

NMR-based Metabolite Profiling of Pancreatic Cancer

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Abstract: Metabolite profiles of serum from pancreatic cancer (PC) patients (n=51) and non-disease controls (n=47) were measured using ¹H nuclear magnetic resonance (NMR) spectroscopy with a focus on the metabolic changes associated with PC pathology and the development and external validation of the statistical models developed using the metabolite data. Univariate statistical analysis indicated 42 metabolite features showing significant differences between PC and controls (p<0.05). Based on multivariate regression analysis of the data from 38 PC patients and 32 controls, twelve distinguishing metabolites (alanine, choline, citrate, creatinine, glucose, glutamine, glutamic acid, 3-hydroxybutyrate, lactate, lipids, methionine and valine) were determined based on their ranked importance in a partial least square discriminant analysis (PLS-DA) model. A cross-validated regression PLS-DA model built using these metabolites differentiated the cancer and control groups with high accuracy and an area under the receiver operating characteristic curve (AUROC) of 0.95. Notably, external validation of this model using the NMR data from a second, distinct set of samples (13 PC patients and 15 controls) collected approximately 1 year later showed an AUROC of 0.86, which represents very good performance compared to the current approaches for identifying pancreatic cancer patients. Metabolic changes in pancreatic cancer patients compared to healthy controls as shown in this study demonstrate the potential for the development of regression models based on blood metabolites to identify patients with pancreatic cancer.

Keywords: ¹H NMR, early detection, MCCV analysis, metabolite profiling, metabolomics, pancreatic cancer.

INTRODUCTION

Pancreatic cancer (PC) afflicts both men and women, and is the fourth leading cause of cancer related deaths in the United States. The overall 5-year survival rate of patients with PC is dismal, with 95% of patients dying within five years of diagnosis [1]. According to National Cancer Institute statistics, approximately 47,000 men and women will be diagnosed with PC and 40,000 are expected to die of this disease in 2014 [1, 2]. The number of PC deaths is projected to increase by 55% by the year 2030, due in part to PC's poor prognosis. PC is asymptomatic until late in the disease process and late diagnosis leads to the alarmingly high mortality rate. Specific causes for the development of PC are unknown but major risk factors include age, smoking, diabetes, pancreatitis, obesity, and rarely, genetic predisposition [2]. Tests such as computed tomography (CT), ultrasonography, endoscopic retrograde cholangiopancreatography (ERCP), and biopsy are often used for the diagnosis of PC [3]. Resection, or the removal of the affected area remains the best chance for survival but this approach is unfortunately restricted to only 10-15% of patients with early stage

disease [4]. While routine radiographic studies may provide disease information, they are not always useful because of limited performance for early disease detection [5].

Significant interest has been focused on the identification of molecular markers as a more reliable approach for detecting PC. Many blood markers including cancer antigen (CA19-9), carcinoembryonic antigen (CEA), CEA-related cell adhesion molecule 1 (CEACAM1), macrophage inhibitory cytokine 1 (MIC1), alphafetoprotein (AFP), DU-PAN-2, alpha4GnT, cytokeratin-19 (CK-19) mRNA, and tissue polypeptide antigen have been examined for early detection of pancreatic cancer [6-12]. Unfortunately, none of these markers provide the required sensitivity and specificity for routine screening and thus, currently, there is no reliable screening tool for early detection of PC either in the general population or in at-risk patient populations.

An alternative approach is metabolomics, a fast growing area in systems biology that combines data-rich analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy and/or mass spectrometry (MS), with chemometrics, and promises the identification of sensitive metabolite biomarkers associated with disease, drug treatment, toxicity and environmental effects among its many applications [13]. Metabolites are the downstream products of genes, transcripts and protein functions in biological systems and they

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can be especially sensitive to perturbations in a number of metabolic pathways and varied pathological conditions. Recent advances in cancer biomarker discovery promise the development of early disease diagnostics as well as understanding perturbed metabolic pathways [14]. To date, a few metabolomics investigations have focused on the identification of metabolite biomarkers for PC using samples from either animal models or humans [15-24]. These studies have analyzed urine, tissue, blood serum/plasma or saliva metabolic profiles using NMR or MS methods. Notably, each study, owing to the combination of the metabolic complexity of different biological samples and the varied sensitivity, selectivity or resolution associated with each type of analytical method, identified different sets of distinguishing metabolite biomarkers, some of which were unique to each study. In a recent study, Kobayashi *et al.* analyzed serum metabolites using gas chromatography (GC)-MS methods to discover a panel of four metabolites that were used for building a diagnostic model using logistic regression [24]. Validation of this model using a matched and independent set of samples revealed a higher accuracy for detecting pancreatic cancer than the conventional tumor markers, CA19.9 and CEA; the sensitivity, specificity and area under the receiver operating curve (AUROC) were 71.4%, 78.1% and 0.76, respectively, as compared to 69.0%, 85.9%, 0.79 for CA19.9 and 35.7%, 79.7% and 0.66 for CEA.

NMR spectroscopy of blood serum or plasma is an especially attractive analytical approach in metabolomics as it provides highly quantitative and reproducible data for many important, albeit highly concentrated, metabolites. Metabolomics studies focused on establishing human blood serum or plasma metabolite markers of PC using this very quantitative method are of particular interest for a number of reasons including diagnostic and therapeutic applications. Thus far, only three investigations using NMR have been reported in the literature using blood serum or plasma [18, 19, 21]. OuYang *et al.* compared serum metabolic profiles from a small number of PC patients (n=17) with healthy controls (n=23) and reported down-regulation of 3-hydroxybutyrate, 3-hydroxyisovalerate, lactate, and trimethylamine-N-oxide and up-regulation of isoleucine, triglyceride, leucine, and creatinine in PC [18]. A second study compared PC (n=56) with a group of non-malignant patients with various hepatopancreatobiliary etiologies (n=43) and showed that glutamate and glucose were elevated and creatine and glutamine were decreased in PC [19]. A more recent study investigated PC (n=19) and chronic pancreatitis (n=20) patients, and controls (n=20), and showed, among others, decreased levels for histidine, glutamine, glutamate, alanine, valine and lactate in PC [21]. However, additional investigations that provide validation using independent sets of patient cohorts such as the study by Kobayashi *et al.* are still lacking.

In this study, we compare serum metabolite profiles of PC patients with healthy controls, using an NMR-based metabolomics approach, with the ultimate goal of developing a metabolite profile for early PC detection. The healthy controls used in this study were either subjects living in the same environment with the patient (co-inhabiting, not genetically related), or were genetically related (parents, siblings or children) to the PC patients. Using multivariate sta-

tistical methods, we developed a partial least squares discriminant analysis (PLS-DA) model based on 12 metabolites that contributed the most to the model. The results show that external validation of the predication model using an independent patient cohort provides very good performance as compared to other panels of metabolites proposed previously for distinguishing pancreatic cancer patients from healthy controls.

MATERIALS AND METHODS

Chemicals and Serum Samples

Deuterium oxide (D₂O, 99.9% D) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Trimethylsilylpropionic acid-d₄ sodium salt (TSP) was purchased from Sigma-Aldrich (analytical grade, St. Louis, MO).

Blood samples from PC patients (n=51) and healthy controls (n=47) were obtained from the Indiana University School of Medicine. All serum samples were obtained after overnight fasting. The samples were obtained in two different batches approximately 1 year apart. The first batch consisted of 70 samples from 38 cancer patients and 32 controls, and the second batch consisted of 28 samples from 13 cancer patients and 15 controls. Thirteen control samples in the first batch and nine in the second batch were obtained from volunteers genetically related to the patients (but who were not living in the same household as the PC patients), while the remaining samples were obtained from non-genetically related volunteers living in the same household with the patient. The mean age for cancer patients was 65 years (range 48-86), while that for controls it was 56 years (range 28-89). Demographic information is provided in Table 1.

Each collected blood sample was allowed to clot for 45 min and centrifuged at 1500 g for 10 min. The serum samples were separated, aliquoted into separate vials, frozen, and shipped over dry ice to Purdue University, where they were stored at -80 °C until analysis. Protocols approved by the Institutional Review Boards from both Indiana University School of Medicine and Purdue University were followed for collecting and analyzing the samples. All patient information was de-identified, and the recruited subjects provided informed written consent.

¹H-NMR Spectroscopy

All NMR experiments were carried out at 25 °C on a Bruker DRX 500 MHz spectrometer equipped with a cryogenic HCN triple resonance probe with triple-axis magnetic field gradients and operated using XWINNMR software version 3.5. The serum samples were thawed at room temperature and 570 µL was transferred to 5 mm NMR tubes. A coaxial glass insert (OD 2 mm) containing 60 µL 0.012% TSP solution in D₂O was used as a chemical shift reference (δ=0.00 ppm) and field-frequency locking solvent. One dimensional ¹H NMR experiments were performed on each sample using the CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence. The water signal was suppressed by presaturation during the 3 s recycle delay. Spectral widths, time domain data points and the number of transients used were 6000 Hz, 32 K and 32, respectively. An exponential weighting func-

Table 1. Summary of clinical and demographic characteristics of patients used in this study.

Characteristics	Healthy controls	Cancer
Number	47	51
Age Mean (Range) Yrs.	56 (28-89)	65 (48-86)
Gender		
Male	10	29
female	31	20
Unknown	6	2
Race		
Caucasian	34	44
African American	-	3
Unknown	13	4
Cancer Stage		
IB	-	2
IIA	-	10
IIB	-	16
III	-	5
IV	-	10
Unknown	-	8
Diagnosis		
Adenocarcinoma	-	44
Carcinoma	-	1
Unknown	-	6

tion corresponding to a line broadening of 0.3 Hz was applied to the free induction decay (FID), before Fourier transformation. Resulting spectra were phase and baseline corrected and subjected to further data and statistical analysis.

Statistical Analysis and Metabolite Identification

¹H NMR spectra obtained using the CPMG sequence were aligned with reference to the TSP peak at $\delta=0$ ppm. The region between 0.5 and 9.0 ppm was used for analysis after deleting the region between 4 to 6 ppm that contains the residual water and urea peaks. Based on a variable binning approach [25], 70 spectral bins were manually selected from the spectra and integrals were obtained by summing up intensities of all the points within each bin (see Supplemental Table S1). Resulting data from the two sample batches were subjected to univariate and multivariate analysis; Figure 1 depicts the flowchart for statistical analysis. The first batch of samples was used for metabolite selection and developing two statistical models, while the second batch of 28 samples was used for external validation. Initially, a statistical model was built based on PLS-DA using all 70 variables; the model was subjected to Monte Carlo Cross Validation (MCCV) [26, 27] randomly leaving out 30% of the data, and internally

cross-validated using 500 iterations. Variable importance projection (VIP) scores were calculated to estimate the importance of each variable in PLS-DA modeling. A separate statistical model was then built using only the variables with $VIP>1$ ($n=12$) and subjected to Monte Carlo Cross Validation (MCCV), again internally cross-validated using 500 iterations. The two statistical models thus developed were then tested using the independent batch of 28 samples. The performance of the two statistical models on both the training and validation sets of samples was evaluated based on sensitivity, specificity and area under the receiver operating characteristic curve (AUROC). Metabolite identities for the variables with $VIP>1$ were established based on the HMDB NMR database [28]. Separately, the data were analyzed with a focus on distinguishing early stage (I/II) from late stage (III/IV) cancer.

RESULTS

¹H NMR spectra obtained using the CPMG sequence were devoid of macromolecule signals due to their effective suppression by the CPMG sequence and enabled visualization of the low molecular weight metabolites. The differ-

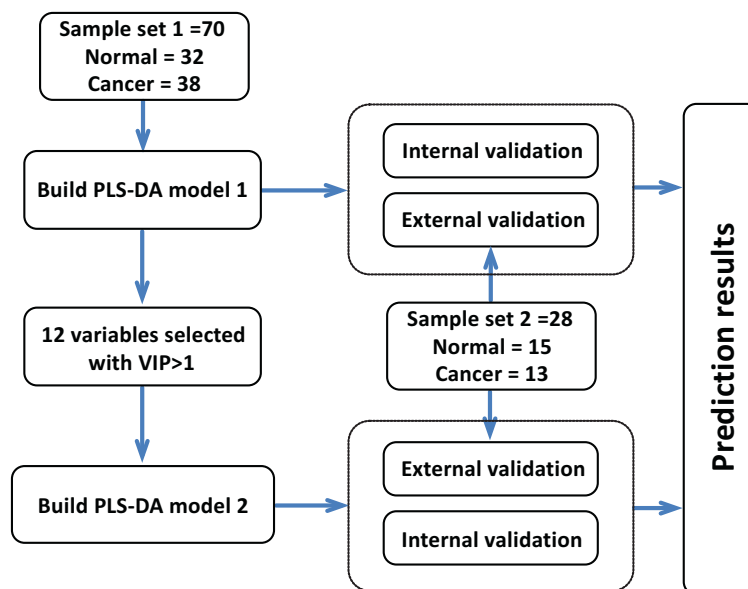


Fig. (1). A flow chart describing the steps of data analysis used to develop and validate the two PLS-DA models based on two independent sets of samples.

ences in metabolic features between PC and controls were clearly visible in the CPMG spectra (see Supporting Information Figure S1).

Univariate analysis of variable binned data showed major changes in the metabolic profiles between PC and controls, which could be visualized through the altered mean levels of the variables: Forty-two of the 70 spectral bins showed statistically significant differences based on the Mann Whitney U-test ($p < 0.05$) between PC and controls (see Supplemental Table S1). The difference in metabolic profiles between genetically related and unrelated subjects was highly insignificant and hence the two groups of control samples within each batch were combined in this study. PLS-DA analysis performed using all 70 variables from the first batch of samples (Batch 1) showed very strong performance with an AUROC of 0.99 (sensitivity 97 %; specificity 94 %; $R^2X = 0.92$). Performance of the model obtained using all 70 spectral variables from Batch 1 is shown as a ROC curve in (Fig. 2).

The calculation of VIP scores showed that 12 metabolites had $VIP > 1$ (see Supplemental Information Table S1). Eight

of these metabolites were also significantly different ($p < 0.05$) between PC and controls. Table 2 lists these metabolites along with their p-values (both before and after correction for multiple comparisons) and fold changes between the cancer and control groups. A separate analysis using only these 12 variables from Batch 1 provided a classification model with a high accuracy with an AUROC of 0.98 (sensitivity 97 %; specificity 88 %; $R^2X = 0.95$) (see Fig. 2). The models obtained from all 70 variables and the reduced set of 12 variables with $VIP > 1$ were separately subjected to internal validation using MCCV. The results show that the model obtained using the 12 variables exhibited better performance when compared to that obtained using all the variables; the AUROC was 0.95 for the reduced model ($R^2Y = 0.67$; $Q^2Y = 0.48$), while the AUROC was 0.93 for the model obtained using all 70 variables ($R^2Y = 0.74$; $Q^2Y = 0.41$) see (Fig. 3). Interestingly, further validation of the models externally using an independent set of samples (Batch 2), which had not been used for variable selection or initial PLS-DA modeling, showed significantly better performance for the model derived using only variables with $VIP > 1$; the AUROC was 0.86 for the variables with $VIP > 1$ (sensitivity

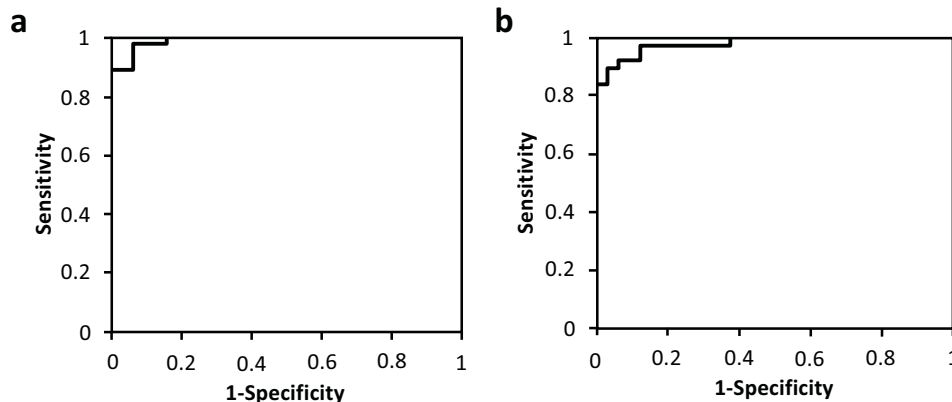


Fig. (2). ROC curves for the PLS-DA models of Batch 1 data set obtained based on the analysis of (a) initial 70 variables; (b) 12 selected variables with $VIP > 1$.

Table 2. Metabolites detected by ^1H NMR used to build a classification model based on partial least squares discriminant analysis.

	Metabolite	<i>p</i> -value*	Bonferroni corrected	FDR corrected	Fold change**
1	Alanine	2×10^{-6}	0.0002	0.0002	-1.3
2	Choline	0.3	32	10	-1.2
3	Citrate	0.06	4	1	1.1
4	Creatinine	0.0004	0.03	0.007	-1.3
5	Glucose	9×10^{-6}	0.0006	0.0001	1.2
6	Glutamic acid	0.04	3	0.4	1.2
7	Glutamine	0.01	0.7	0.1	-1.2
8	3-Hydroxybutyrate	0.002	0.1	0.02	1.5
9	Lactate	0.1	7	0.8	1.1
10	Lipids	0.05	4	0.4	-1.2
11	Methionine	0.3	21	2	1.0
12	Valine	0.003	0.2	0.02	-1.2

*Values determined from the Mann-Whitney U-test.

**Negative values indicate decreased metabolite levels in cancer samples.

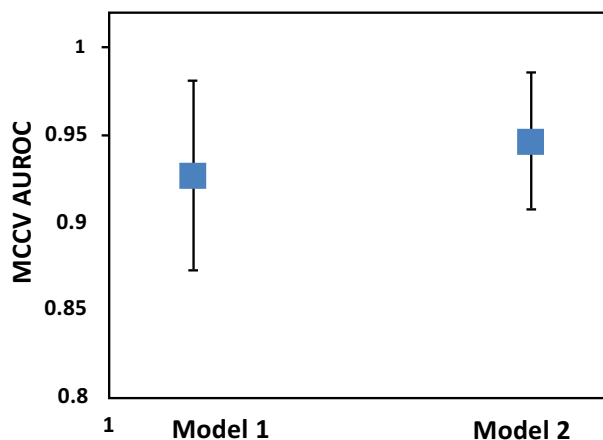


Fig. (3). Internal validation of the two models, Model 1 and Model 2, derived from all 70 and 12 variables with VIP >1, respectively, using the Batch 1 data set. Each model was subjected to MCCV with 70% of the data used for training the model while the remaining 30% served as the testing set. 500 iterations were used for each model.

70%; specificity 93%) and 0.72 for all variables (sensitivity 72%; specificity 85%) (see Fig. 4). Similar analysis focused on distinguishing cancer stages showed no significant difference between early stage (I/II) and late stage (III/IV) patients.

DISCUSSION

The long term goal of this study is to develop a simple metabolomics-based screening tool for PC. Current diagnostic tools including radiographic studies and blood based molecular markers exhibit limited performance for detecting PC, and thus the development of a reliable and accurate screening tool would represent an important step for improving PC treatment and survival. In this study we present the development of a classification model for distinguishing PC from healthy controls based on an altered serum metabolite

profile. Considering that highly quantitative and reproducible metabolic profiles can be obtained by NMR, the establishment of a biomarker panel for PC using this method would promise novel avenues for the detection of PC, as well as insights into the altered biochemistry of PC. Therefore, a combination of high resolution NMR spectroscopy and multivariate statistical analysis was employed in this study to achieve this goal. The development of a robust classification model involved multivariate analysis using PLS-DA (Fig. 2) and cross validation using MCCV (Fig. 3). The model was developed based on a panel of 12 highly ranked metabolites (Table 2) shortlisted based on VIP scores, and provided a high accuracy for distinguishing PC from controls (Fig. 2). From an evaluation of the 12 highly ranked metabolites it is clear that individual metabolite biomarkers are not sufficiently different in PC patients compared to healthy controls to separate the two groups accurately; however, their

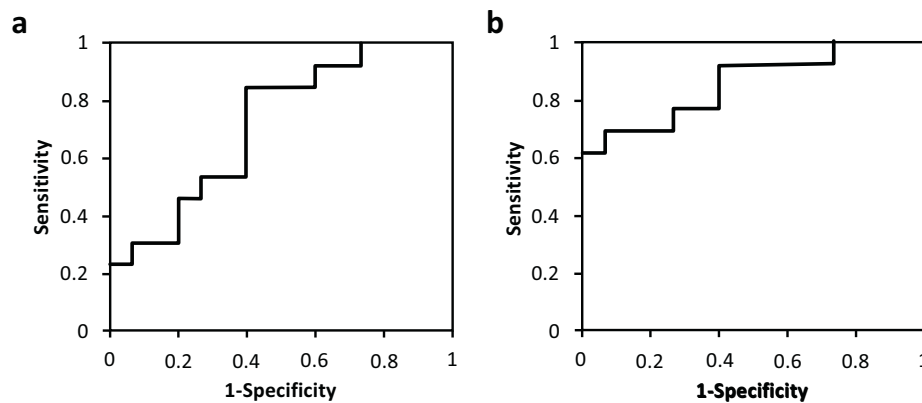


Fig. (4). ROC curves for validation of models developed (see Fig. 2) using an independent set of samples from Batch 2. (a) Results for an independent validation of the model developed using all the 70 variables and (b) results for the validation model developed using 12 spectral variables with VIP >1.

combination in a statistical model provides a good separation with high diagnostic accuracy.

An important aspect of the current study is that the resulting model could be validated using an independent set of samples (Batch 2), and the classification accuracy for validation, although somewhat poorer compared to the performance of the prediction model, was still the highest for any panel of metabolites proposed thus far for distinguishing pancreatic cancer patients (Fig. 4). The lower performance of the validation data set indicates some overtraining of the model, which can be produced by a number of factors un-

connected with the disease. In the current study, to minimize the confounding effects of diet on the metabolite levels, we collected serum samples from overnight fasted patients and healthy controls. Clearly, expanding the number of patients and their diversity would help to reduce overtraining. Additional factors, such as age and gender, which are not matched perfectly in this study, may also contribute to the outcome.

The metabolites identified in this study represent various biologically significant processes connected with the pathogenesis of PC. In Figure 5, we highlight the pathways associated with metabolites that were altered in the development

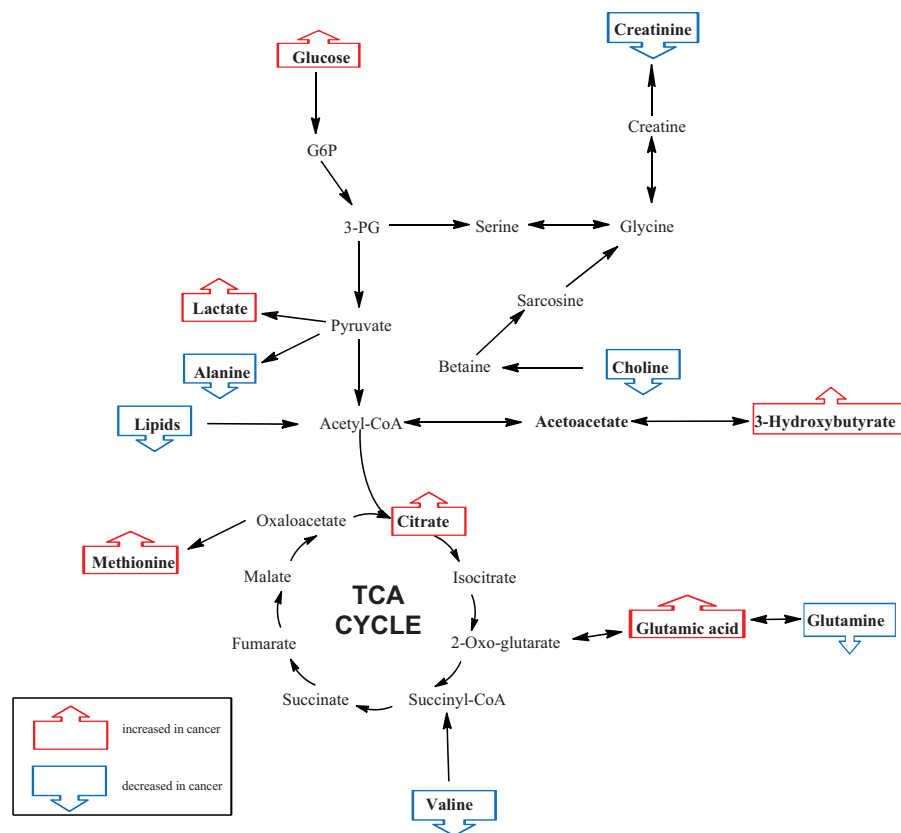


Fig. (5). A summary of the altered metabolic pathways associated with metabolites that distinguish PC and control samples (with VIP>1). The metabolites indicated with borders with upward arrow showed an increase in concentration in cancer patients while those with downward arrow showed a decrease in concentration.

of PC. Altered levels of glucose and lactate are consistent with increased glycolysis in malignancy; altered glycolysis is common in growing cancers where the cells derive energy through the conversion of glucose to lactate even in the presence of oxygen, i.e., the well-known Warburg effect [29, 30]. Uptake and catabolism of amino acids are also enhanced to support the proliferation of cancer cells. Thus, the decreased blood levels of alanine, glutamine and valine observed in this study are consistent with an increased utilization of these amino acids in PC, which arises from the increased demand for these metabolites for tumor growth. The decrease in levels of these amino acids is also consistent with an earlier NMR-based metabolomics study of PC using plasma samples [21].

Interestingly, unlike other amino acids, glutamic acid increased significantly and methionine increased marginally in PC. One of the major findings in cancer metabolism is the metabolic reprogramming in which cancer cells derive energy from TCA cycle fueled by glutamine instead of glucose [31]. This phenomenon in which the cancer cells depend on glutamine as an alternative source of energy as well as nitrogen and carbon is known as glutamine addiction. Glutamine is utilized to form glutamic acid, which then enters TCA cycle through 2-oxoglutarate. Myc activation is associated with increased activity of the enzyme glutaminase, which is responsible for converting glutamine to glutamic acid, the source of energy production via TCA cycle. In fact numerous investigations have shown Myc overexpression in pancreatic cancer [32-34] and pharmacological inhibition of glutaminase is reported to be a promising target for pancreatic cancer [32]. Thus the increased levels of citrate, methionine and glutamic acid, and decreased level of glutamine as determined in the current study agree with the altered TCA cycle metabolism. Significant increase in the level of glutamic acid in serum in PC patients is also consistent with the previous investigations of PC [19, 24].

Altered lipid metabolism is one of the early findings in cancer [13(c)]. Lipids are needed for cell growth and proliferation, and therefore there is a high demand for lipid components in rapidly growing cancer cells. Creatinine is also associated with choline and lipid metabolism pathways [35]. Thus, the decreased levels of choline, creatinine and lipids observed in the blood are consistent with altered lipid metabolism and also with results obtained in previous investigations of PC [17, 20, 24]. In our study, the level of 3-hydroxybutyrate is enhanced significantly, indicating an increased synthesis of ketone bodies in PC. A recent study has shown that ketone bodies drive tumor growth and their inhibition potentially opens new therapeutic routes to treating cancer patients [36]. The elevated levels of 3-hydroxybutyrate in PC was also reported in earlier investigations [19, 24].

Finally, it is important to note the panel of highly ranked metabolites determined and used for developing the classification model in this study is not completely unique, as the identification of metabolite biomarkers can depend somewhat on the type of the analytical technique used. For example, in an earlier metabolomics study of PC based on GC-MS, an entirely different panel of metabolites, including xylitol, 1,5-anhydro-D-glucitol, histidine and inositol, were shown to be highly predictive of PC [24]. Such differences in

metabolite panels occurs in part because diseases such as cancer affect multiple metabolic pathways and thus many metabolites are likely to be affected by disease development. Different analytical platforms detect metabolites with varying degrees of sensitivity. In addition, the complexity of biological samples including blood serum/plasma is extremely high, such that current analytical techniques detect only a small fraction of the enormously diverse metabolite pool. The analytical methods are largely complementary in nature. In the current study, we show that NMR provides a good and reliable platform (which is complementary to MS methods) for the detection of a number of metabolites that are related to PC. Future studies would benefit from the use of methods to uncover additional metabolites, such as NMR-based isotope tagging [37, 38] as well as the combination of such advanced methods and multiple analytical platforms to enhance the pool of metabolite biomarkers and further improve the model [39, 40]. As an example, targeted MS methods [41] can provide access to a larger number of metabolites while providing good reproducibility. A further limitation of this study is the lack of tumor marker CA19.9 data for all patients. Future studies could benefit from incorporating this marker into the model if it is available.

CONCLUSION

PC is usually diagnosed in late stages, typically when it has metastasized, and is due to the lack of reliable early detection methods, which results in high mortality rates. The need to develop highly sensitive and selective methods for routine screening of patients at risk for PC, therefore, continues to be a high priority. The identification of molecular markers of PC holds significant potential for developing a reliable method for early detection of the disease. As part of such efforts, detection of metabolite-based biomarkers using advanced metabolomics tools is a promising approach, because of the likelihood that metabolic markers will be highly correlated with PC disease development. Metabolite biomarkers are also easily detected, and thus provide practical utility for clinical diagnostics. Despite significant advancements in metabolomics methods, a challenging issue is the presence of confounding factors that can challenge biomarker detection and the ability to validate initial findings. To minimize effects from such factors metabolic profiles of blood from fasting patients with reasonably matched characteristics were investigated in this study. Investigations combining NMR and multivariate statistical methods identified twelve metabolites as potential distinguishing biomarkers of PC. Further, focusing on exploring these metabolites for screening PC patients, a prediction model was developed by internal validation of the model and then external validation using an independent set of samples. The resulting classification of PC patients and controls, based on NMR detected serum metabolite profiles, demonstrates the potential utility of the metabolite markers for early detection as well as better understanding the molecular events in the development of PC.

CONFLICT OF INTEREST

The authors declare the following competing financial interests: Dr. Raftery reports holding equity and an executive

position at Matrix-Bio, Inc.; Dr. Shanaiah holds equity in Matrix-Bio.

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SUPPLEMENTARY MATERIALS

Supplementary material is available on the publisher's web site along with the published article.

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