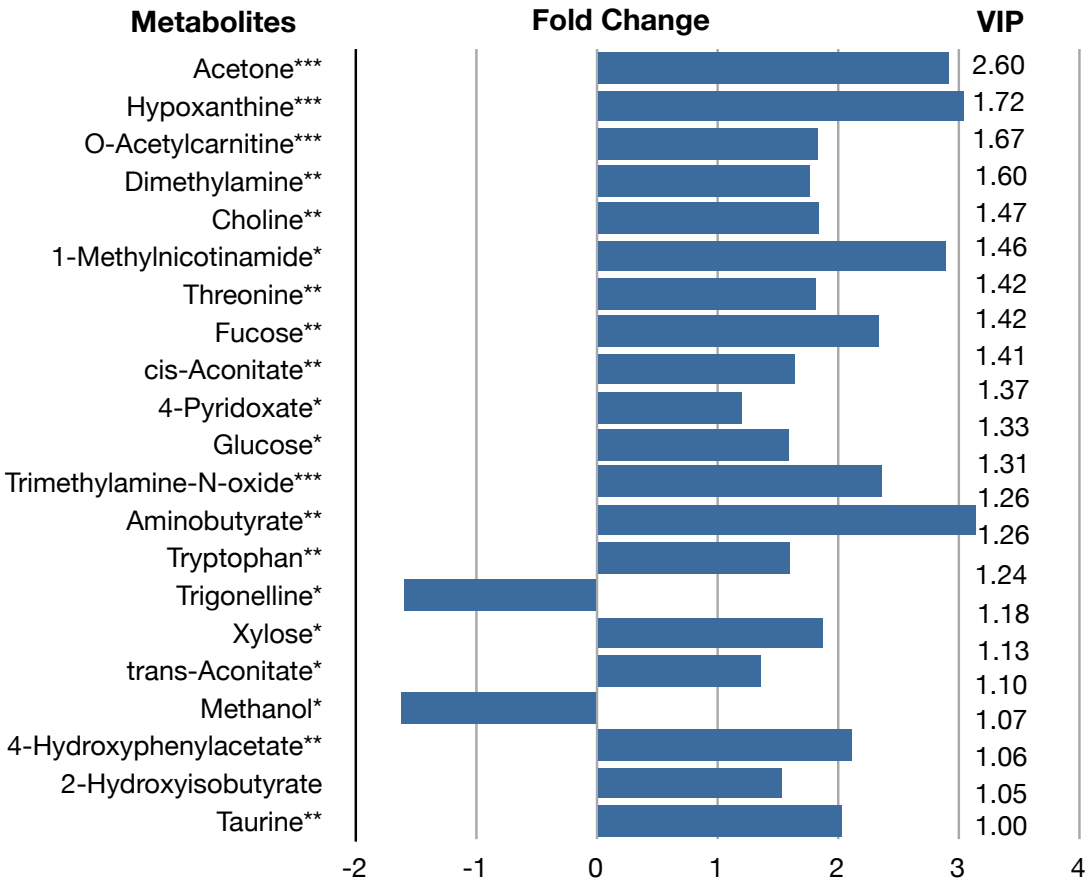

Table 3.2. Key Metabolite Differences Between Pancreatic Ductal Adenocarcinoma and Healthy Controls.

Key metabolites involved in OPLS-DA model generation according to the VIP-parameter and p-value significance. Only those metabolites with significant concentration differences or a VIP-parameter ≥ 1 are displayed. p-values were obtained using Mann-Whitney nonparametric statistical analysis. * $p < 0.05$, ** $p < 0.01$, ***$p \leq 0.001$. Fold change was calculated by dividing the median metabolite concentration in cancers by controls. Those metabolites increased in cancers are projected to the right, while those decreased in cancers are projected to the left.

Additionally, a sub-analysis was carried out excluding exogenous metabolites to minimize dietary and environmental influences on model generation. These included 1,6-anhydro-D-beta-glucose, adipate, 2-hyroxyisobutyrate, ascorbate, ethanol, sucrose and xylose. Methanol (a microbial metabolite) was also excluded in the secondary analysis. The OPLS-DA model (results not shown here) achieved comparable class separation, with similar model parameters (R2=0.79, Q2=0.52). These results suggest that exogenous metabolites did not contribute significantly to class discrimination.

3.5.4 Model Validation and Prediction Accuracy

Two separate methods of internal validation, permutation testing and cross-validation, were used to confirm model validity. Permutation tests involve random assignment of class labels to cases and controls. **Figure 3.2** (see below) demonstrates that the R^2/Q^2 values of the original model were always higher than those of the 100-permuted models.

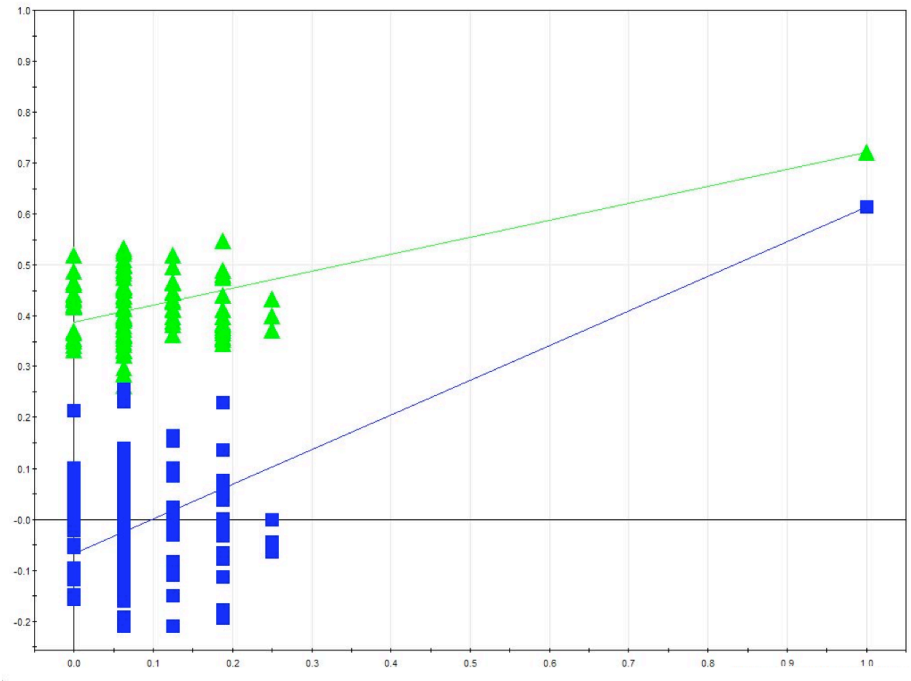

Figure 3.2. Permutation Analysis of OPLS-DA Model.

Statistical validation of OPLS-DA model by permutation analysis using 100 different model permutations. The goodness of fit (R^2) and predictive capability (Q^2) of original model are indicated on far right and remain higher than those of the 100 permuted models to the left.

OPLS-DA model generation employed a seven-fold cross validation step. This involves omitting a portion of the data from model development, developing parallel models from the reduced data, predicting the omitted data from the different models, and then comparing predicted with actual values, providing an estimate of overall predictive power. Using the cross-validated Y-predicted values, model sensitivity and specificity were summarized with an ROC curve

(Figure 3.3). The calculated AUROC of 0.988 is indicative of strong predictive power.

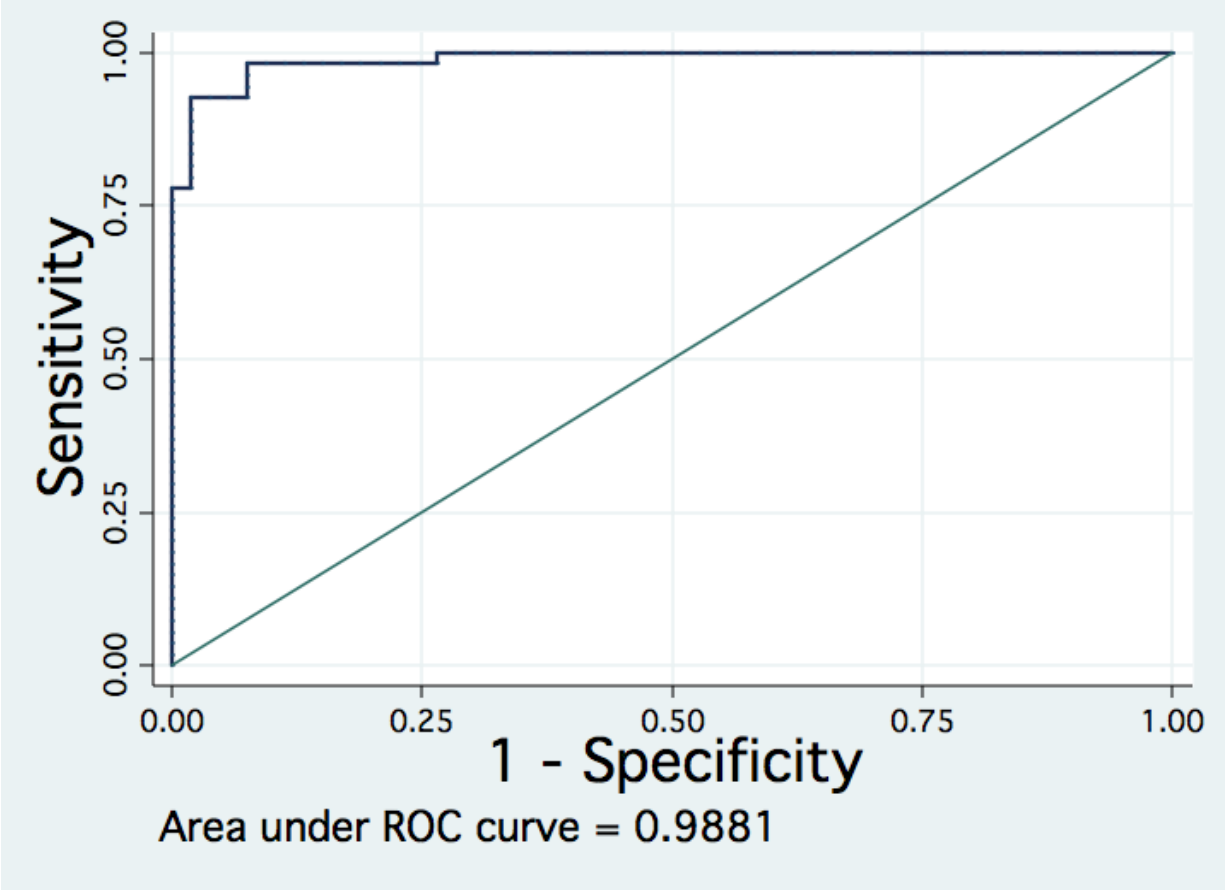

Figure 3.3 Measure of Model Predictive Ability Using ROC Curve Analysis.

ROC curve analysis using cross-validated predicted-Y values of the OPLS-DA model discriminating PDAC patients from controls. AUROC= 0.9881 indicating strong predictive ability.

3.5.5 Effects of Surgical Resection on Metabolomic Profile

PCA and OPLS-DA pattern recognition techniques were applied to paired, pre and postoperative metabolite concentrations after complete R0 surgical resection. While no group clustering was observed with PCA, OPLS-DA supervised methods revealed distinct class separation following surgical resection (model parameters R^2=0.86, Q^2=0.30, sensitivity 95.2%, specificity 85.7%) (**Figure 3.4)** (see below)

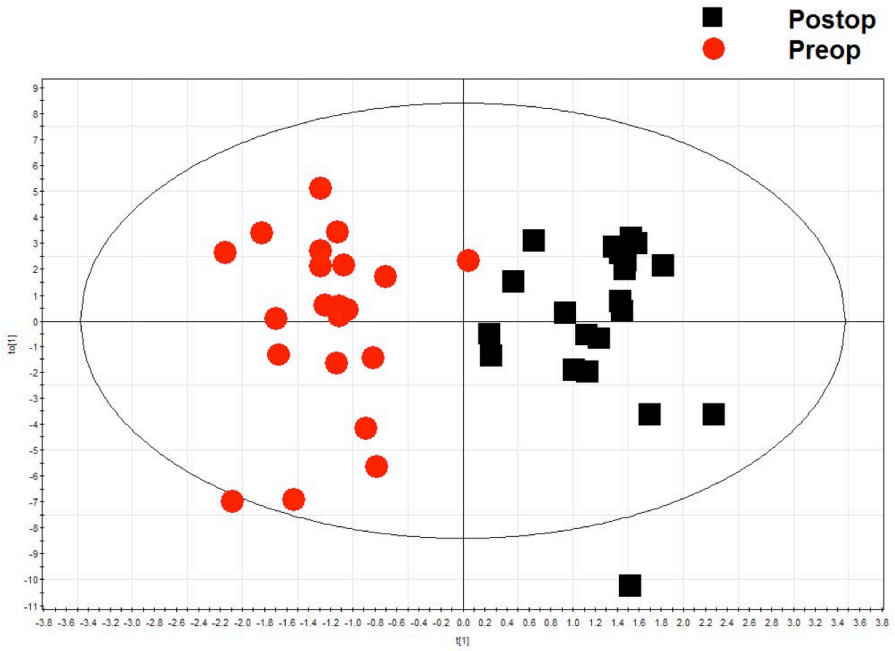

Figure 3.4. OPLS-DA Score Plot: Effect of Surgical Resection on Urinary Metabolomic Profile Associated with PDAC

Urinary Metabolite Profiles Derived from Preoperative and Postoperative PDAC Patients. Red circles represent preoperative PDAC samples and postoperative PDAC samples are represented by black squares. 2 component model based on 59 measured metabolites. Abbreviations: *OPLS-DA* Orthogonal Partial-Least Squares Discriminant Analysis*, PDAC* Pancreatic Ductal Adenocarcinoma

3.6 Discussion

PDAC remains an aggressive malignancy with a high degree of biological heterogeneity and dismal prognosis. Delayed diagnosis precludes the majority of patients from curative intent surgery since up to 80% of patients present at an advanced, incurable stage [26]. While a wide array of molecular markers have been developed to help with early and accurate diagnosis of pancreatic pathologies, they lack adequate sensitivity and specificity to provide useful clinical resolution [27]. Additionally, despite many advances, current imaging and diagnostic technologies are frequently unable to discriminate benign from malignant pancreatic disease often resulting in unnecessary or inadequate surgery. Furthermore, between 10-25% of patients undergoing radical pancreatic surgery are found subsequently to have benign disease on pathology [3, 28-30].

Unlike advances in the screening and early detection of other malignancies such as breast and colorectal cancers, reliable and sensitive population-based screening modalities for PDAC do not yet exist. Given the relatively low prevalence of disease and limited accuracy of current testing modalities, presently population-wide screening is not practical. However, high-risk patients, accounting for up to 17% of all cases of PDAC, represent a potentially important target population where effective screening tools are needed [4, 31-35]. While several institutions have screening protocols for patients with familial or hereditary predispositions, guidelines are sometimes conflicting and diagnostic

tools invasive and inadequate [35]. Timely diagnosis alone however, will unlikely improve outcomes but may enhance lead-time bias. Survival remains dismal even among those patients in whom complete resection (RO) is achieved. Improved understanding of the molecular basis of PDAC is needed to provide avenues for advancements of targeted therapeutics.

Urinary metabolomics offers a reliable, non-invasive means of identifying tumor-associated perturbations of cellular metabolism. By comprehensively assessing overall metabolic profiles of biological samples, metabolomics may establish the missing link between gene/protein expression profiles and final cellular phenotypes in normal and diseased states. Metabolomics has the potential to drastically alter the field of surgical oncology in the diagnosis, treatment and understanding of tumor biology.

To date several studies have explored the role of metabolomic profiling and diagnostics in pancreatic cancer. Using an NMR-based approach, Bathe *et al*. recently established a serum metabolomic profile capable of discriminating pancreatic cancer patients from patients with benign pancreaticobiliary disease [3]. These results were congruent with previous serum studies which were limited in sample size (n=5/17) and analyses technique, using unsupervised methods alone [9]. One study reported successful discrimination of malignant from benign biliary disease using bile samples [12]. Using ^{1}H-NMR, Nishima *et al*. discovered significantly elevated lactate levels in the serum and bile of patients with

malignant hepatobiliary disease [8]. However invasive methods are required for collection of bile. While it is still unclear which biofluid is optimal for metabolomics processing, urine has the distinct advantage of being easy to collect, simple to handle and process, and avoids issues of degraded spectral resolution resulting from hydrophilic lipid compounds present in serum samples [36]. Recently, Napoli *et al.* identified a distinct urinary metabolomics signature of PDAC, distinguishing patients from healthy controls. However, a large proportion of patients had advanced stage disease and gender-effects were not examined as profiles were developed from a cohort of male patients alone [7].

Using a representative sample of male and female patients with early stage or locally advanced disease, we have identified a urinary metabolomic signature of PDAC with strong predictive accuracy. In comparing metabolite expression profiles of PDAC patients and healthy controls, it is evident that a number of pathophysiological processes may contribute to the differences observed. We suspect that the spectrum of metabolic perturbations observed is reflective of changes occurring both at the tumor microenvironment level and in global metabolism. Cancer-specific elevations of hypoxanthine, choline, trimethylamine-N-oxide, o-acetylcarnitine and acetone, may be indicative of metabolic disturbances associated with tumor metabolism. These changes possibly represent enhanced capacity for DNA synthesis and energy production (hypoxanthine), cell membrane formation (choline, trimethylamine-N-oxide) as

well as increased rates of lipolysis and fatty acid metabolism (o-acetylcarnitine, acetone) seen in rapidly proliferating tumor cells [37-40].

Cancer-specific increases in a number of amino acids and amino acid derivatives were also noted; threonine, tryptophan and 4-hydroxyphenylacetate were highly significant, while alanine, isoleucine, leucine and valine showed trends towards significance. This pattern may be reflective of muscle protein breakdown, during which all constituent amino acids enter oxidative pathways [14]. PDAC has one of the highest incidences of cachexia among patients with solid-epithelial malignancies [41]. A majority of our patients had ≥ 5% weight loss at diagnosis. Representing a complex metabolic syndrome characterized by anorexia and high rates of fat and skeletal muscle degradation, cachexia results in severe metabolic disturbances in energy and protein metabolism [41]. Muscle wasting can occur independent of changes in fat mass, and often occurs as an occult phenomenon in early stages of malignancy, before becoming clinically apparent [14, 42]. A noninvasive means of detecting early muscle wasting in patients harboring occult malignancy could have significant clinical utility. The above postulations are speculative however and further experimental *in vivo* modeling is required to confirm their relevance.

Complete surgical resection successfully eliminated the observed PDAC associated metabolic perturbations. The sensitivity and specificity of the surgical model were high (95.2% and 85.7%, respectively). These findings are

preliminary, and will require further validation in a larger patient subset. This data suggests however that the PDAC-associated profile is driven primarily by altered tumoral metabolism, rather than epi-phenomenal cachexia related change.

Using NMR and multivariate statistical techniques, we were able to define a discrete metabolic signature associated with early and locally advanced PDAC with a high degree of accuracy. Such a tool could allow for mass-screening of at risk populations, and facilitate early intervention at a curable stage as well as potentially uncovering distinct molecular targets for future interventional therapies. Limitations of this study include small sample size, and future investigations should include model validation using an external, independent cohort of patients. Additionally, further exploration of underlying molecular mechanisms of metabolic change are required through the use of *in vivo* studies/animal models. Integration of results from diverse biofluids and analytical platforms including mass spectrometry, will also further refine these preliminary results.

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Chapter 4: Summary and Concluding Remarks

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4.1.1 Summary of Research

4.1.2 Metabolomic Signature of Pancreatic Cancer

Metabolomics has been defined as *"the systematic study of metabolic responses of multicellular systems to pathophysiological stimuli or genetic modification* [1]." Application of metabolomic tools in oncology offers a potential to uncover new pathways in cancer control and signaling, and could potentially facilitate non-invasive screening for early cancer detection for broader application in high-risk populations. In contrast to genomics and proteomics research, metabolomics science involves a comprehensive study of endpoint metabolites, representing a more mature, downstream response of biological systems to genetic or pathophysiologic insults. Metabolomic screening would gain considerable traction if signals of altered cancer cell metabolism were detectable prior to the surfacing of gross phenotypic change and clinical presentation, providing an opportunity for earlier surgical, chemotherapeutic or other neoadjuvant therapies with a consequent increased likelihood of cure.

PDAC remains one of the most aggressive cancers to manage. Surgical resection of early stage disease provides the only chance for cure, however disease remains undetected in the vast majority of patients until advanced and

inoperable. The opportunity to diagnose and intervene at a much earlier stage would therefore have profound clinical consequence, especially if disease detection occurred at early or even pre-invasive stage. A wide array of molecular markers have been explored for potential use in early diagnosis and accurate detection of pancreatic pathologies, however to date none have had clinical utility. Targeted screening tools could prove to be transformative in patients with higher risk of pancreatic cancer. Timely diagnosis must occur in concert with improved therapeutics since outcomes remain dismal even when margin negative resection is achieved. A greater understanding of the molecular pathogenesis of PDAC and the downstream functional significance of genomic/proteomic discoveries will provide an avenue for advances in effective, targeted therapeutics.

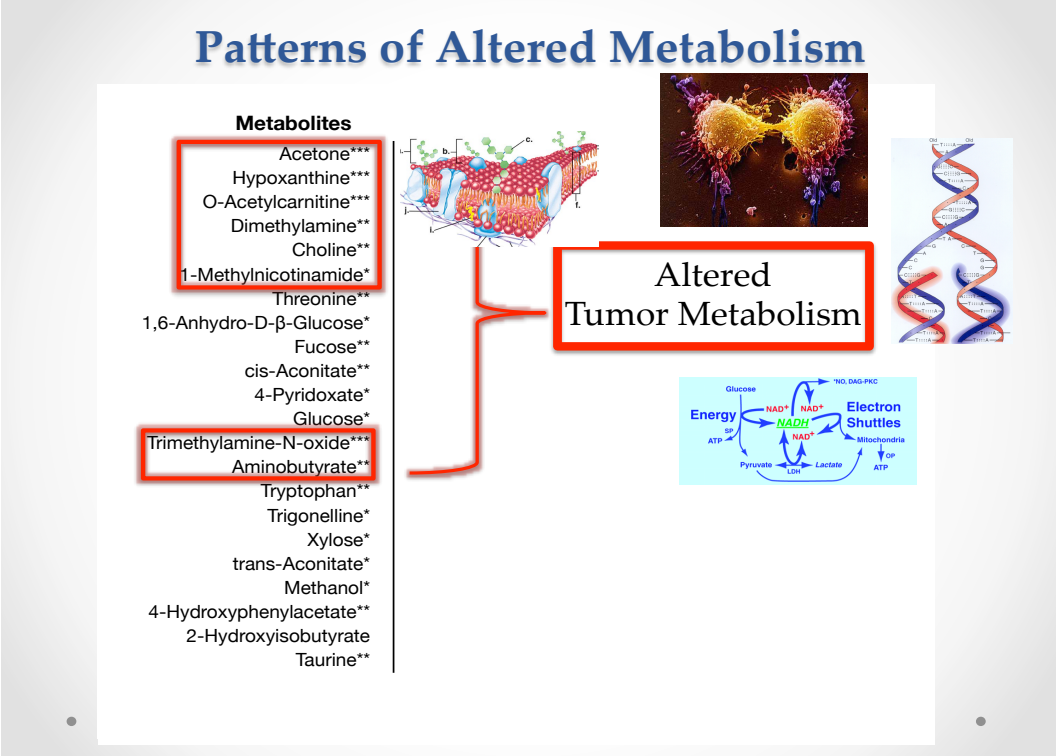
Using ^{1}H-NMR and multivariate statistical analysis, we have successfully identified a discrete, highly predictive urinary metabolomic signature associated with early stage and locally advanced PDAC. Class separation was apparent between PDAC patients and controls even at the unsupervised phase of analysis, which to date, has not been consistently demonstrated in metabolomics-based studies of PDAC. Given that a spectrum of metabolic perturbations appear to be responsible for the metabolomic PDAC signature, we have identified a multi-molecular biomarker capable of distinguishing patients with PDAC from normal healthy controls. The strong predictive accuracy of this multi-molecular biomarker is promising, in light of the shortcomings of CA19-9

which is currently the only clinically available biomarker for pancreatic cancer. Such a multi-molecular biomarker might in fact be more relevant and avoid the pitfalls of single-marker studies, given the molecular heterogeneity of pancreatic cancer [2-4]. It is apparent from our analysis that simultaneous perturbations to multiple metabolic pathways are likely responsible for the class separation achieved (cancer vs. normal). We hypothesize that metabolite expression patterns detected in urine are reflective of a convergence of metabolic disturbances associated with tumor metabolism as well as alterations in global metabolism. Various analytical methods were applied to ascertain which metabolites were most responsible for class discrimination. These included non-parametric statistical analyses of metabolite concentrations, fold-change calculations, and VIP parameters specific to each metabolite.

Attempts to link the metabolic signature of PDAC with underlying molecular pathways involved in pancreatic carcinogenesis remain speculative at this exploratory stage. The metabolic signature presented here provides a starting point for more in-depth interrogation of pancreatic cancer biology, where further integration of genomic and proteomic discoveries and use of *in vivo* investigations with appropriate model systems may uncover definitive cancer pathways [4].

A brief, preliminary discussion of the patterns of metabolite expression will be largely limited to those metabolites thought to be key contributors of the PDAC-

specific metabolic profile, at this exploratory phase of analysis. Patterns of metabolite disturbance have been segregated into those associated with altered tumor metabolism and those associated with more global metabolic disturbance (**Figure 4.1**) (see below).



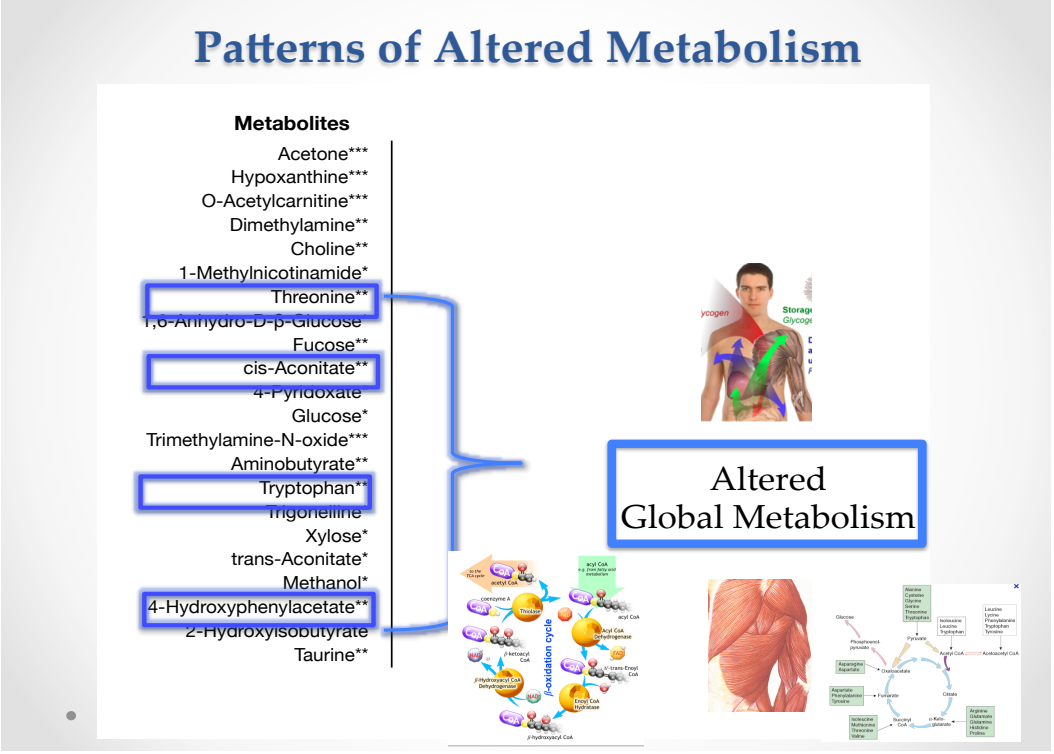


Figure 4.1 Patterns of Altered Tumoral and Global Metabolism

O-acetylcarnitine was significantly elevated in PDAC patients in comparison with controls. An acetylated form of carnitine, this metabolite facilitates transport of acetyl-coA into mitochondrial matrices during the oxidation of fatty acids via the beta-oxidation cycle, which is necessary for generation of reduced energy carriers [5]. High rates of energy expenditure are needed for tumor growth and proliferation; increased rates of lipolysis and fatty acid metabolism are required to meet this increased metabolic demand. Accordingly, acetone, one of three by-products of fatty acid breakdown was also significantly elevated in PDAC patients, further validating the hypothesis of high-energy expenditure associated with tumor metabolism.

Altered expression of **choline** and choline containing compounds have been the focus of much attention in metabolomics research, most notably breast-related metabolomics studies. Elevated levels of choline and choline derivatives have been used for diagnostic purposes as well as a means of optimizing neo-adjuvant chemotherapy by monitoring variations in choline concentrations [6-9]. Choline and choline metabolites are known to be markers of cellular proliferation, representing key constituents in phospholipid metabolism of cell membranes [10, 11]. In addition to its role in phospholipid metabolism, choline acts as a methyl donor, important in a number of chemical processes including cell replication [12].

Trimethylamine-N-oxide, also elevated in PDAC, is an oxidation product of trimethylamine, which is derived from choline [5]. This pattern of elevated choline expression was observed among PDAC patients, in concordance with previous findings and is likely representative of rapid tumor cell proliferation, characteristic of PDAC. While a number of features of carcinogenesis are common among cancers of different origin, PDAC is unique in its aggressiveness, tendency to form large tumors and rapidity of growth [4, 13].

In concordance with the phenotypic observation of rapid tumor growth characteristic of PDAC, cancer-specific elevations of **hypoxanthine** could represent an underlying enhanced capacity for DNA synthesis and energy production. Hypoxanthine is a purine derivative; purine and pyrimidines are building blocks of RNA and DNA, making up the two groups of nitrogenous bases, including the two groups of nucleotide bases [5, 14]. Hypoxanthine is also an intermediary metabolite of adenosine metabolism and a reaction intermediate in the formation of nucleic acids [5]. Purines not only have a critical role in formation of RNA and DNA, but are involved in energy metabolism and are constituents of a number of important cellular molecules such as ATP, GTP, cyclic AMP and NADH. Cancer-specific elevations of purine derivatives clearly points to altered energy metabolism and possibly an enhanced capacity for DNA/RNA synthesis.

Cancer-specific elevations of a number of **amino acids and amino acid derivatives** were evident among PDAC patients; threonine, tyrosine, tryptophan and 4-hydroxyphenylacetate were highly significant, while alanine, isoleucine, leucine valine and glutamine, showed trends towards significance. It is possible that this pattern may in part be reflective of muscle protein breakdown, during which all constituent amino acids enter oxidative pathways [15].

Cancer cachexia, is a complex, inflammatory metabolic syndrome associated with severe illness and is characterized by an unintended loss of weight exceeding 5-10% of stable weight. This phenomenon is associated with nearly 80% of upper gastrointestinal tract malignancies and PDAC specifically, has one of the highest incidences of cachexia of all solid-epithelial cancers [16-18]. Cancer-related cachexia features severe disturbances in energy and protein metabolism, the etiology of which is still largely unknown [18]. Muscle wasting occurs with or without the loss of fat mass and is often an early, occult phenomenon, clinically undetectable until advanced stages of disease [15, 18]. A majority of patients with early stage/locally advanced disease included in this thesis reported a minimum of 5-10% weight loss at the time of diagnosis, and were likely suffering from some degree of early, cachexia related muscle protein breakdown long before becoming clinically apparent. A noninvasive means of detecting early muscle wasting in patients harboring occult malignancy could have significant clinical utility and urinary NMR spectroscopy combined with

multivariate techniques may provide an avenue for the potential development of such a tool [4].

The pattern of metabolic derangements associated with the metabolomic signature of PDAC appear to include altered lipolysis, enhanced capacity for DNA synthesis and energy synthesis and amino acid catabolism (possibly related to cancer cachexia). This however represents only a preliminary analysis and further exploration and in-depth pathway analysis using applications such as Ingenuity Systems Pathway Analysis followed by confirmation of these results with *in vivo* studies using appropriate model systems are needed to fully characterize the patterns of altered metabolite expression profiles observed here. Conclusive discernment of whether the metabolic signature associated with PDAC is a direct phenomenon related to cancer cachexia or rather an epiphenomenon requires prospective monitoring of a high-risk pancreatic cancer cohort to see if the distinctive metabolic signature emerges prior to the onset of visible cachexia. Collaborative integration of these findings with the other 'omic' sciences are needed to provide complete a picture of malignant phenotype and the cellular changes associated with malignant transformation in pancreatic cancer.

4.1.3 Effects of Surgical Resection

Effects of complete, RO surgical resection were examined in a subset of patients. Preliminary analysis suggests that the PDAC metabolomic signature was extinguished following surgical resection given that clear separation was observed when supervised multivariate statistical methods were used to compare metabolite expression profiles of PDAC patients pre and postoperatively. While recognizing the limitations of this analysis given the small sample size, these preliminary results appear promising (sensitivity 95.2%, specificity 85.7%). When the inciting tumoral burden is surgically removed, the cancer- associated metabolite expression profile is no longer detectable. If the metabolomic signature is in fact reflective of altered tumor metabolism as well as alterations in global metabolism, including muscle protein breakdown related to cachexia, these results support that the thought that the cachectic process is being driven by multiple inflammatory mediators associated with the tumoral environment, including known catabolic cytokines such as IL-6, TNF- α, IL-1, and IFN-γ [19-22]. Future investigations could involve prospective monitoring of patients following R0 resection in order to uncover the pre-emptive re-emergence of the PDAC signature ahead of clinical evidence of recurrent disease. Additional potential applications of this urinary molecular biomarker could include a novel means of surveillance post-resection or adjuvant therapy, providing an opportunity for early intervention when effective therapies become available. Furthermore, prospective monitoring of patients following curative resection could

lead to identification of a molecular signature associated with poor prognosis and early metastases. Such a prognostic tool could guide treatment, helping to minimize futile surgical intervention among patients who are early progresors.

4.2.1 Challenges and Future Directions

4.2.2 Sample Size Limitations

Sample size remains a limiting factor not only in the results presented here, but in pancreatic cancer research of all types given the low incidence of disease. Coordinated collaboration with other surgical centers could help in this regard, however, methods must be in place to assure standardized sample collection and processing procedures in order to avoid confounding results. Standardization of sample handling, storage and processing stages are necessary to minimize the confounding effects of protocol variation on sample recovery. While multivariate statistical techniques are able to deal with highly correlated, multidimensional data matrices, caution must be used when interpreting results of smaller studies where the number of metabolites greatly exceeds the number of samples, a common feature of omics studies in general [23]. False discovery rates (FDRs), referring to the frequency of Type I error, are more likely to occur in studies of inadequate sample size. In these situations, model validation steps

are increasingly important to ensure the veracity of results. While the results presented here are both novel and compelling, they are still exploratory and require further validation in larger patient cohorts. Preliminary results such as these provide an important path for further validation and are a necessary step for incremental discovery in any new scientific arena.

4.2.3 Multiple Hypothesis Testing

Another challenge that should be addressed is the issue of multiple hypothesis testing, an inherent part of omics research. Metabolomics-based experiments are no exception and involve the simultaneous examination of expression pattern differences of a vast array of metabolites under varying pathophysiological conditions. Bonferonni correction, in which the *p* value for statistical significance is divided by the number of metabolites examined, is one of a number of methods aimed at minimizing the risk of false discoveries [23-25]. While providing protection against the risk of Type I error (false positives), such stringent criteria also increase the risk of Type II error (false negatives), and are arguably too conservative especially at the exploratory phase of analysis [25]. Bonferonni correction also assumes that all variables are independent of each other, a false assumption in any metabolomics experiment. More appropriate multiple testing correction techniques for highly correlated data aimed at limiting the rate of false positives without unnecessarily increasing the rate of false

negatives are being developed and include Benjamini-Hochberg correction or bootstrap sampling [24, 26]. Future work is needed examine the appropriateness of such techniques in the setting of metabolomics-based research.

Post hoc stratification is another form of multiple hypothesis testing which was avoided in this thesis by establishing hypotheses *a priori. Posthoc* stratification, sometimes referred to as or 'data dredging' involves the performance of additional analyses at the end of the primary experiment with the aim of discovering patterns, or relationships in subgroup populations. While these types of *posthoc* analysis were not performed here and must be interpreted with caution, they are also arguably a valuable technique at the exploratory stage of analysis.

4.2.4 Spectrum Bias

In planning future studies to further examine the role of urinary metabolomics as a diagnostic or screening tool in PDAC, the issue of spectrum bias must be addressed. While the use of healthy, asymptomatic volunteers may be appropriate in early stages of diagnostic test development, the use of control subjects lacking diagnostic uncertainty may produce a biased estimate of a tests true performance [31, 32]. Ideally, in order to avoid unrepresentative patient selection, both cases and controls should present a diagnostic dilemma. While

study enrollment was limited to those patients with earlier stage disease, excluding those with stage IV disease, a more representative control population is needed in future analysis to avoid the risk of biased estimates of model performance as a diagnostic tool. Bathe *et al*. were the first to examine the role of metabolomics in accurately identifying PDAC patients from a representative cohort of patients with benign pancreaticobiliary disease, presumably for whom there was some degree of diagnostic uncertainty. We have begun preliminary work (not included in this thesis) comparing early stage disease PDAC patients to a more representative control group consisting of patients with benign pancreaticobiliary disease (n=25). Early results are promising and consistent with our primary findings. Future analyses with larger patient cohorts are necessary to further explore these results and going forward, should include cases and controls more representative of day-to-day clinical practice where diagnostic uncertainty exists.

4.2.5 Biofluids and Analytical Platforms

The majority of clinical metabolomic studies have been serum or urine-based experiments. Urine has been the most frequently used biofluid in toxicology-related research, however a growing number of studies have examined its utility in cancer diagnosis [27-29,[4, 27]. While it is still not clear which biofluid is optimal, urine has several clear advantages including its non-invasive nature of

sample procurement and ease of collection at multiple time points permitting a temporal based analysis [28]. While even slight changes in sample handling and processing of serum samples can alter metabolite recovery rates, urine processing steps are simple and easily reproducible [28]. Nevertheless, serum metabolite profiles have shown decreased diurnal variation as well as decreased inter-and intra-subject variability compared with urine. Further studies are needed linking the results from urine, serum and bile in order to provide the most comprehensive picture of overall global metabolic perturbations. In addition to integration of results from an array of biofluids, integration of varying analytical platforms will also provide further refinement to the metabolic signature of pancreatic cancer presented here.

It is increasingly apparent that no one analytical platform will alone provide a truly complete molecular fingerprint of disease. While NMR is fast, inexpensive, reproducible, low abundance metabolites are not detectable. MS used in combination with a separation technique has a higher overall sensitivity than NMR thereby providing a more comprehensive picture of the overall metabolome [28]. As such, an increasing number of metabolomics studies are attempting to integrate results from multiple analytical platforms (i.e. NMR and MS), in order to provide the most comprehensive picture of the metabolic milieu underlying a given disease state.

4.2.6 Improving Diagnostic Accuracy

The diagnostic accuracy of the metabolic signature of PDAC presented here may be further improved by minimizing influences of external, potentially confounding factors not related to disease [4, 15]. Environmental and physiologic factors (i.e. diet, hormonal milieu, stress, diurnal cycles) which influence the overall expression profile run the risk of confounding or at least clouding the results of any metabolomics experiment [28]. Innate physiologic variations of biofluid spectral profiles present a significant analytical challenge. In this thesis, steps were taken to minimize influences of age and gender by matching PDAC patients to controls according to these factors. Further studies should include additional steps to abrogate or minimize the influence of these external effects, allowing for a clearer picture with improved interpretability. Repeated sample collection from the same individual at different time points is an example of one such step. OCS and VAST are both examples of techniques used at the pre-processing stage of sample analysis that minimize the influence of extraneous confounders [29, 30]. Scaling and data filtering may also enhance the attainment of class-specific information [29]. While OCS and data scaling were used in this thesis, further analysis should include additional techniques to maximize relevant information recovery.

4.2.7 Model Validation

Internal validation techniques provide an alternative method of testing model validity in the setting of small sample size, however, the most rigorous method of model validation is through external validation which involves making predictions for an independent set of data not involved in model generation [33]. While model validity was confirmed using internal validation techniques, the preliminary results presented here must next be validated on an external patient population. Low disease incidence and delayed presentation in the majority of patients with PDAC are limiting factors however in recruiting patients with early stage disease suitable for further analysis and therefore recruitment will take time. Registries such as the NFPTR are essential for continued research into the early detection of pancreatic cancer. A cohort of high-risk patients such as these represent an ideal, truly representative external population for further validation of these results. Further refinement to the proposed metabolic signature of PDAC is required, including validation with larger sample sizes prior to external validation using such a population however. While further refinement is needed, the highly predictive, discrete urinary metabolic signature of PDAC established could conceivably advance programs for early detection and population-based screening for PDAC.

4.2.8 Potential Future Applications

Metabolomics based research not only has the potential to bring advances in the area of diagnosis and screening as outlined in this thesis, but could also conceivably play a role in advancing current systems of prognostication, targeted therapeutics, and methods for therapeutic response monitoring. **Figure 4.2** (see below) outlines potential target areas of future metabolomics-based research in pancreatic cancer.

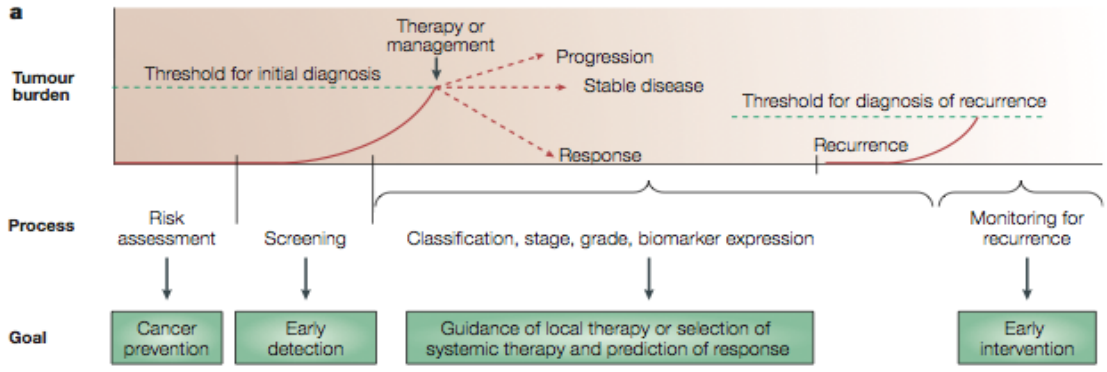


Figure 4.2 Future Potential Target Areas for Metabolomics-Based Research in Pancreatic Ductal Adenocarcinoma
(*reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, Ludwig et al., Biomarkers in Cancer Staging, Prognosis and Treatment Selection 2005; 5: 845-856)*

The current TNM staging system for pancreatic cancer has little clinical value in the prognosis and staging patients for treatment given the poor overall outcomes associated with this disease [34]. Continued advancements in delineating

tumorigenesis are necessary in bringing improvements to such classification systems which rely on tumor morphology alone. Complementary metabolic information may help portray a more inclusive tumor portrait, providing a more individualized estimate of prognosis [28]. A method of more personalized prognostication, incorporating molecular information with gross tumor morphology is especially important in pancreatic cancer that exhibits a high degree of molecular and phenotypic heterogeneity. Prognostication methods capable of stratifying patients into subgroups whose disease metastasizes early versus those who tumor biology displays features of preferential local growth could allow for more tailored treatment. A combination of improved diagnostic and prognostic tools could help avoid aggressive surgical treatments with a high likelihood of futility, while also conceivably broadening the scope of surgical intervention through early detection at pre-invasive or early stages of disease. Metabolomic-based techniques offer a means of non-invasive preoperative phenotyping, providing information critical in subsequently guiding both surgical and medical treatments [28].

An increased understanding of the molecular pathogenesis of PDAC will also lead to advancements in targeted therapeutics tailored to molecular processes of underlying disease. Therapy guided by metabolic endpoints rather than gross tumor morphology and imaging alone will allow for personalized treatment of this biologically heterogeneous disease in contrast to current one-size -fits all surgical and chemotherapeutic treatment regimes [28]. Personalized therapy guided by

early metabolic responses rather than waiting for evidence of gross phenotypic change based on comparatively insensitive imaging modalities could improve outcomes by helping to individualize therapy. Going forward, new holistic approaches involving the correlation of genomic, proteomic and metabolomics data are required in order to provide a truly comprehensive view of the malignant phenotype of PDAC and the molecular mechanisms of malignant transformation, necessary for improvements in both diagnosis and treatment of this devastating disease.

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