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# Early Detection of Pancreatic Cancer in Type 2 Diabetes Mellitus Patients Based on $^{1}$H NMR Metabolomics

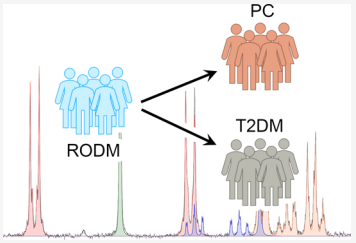
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**ABSTRACT:** The association of pancreatic cancer with type 2 diabetes mellitus was investigated by $^{1}$H NMR metabolomic analysis of blood plasma. Concentration data of 58 metabolites enabled discrimination of pancreatic cancer (PC) patients from healthy controls (HC) and long-term type 2 diabetes mellitus (T2DM) patients. A panel of eight metabolites was proposed and successfully tested for group discrimination. Furthermore, a prediction model for the identification of at-risk individuals for future development of pancreatic cancer was built and tested on recent-onset diabetes mellitus (RODM) patients. Six of 59 RODM samples were assessed as PC with an accuracy of more than 80%. The health condition of these individuals was re-examined, and in four cases, a correlation to the prediction was found. The current health condition can be retrospectively attributed to misdiagnosed pancreatogenic diabetes or to early-stage pancreatic cancer.



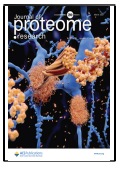

**KEYWORDS:** *pancreatic cancer, diabetes mellitus, pancreatogenic diabetes, NMR metabolomics*

## INTRODUCTION

Pancreatic cancer (PC) is the most aggressive type of gastrointestinal cancer with a very low 5-year survival rate ranging between 3 and 6%. The disease is increasing in incidence constantly and has been recognized as the fourth leading cause of cancer related deaths in the USA.[1−3] The high mortality rate is mainly caused by unspecific symptoms at the early stage, for example, nausea, fatigue, loss of body weight, abdominal and back pain, and so forth. Clinical diagnosis relying on high-resolution imaging methods, such as computed tomography (CT), magnetic resonance imaging (MRI), endoscopic retrograde cholangiopancreatography (ERCP), and endoscopic ultrasound (EUS), usually comes too late.[1,2] After the diagnosis, a radical surgery is often the only possible cure. Unfortunately, it is suitable just for 15−20% of patients. Subsequent relapses are frequent, and chemotherapy is basically only palliative.[4] The early diagnosis by classical tumor biomarkers used in clinical practice suffers from low specificity and sensitivity.[5] Protein biomarkers, namely, CA 19-9, can reach up to 80% sensitivity, but the specificity is decreased due to a similar effect of a myriad of non-neoplastic conditions, for example, pancreatitis, hepatitis, or biliary obstruction.[1,4,5] Therefore, the use of such biomarkers is not reliable for screening and/or early detection of PC. Recent studies[4−6] also describe chromosome mutations, genetic alterations, or epigenetic changes in relation to malignant progression of PC. However, the clinical utility of the aforementioned approaches in the diagnosis of early PC is limited.

On the other hand, diabetes mellitus or impaired glucose tolerance has been observed in 80% of PC patients.[3,7] The association between diabetes mellitus and PC has been repeatedly mentioned,[2,3,7,8] yet the role of diabetes mellitus as a cause or effect of PC is still controversial. On the one hand, long-term type 2 diabetes mellitus (T2DM) lasting more than 5 years is considered an etiologic factor of PC. On the other hand, recent-onset diabetes mellitus (RODM) might be considered the first manifestation of PC.[2] According to recent studies,[2,3,7,9] diabetes developed 24 months prior to PC diagnosis in almost 90% of patients. Therefore, patients with recently diagnosed diabetes are associated with an up to 7-fold increased risk of PC.[7] This specific type of diabetes is called pancreatogenic diabetes (T3cDM), and it probably develops as the first symptom of cancer. T3cDM is a secondary type of diabetes associated with endocrine pathology.[9,10] Unfortunately, the pathogenic mechanism of T3cDM development has not been fully elucidated, although specific substances originating in the tumor cells are supposed to be the cause of $\beta$-cell dysfunction and insulin resistance.[2] Thus, tumors can be considered a cause of diabetes, which is then the first clinical manifestation of PC.[8] Globally, T3cDM represents up to 8% of all patients diagnosed with diabetes mellitus, and almost 30% of recently diagnosed diabetes patients might suffer from T3cDM.[9]

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The prevalence of diabetes in PC patients is much higher when compared to diabetes that develops in association with other cancers.[8] Since T3cDM develops similar to the most prevalent T2DM, it is often misdiagnosed.[9,10] Therefore, the main task for physicians remains to distinguish T3cDM from the more common T2DM.[2] The identification of new specific biomarkers in recently diagnosed diabetes patients seems to be necessary for the early diagnosis of cancer. For this purpose, development of a simple blood test with satisfactory specificity and sensitivity would be the most beneficial. Likewise, T3cDM-T2DM discrimination may be provided by a metabolomic analysis of blood-based samples. Metabolomics is defined as a comprehensive measurement of the metabolome, the complete set of metabolites present in a certain biological system, such as a cell, tissue, or an organism.[11,12] The metabolome is a dynamic and evolving entity, which can be thought of as the downstream endproduct of the interaction of genetic expression, transcriptional changes, post-translational modification of proteins, and various external factors, such as environment, diet, drug administration, diseases, lifestyle, and age.[1,11,12] In metabolomics research, there are two main analytical techniques, mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. Both techniques are complementary, both having advantages and limitations.[1,11,12] Mass spectrometry is a well-established technique in metabolomic analysis mainly due to its high sensitivity, while the less sensitive NMR spectroscopy provides high reproducibility, is quantitative, and requires only a simple sample preparation. NMR-based metabolomics has been applied to many areas of biomedical research.[1,11] It has been used for characterization of metabolomic profile or disease fingerprinting for diagnostic and prognostic purposes and/or to reveal specific biomarkers or fingerprint of drug response.[1,11−14]

In this work, $^1$H NMR spectroscopy was used in a metabolomic study to investigate the metabolic changes in blood plasma induced by PC and diabetes mellitus and to establish a specific panel of biomarkers. The main aim of the work was to propose a screening method for the detection of early-stage PC or misdiagnosed T3cDM.

## MATERIALS AND METHODS

### Patient Recruitment and Plasma Sample Collection

This study was approved by the Ethics Committee at the Military University Hospital Prague (108/8-44/2015). All procedures were performed following the Helsinki Declaration and the Collection of Laws of the Czech Republic. All participants of this study signed an informed consent.

Subjects of interest were recruited at the Department of Gastrointestinal Endoscopy at the Internal Clinic of the Military University Hospital and the First Faculty of Medicine of Charles University in Prague. Plasma samples were collected from four groups: healthy controls (HC, age range 49−85, average age 63, 19 females, 10 males), PC patients (PC, age range 49−85, average age 67, 17 females, 26 males), long-term T2DM patients (T2DM, age range 50−82, average age 68, 16 females, 18 males), and RODM patients or patients with recently developed impaired glucose tolerance (age range 50−85, average age 65, 32 females, 27 males). T2DM patients had suffered from diabetes for more than 4 years (median 12 years); the patients in the RODM group had been diagnosed with diabetes less than 3 years prior to their recruitment. The PC patients included in the study underwent the examination following a clinical protocol, that is, EUS, MRI, and/or CT. In addition, all PC cases were diagnosed by histopathologic examination. A list of acute and chronic medication of PC and T2DM patients can be found in the Supporting Information (Table S2, S3).

Venous blood (9 mL) from the studied subjects was collected using sterile blood collection tubes with $K_3$EDTA as an anticoagulant. Blood samples were also subjected to a routine biochemical test, assessing basic physiological indicators, such as fasting plasma glucose (FPG), glycated hemoglobin ($Hb_{1c}$), human serum albumin (HSA), and protein or cancer biomarker (CA 19-9, CEA) levels. The clinical characteristics of the participants are summarized in Table S1 in the Supporting Information The plasma fractions were obtained by centrifugation at 15,000$g$ for 10 min and frozen immediately afterward (−80 °C).

### Sample Preparation

The plasma samples were thawed at room temperature, and aliquots of 300 $\mu$L were centrifuged through an Amicon 3-kDa cutoff filter (Merck, Germany) for 30 min at 12,000 rpm to isolate low-molecular metabolites. After ultrafiltration, 300 $\mu$L of phosphate buffer in $D_2O$ [0.1 mol•L$^{-1}$; pH = 7.4; 0.1 mmol•L$^{-1}$ sodium salt of trimethylsilyl-[2,2,3,3-$d_4$]-propionic acid (TSP); 38 mmol•L$^{-1}$ $NaN_3$] was mixed with the ultrafiltrate. The prepared mixture (600 $\mu$L) was transferred into a 5 mm NMR tube for the analysis.

### Acquisition

One-dimensional proton NMR spectra for all samples were acquired using a Varian INOVA 500 MHz spectrometer (Varian Instruments Inc., USA) operating at 499.87 MHz proton Larmor frequency equipped with the probe having an inner $^1$H coil for higher sensitivity. Before measurements, the samples were kept for at least 10 min inside the NMR probe for temperature equilibration (298.15 K). For each plasma sample, a water-suppressed Carr−Purcell−Meiboom−Gill (CPMG) spin echo pulse sequence with 1000 scans, 65,536 data points over a spectral width of 8000 Hz, with a relaxation delay of 2 s, and an acquisition time of 2.7 s was used to acquire the $^1$H NMR spectra.

### Data Processing

The Fourier transform spectra were manually corrected for phase and baseline distortions using Chenomx NMR Suite 8.0 (ref 15, NMR Suite program, Canada). The experimental spectrum was referenced to TSP. The solvent signal residuum was subtracted. Finally, the TSP signal linewidth was determined, and pH was set to 7.4 based according to buffer use.

Subsequently, untargeted profiling was performed in the Chenomx Profiler. In each sample, 59 metabolites were identified according to a precise fitting of the compounds from the Chenomx library to the experimental spectrum. One of the metabolites, glycerol, had to be removed from the list of metabolites because of its presence in the membrane of the filters used. The quantification was performed according to the known concentration of TSP; the output was represented by the list of assigned compounds and their concentrations.

In addition, each spectrum in the range of 0−10 ppm was segmented into 0.02 ppm chemical shift bins, which is a standard size for metabolomics. Regions containing residual water signal (4.70−4.85 ppm) and signals of EDTA and its


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metal ($Mg^{2+}$, $Ca^{2+}$) complexes (4.70−4.85, 3.57−3.60, 2.52−2.57 ppm) were excluded from the binning. Binning reduces the number of total variables and compensates for small differences in shifts in the spectra. Both the data inputs (concentrations, bins) were normalized to the total area to reduce effects of sample dilution prior to statistical analysis.

### Statistical Analyses

All data analyses were performed using the open-source software R (ref 16) and Metaboanalyst 4.0 (ref 17). Multivariate data analyses were conducted on processed concentration data and binned data separately. For crude overview and initial exploratory analysis, principal component analysis (PCA) was used to check for outliers from the score plots and to investigate important features from the loading plot. For classification, orthogonal partial least-squares discriminant analysis (OPLS-DA) was applied, retaining in each model the minimum number of components with maximized accuracy. All reported values of accuracy, sensitivity, and specificity were assessed by means of 100 cycles of a Monte Carlo cross-validation scheme (90% of the samples were randomly selected at each iteration as a training set to build the model; the remaining 10% were subsequently tested on performance characteristics for the classification).

A complementary univariate analysis, which is easier to interpret compared to multivariate models because univariate models work with one metabolite at a time, was conducted only on the concentration data set. Based on the biological assumption that metabolites are not normally distributed, a statistical significance was assessed using the nonparametric Wilcoxon rank sum test, also known as the Mann−Whitney test[18] (Tables S4−S6 in the Supporting Information). In order to identify the most influential and statistically significant compounds, the Wilcoxon test was paired with fold change. Fold change is defined as a logarithm of base 2 of a ratio of the median concentration of an individual compound in the patient group and the median concentration of an individual compound within the control group. The obtained P values were adjusted using the Benjamini and Hochberg correction[19] for multiple comparison, and the threshold of adjusted *P*-values was set to <0.05 for statistical significance, generally used in metabolomics as a cutoff value.

Significant metabolites were described by *P*-value and the value of area under the receiver operating characteristics curve (AUC). In addition, a set of potential biomarkers from the Wilcoxon test was evaluated by a multivariate algorithm random forest.[20] The results were presented as the AUC value, 95% confidence interval, and average accuracy based on 100 cross validations. Metaboanalyst 4.0 was also used to detect altered metabolic pathways, and the metabolite ID was matched with the Human Metabolome Database.[21] Metabolic pathway analysis was performed to reveal the biological impact of obtained metabolomics data. A plot of affected pathways (Figure S1 in the Supporting Information) contained 43 nodes, each representing one pathway, with color and size coding corresponding to pathway significance and impact, respectively. The significance was generated from betweenness centrality and out-degree centrality measurements. The pathway impact was generated by the summation of importance measures of matched metabolites to all metabolites present within the pathway. Pathways with impact values exceeding 0.5 were considered as the key pathways.

The prediction models were based on an OPLS-DA discrimination analysis of PC and T2DM groups. Individual models varied by the number of samples used in the models. The samples involved in a given model were selected using a random number generator. Overall, 100 OPLS-DA prediction models were constructed using two types of normalization: normalization to total area and probabilistic quotient normalization (PQN).[22] Prediction models were tested for the classification of RODM samples. Each prediction was verified by a Monte Carlo validation using 100 repetitions.

## RESULTS AND DISCUSSION

In this study, $^1$H NMR spectra of 165 blood plasma samples were measured. The samples were divided into four groups: patients with PC (43 samples), T2DM patients (34 samples), HC (29 samples), and RODM patients (59 samples). The definition of individual groups can be found in Materials and Methods. In each $^1$H NMR spectrum of blood plasma, 58 metabolites were identified using the Chenomx spectrum library and quantified according to the internal standard (for details, see Materials and Methods). The spectral differences between pathophysiological groups were analyzed by multivariate statistical analysis. The multivariate statistical analysis was performed on concentrations of all 58 profiled low-molecular metabolites. The obtained results were supported by untargeted fingerprinting and subsequent statistical analysis of binned spectra.

The unsupervised statistical analysis by PCA was performed on each group to examine the definition and homogeneity of the group and to identify outlying samples. The analysis of PCA results in combination with clinical data input resulted in the exclusion of seven samples; specifically, three PC samples, one HC sample, and three samples from the T2DM group. The T2DM and HC excluded samples revealed increased methanol or ethanol exceeding possible physiological values. This may be associated with some issues during sample collection or its handling. In one case, smaller plasma volume was obtained, which resulted in a poor signal-to-noise ratio. While two patients excluded from the PC group suffered also from another carcinoma, one patient was diagnosed with early-stage mucinous carcinoma. PCA was subsequently tested in group discrimination. However, this analysis was not able to completely discriminate the groups studied; it only indicated some trends in group clustering (Figure S2 in the Supporting Information). The group discrimination was performed using OPLS-DA. The obtained results are divided into individual sections where the examined groups were mutually compared.

### PC Patients vs HC

Data of 40 PC and 28 HC samples were submitted to OPLS-DA. A remarkable separation (Figure 1) of these two groups was obtained using nine components yielding 87.3% sensitivity, 92.3% specificity, and 89.8% accuracy after Monte Carlo cross-validation using 100 repetitions. Likewise, the group discrimination was performed using binning, where the nine-component model provided discrimination with 86.4% overall accuracy (Figure S3 in the Supporting Information).

The nonparametric Wilcoxon test revealed 26 statistically significant metabolites (*P*-value < 0.05). Moreover, 14 metabolites showed a statistical significance (*P*-value < 0.001) (Table S4 in the Supporting Information). Fold change analysis (Figure 2) shows significantly higher levels of 3-hydroxybutyrate and mannose in PC patients, while decreased


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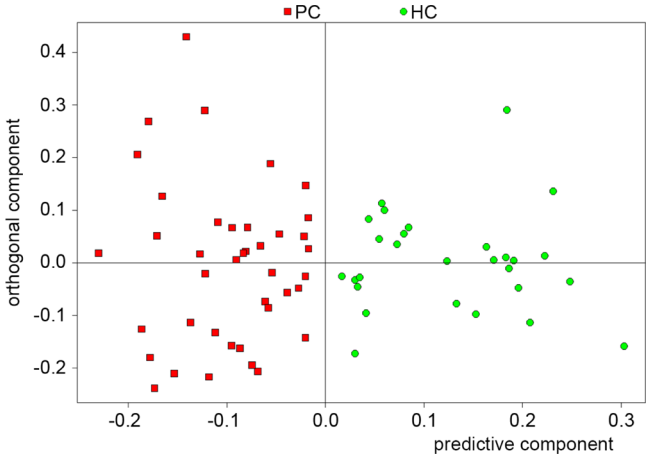



**Figure 1.** OPLS-DA of PC ($n$ = 40, red squares) and HC ($n$ = 28, green circles) using 58 normalized metabolites; Acc. 89.8%, Sen. 87.3%, and Spe. 92.3%.

levels were found for creatine, ornithine, alanine, uridine, serine, histidine, carnitine, glutamine, glycine, threonine, lysine, and methionine.

These findings are consistent with our previous pilot study performed on a limited cohort.[23] Among 12 metabolites, which were selected for the unsupervised statistical analysis, 3-hydroxybutyrate, lysine, glutamine, histidine, and alanine were also present.

An extensive literature survey revealed a match in several metabolites that were described as possible biomarkers of PC. Elevated levels of 3-hydroxybutyrate in blood samples of PC patients were found by Lin et al.,[24] Mehta et al.,[25] and Bathe et al.[26] On the other hand, OuYang et al.[27] and Zhang et al.[6] described a decrease thereof. Inconsistent results were also found for lysine.[6,25,28,29] Decreased histidine level was confirmed by Zhang et al.[6] using $^1$H NMR analysis of plasma and by the GC−MS analysis of serum done by Kobyashi et al.[29] A reduction in plasma alanine was observed by Zhang et al.,[6] Mehta et al.,[25] and also Fukutake et al.,[30] who compared 360 PC patients and 8372 controls using HPLC−ESI−MS. A reduced glutamine content in blood samples was observed in many studies using several analytical techniques, for example, $^1$H NMR,[6] GC−MS,[29] HILIC−LC−MS, and RP−LC−MS.[28] The observed decrease of methionine is consistent with the work of Fukutake et al.[30] and Kobyashi et al.[29] A study performed in two geographically different cohorts (USA and China) showed similar trends for nine low-molecular metabolites (glutamine, creatinine, valine, proline, choline, betaine, urea, tryptophan, and glutamate).[31]

Based on these results, four key metabolic pathways were identified (Figure S1 in the Supporting Information). The pathways mainly impacted included the biosynthesis of phenylalanine, tyrosine, and tryptophan, in which tyrosine

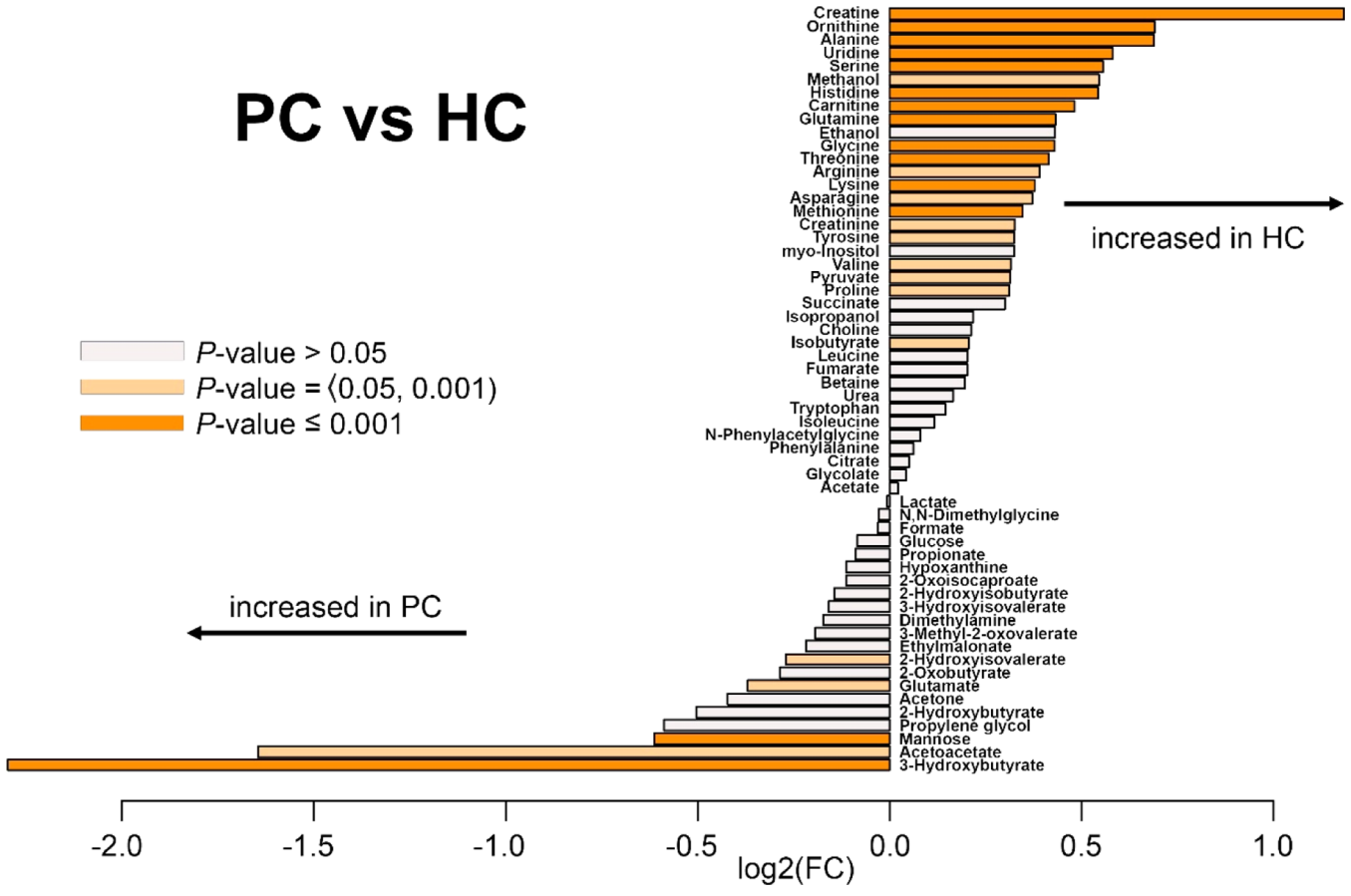



**Figure 2.** Fold change of 58 metabolites depicts key metabolite differences between PC and HC. Adjusted $P$-values for individual metabolites were obtained using the Wilcoxon nonparametric statistical test. Different color coding was used for presentation of metabolite significance ($P$-value < 0.05 and $P$-value < 0.001).



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exhibited the highest *P*-value, and the synthesis and degradation of ketone bodies, in which the major significance was observed for 3-hydroxybutyrate. In a similar manner, glycine, serine, and threonine metabolism and D-glutamine and D-glutamate metabolism were found significantly dysregulated.

### Long-Term T2DM Patients vs HC

Data of 31 T2DM and 28 HC samples were submitted to OPLS-DA. The seven-component OPLS-DA provided clear separation of the T2DM patients and HC (Figure 3) with

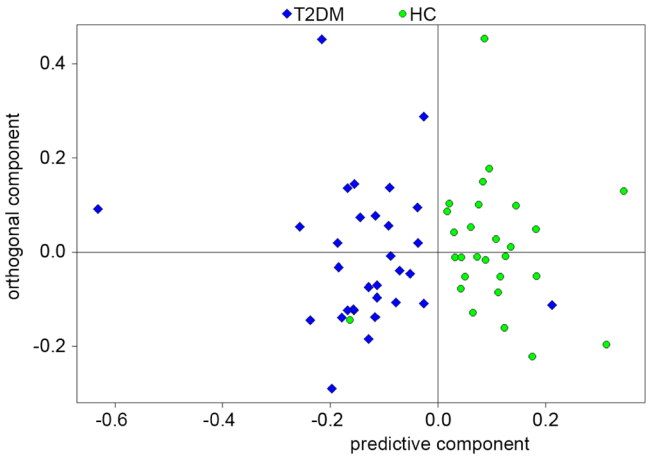



**Figure 3.** OPLS-DA of seven-component model of T2DM ($n$ = 31, blue diamonds) and HC ($n$ = 28, green circles) discrimination using 58 metabolites; Acc. 88.3%, Sen. 85.1%, and Spe. 91.8%.

85.1% sensitivity, 91.8% specificity, and 88.3% overall accuracy after Monte Carlo cross-validation. For the purposes of comparison, binning confirmed the discrimination of these two groups using a higher number of components yielding lower accuracy (83.7%, see Figure S3 in the Supporting Information).

The nonparametric Wilcoxon test showed 19 metabolites as statistically significant (*P*-value < 0.05), 4 with *P*-value < 0.001 (Table S5 in the Supporting Information). Among the metabolites with increased levels in T2DM patients, only glucose was marked with statistical significance. The remaining 18 significant metabolites were found with decreased concentration in T2DM samples. In particular, altered concentrations of ornithine, uridine, histidine, and glutamine substantially contributed to the group separation (Figure 4).

These findings are consistent with a number of studies using $^{1}$H NMR metabolomic analysis of blood samples to distinguish type 2 diabetes patients and controls with normal glucose tolerance. In the literature,[3,7] an increased glucose level was found common to the group of diabetics despite the different diet requirements and level of medication. A decrease of histidine, methionine, glutamine, lysine, threonine, phenylalanine, tyrosine, formate, serine, and glycine levels has been reported in several studies.[32−35] Furthermore, Van Doorn et al.[32] and Zhang et al.[33] also mentioned a decrease of leucine, isoleucine, valine, alanine, and acetate in diabetes samples. Contrary to their findings, leucine, isoleucine, and alanine were found increased in T2DM in this current study but without any statistical significance. The levels of valine and lactate were found similar in both groups. Crucial alterations in metabolite levels indicate a malfunction of several biochemical pathways, notably in the biosynthesis of phenylalanine, tyrosine, and tryptophan, metabolism of glycine, serine, and threonine, metabolism of glutamine and glutamate, and also in the metabolism of alanine, aspartate, and glutamate (Figure S1 in the Supporting Information). Metabolomic studies[3,7,36] suggest that diabetes mellitus affects the metabolic networks of primary biomolecules, such as carbohydrates, lipids, and amino acids. Hyperglycemia and glycosuria are the major manifestations of uncontrolled diabetes mellitus. Abnormal levels of other metabolites also reflect a dysregulation of the carbohydrate metabolism, such as mannose and fructose.[36]

### PC Patients vs T2DM Patients

The discrimination analysis of 40 PC and 31 T2DM patients and 28 HC using a nine-component OPLS-DA model indicates that both diseases exhibit different plasma metabolite profiles. Therefore, PC and T2DM can be distinguished from HC simultaneously and also from each other (Figure 5A). For this purpose, PC and T2DM groups were subjected to the discrimination analysis on their own. The eight-component OPLS-DA of PC and T2DM samples provided a clear separation (Figure 5B) with an outstanding overall 85.4% sensitivity, 93.2% specificity, and 88.8% accuracy after Monte Carlo cross-validation. As in the previous cases, binning results with an equal number of components provided lower values of accuracy, sensitivity, and specificity (Figure S3 in the Supporting Information).

Despite the slight variations in metabolite levels found in the plasma samples of PC and T2DM groups, the nonparametric Wilcoxon test showed 11 statistically significant metabolites (*P*-value < 0.05), 3 with a *P*-value < 0.001 (Table S6 in the Supporting Information). The PC patients were characterized by significantly increased levels of 3-hydroxybutyrate, propylene glycol, mannose, propionate, glutamate, and tryptophan and decreased levels of creatine, alanine, valine, proline, and lysine (Figure 6). The increased levels of propylene glycol in the PC group can be attributed to the acute medication prior to sample collection, which in vast majority followed EUS examination (Table S2 in the Supporting Information).

The metabolic pathway analysis (Figure S1 in the Supporting Information) again indicated altered biochemical pathways mainly linked to gastrointestinal malignancies, such as the biosynthesis of phenylalanine, tyrosine, and tryptophan, synthesis of ketone bodies, metabolism of glycine, serine, and threonine, and metabolism of glutamine and glutamate.

Interestingly, the levels of creatine, alanine, valine, proline, and lysine were previously found decreased also in the group discrimination of PC versus HC and T2DM versus HC. The levels of 3-hydroxybutyrate, mannose, and glutamate were found more pronounced with the development of PC. Unexpectedly, the levels of tryptophan and propionate moved from deficiency to a surfeit when going from the group discrimination of DM versus HC to PC versus HC (Figure S4 in the Supporting Information). These observations indicate that PC significantly alters the metabolic pathways involving these two metabolites. In the case of tryptophan, it was reported that metastatic pancreatic ductal adenocarcinoma increases the T cell number, which is associated with an overexpression of indoleamine 2,3-dioxygenase 1, an enzyme initiating tryptophan metabolism.[37,38] A link between PC and propionate has not been described yet.

The increased levels of 3-hydroxybutyrate, mannose, and glutamate simultaneously with decreased levels of creatine, alanine, valine, proline, and lysine were significant for the


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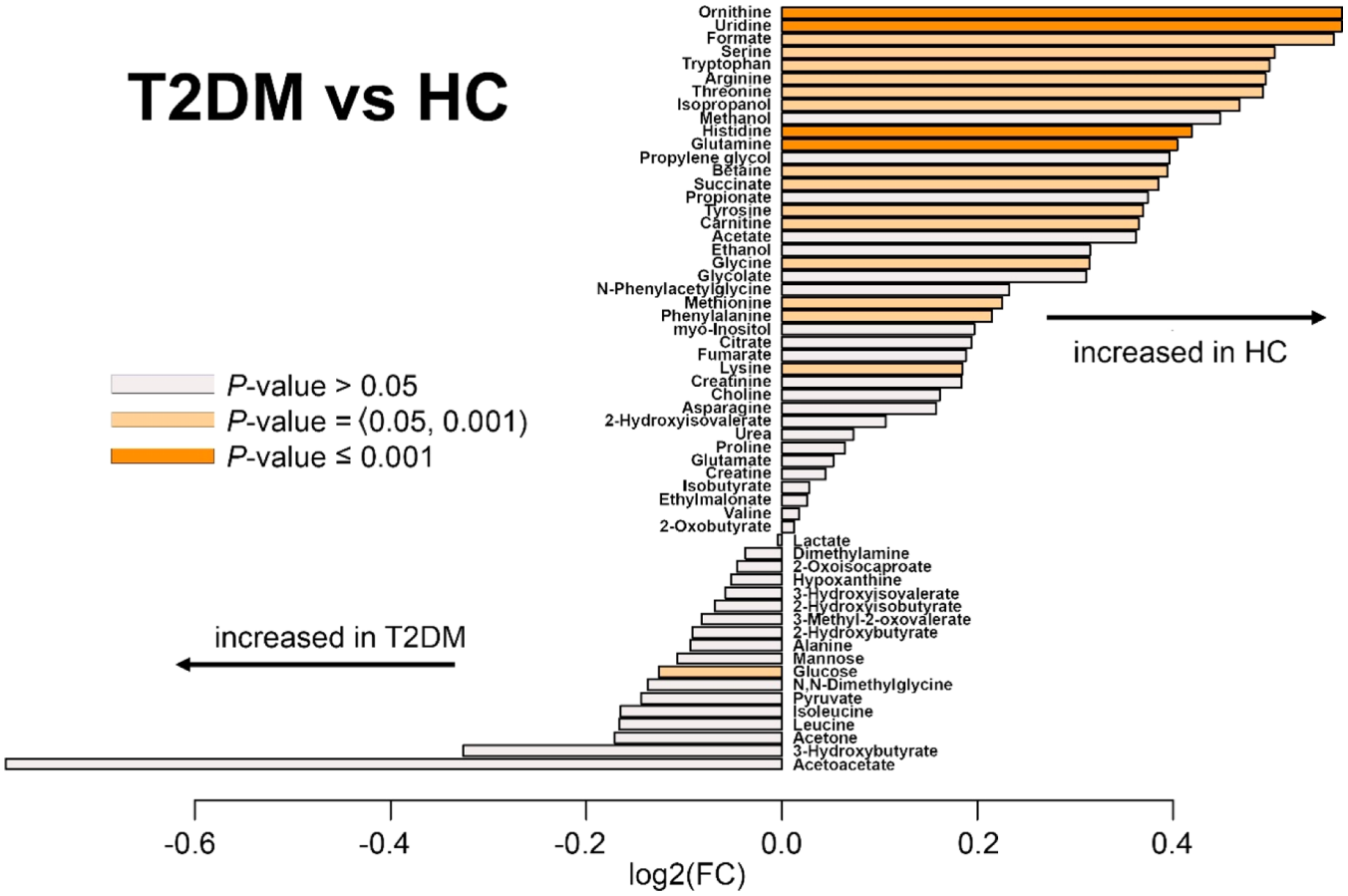



**Figure 4.** Fold change of 58 metabolites depicts key metabolite differences between T2DM and HC. Adjusted *P*-values were obtained using the Wilcoxon nonparametric statistical test. Different color coding was used for presentation of metabolite significance (*P*-value < 0.05 and *P*-value < 0.001).

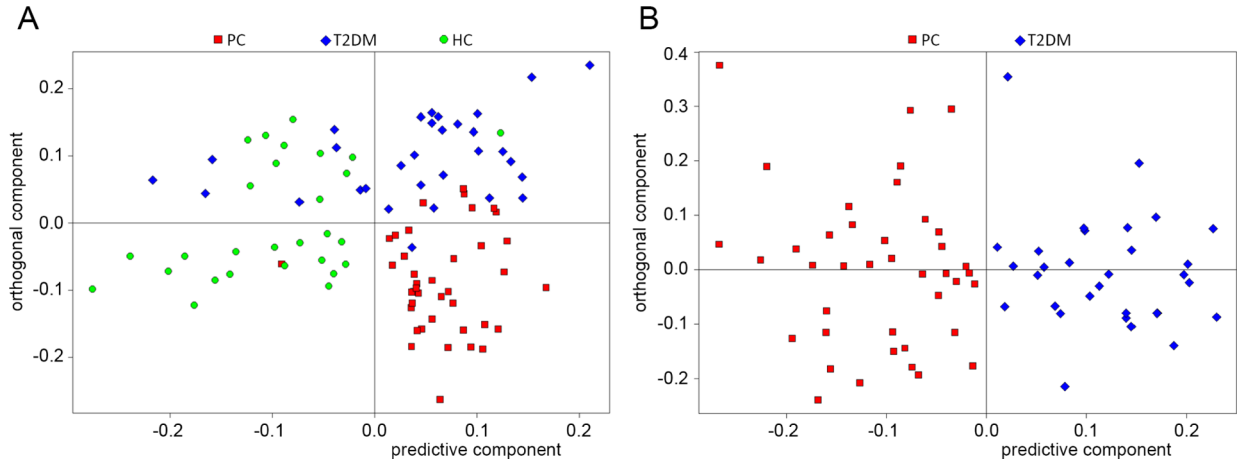



**Figure 5.** OPLS-DA score plots based on 58 normalized metabolite concentrations. Left/A: nine-component discrimination of all three groups: PC ($n$ = 40, red squares), T2DM ($n$ = 31 blue diamonds), and HC ($n$ = 28, green circles); confusion matrix can be found in the Supporting Information (Table S7). Right/B: eight-component discrimination only of PC ($n$ = 40, red squares) and T2DM ($n$ = 31 blue diamonds); Acc. 88.8%, Sen. 85.4%, and Spe. 93.2%.

discrimination of the PC group from T2DM and also from HC. Therefore, these eight metabolites can be considered a specific panel of biomarkers for PC manifestation. The suitability of this plasma metabolite signature as a noninvasive biomarker for the diagnosis of PC was assessed by receiver operating characteristic (ROC) curves generated by the random forest algorithm. The ROC analysis revealed that the 8 proposed plasma metabolite biomarkers were robust in the discrimination of PC patients from HC and from T2DM patients with similar or slightly higher AUC values in comparison to all 58 metabolites or only statistically significant metabolites (*P*-value < 0.05) in a corresponding discrimination (Figure S5 in the Supporting Information).


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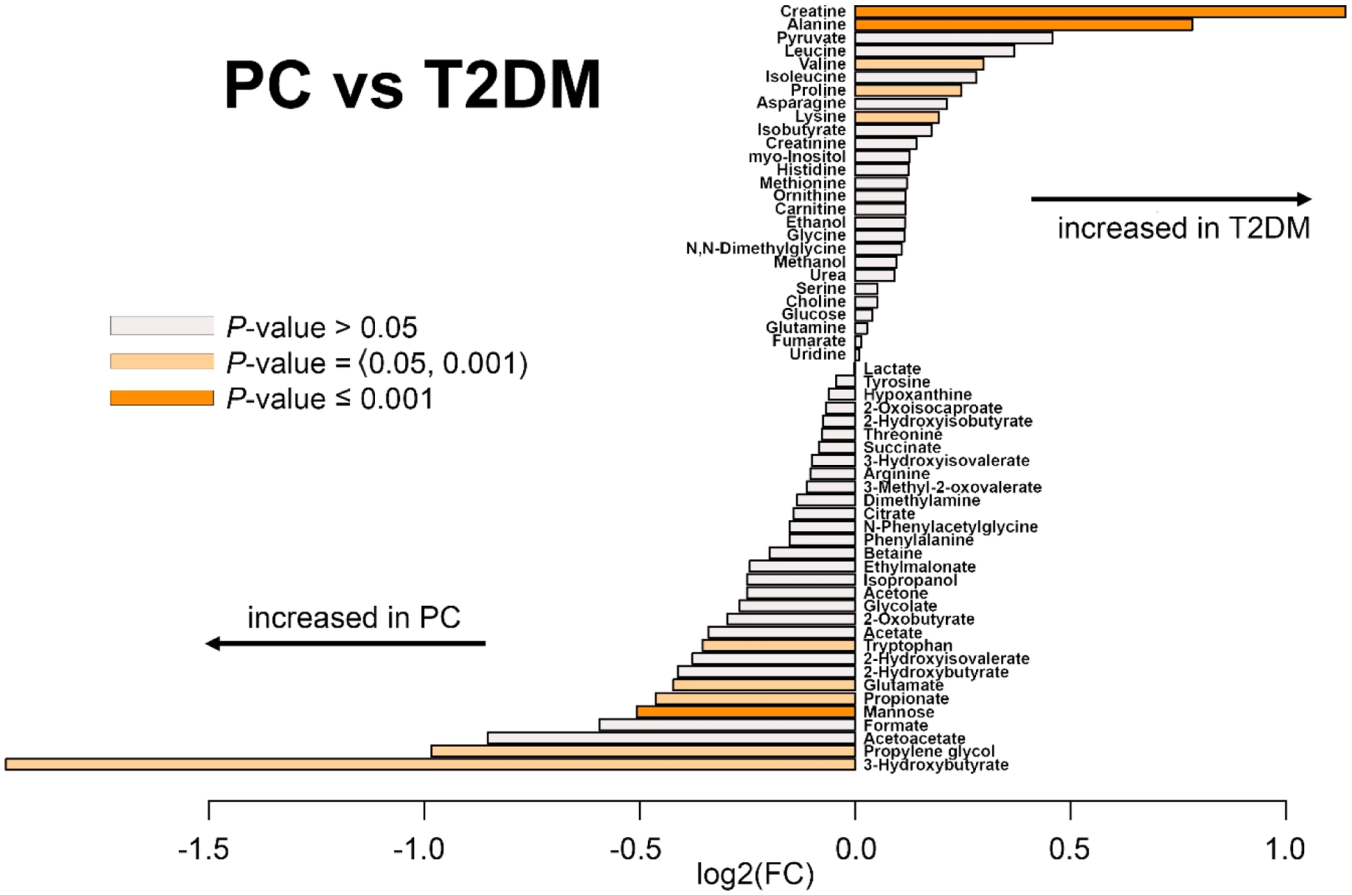



**Figure 6.** Fold change depicts key metabolite differences between PC and T2DM. Adjusted *P*-values were obtained using the Wilcoxon nonparametric statistical test. Those increased in cancer are projected to the left, whereas those decreased in cancer are projected to the right. Different color coding was used for presentation of metabolite significance (*P*-value < 0.05 and *P*-value < 0.001).

According to the Human Metabolome Database, these eight metabolites were already associated with PC in the past. The amino acids (creatine,[6,24,26] alanine,[6,30,39] valine,[6,24,29,30,39,40] proline,[26,30,39,41] and lysine[6,26,28−30,39]) were mentioned as metabolic biomarkers of PC. Products of ketogenesis (3-hydroxybutyrate, acetate, and acetoacetate) may reflect the presence of latent diabetogenic changes, perhaps resulting from the PC.[26] Diabetic ketoacidosis is an example involving the overproduction of ketone bodies, which occurs when there is a lack of, or a resistance to, insulin.[42] Usually, the production of insulin is sufficient to prevent excessive ketogenesis in most diabetes cases. When insulin production decreases, the production of ketone bodies increases due to increased HMG-CoA activity, which is accompanied by increased levels of free fatty acids and by increased uptake of free fatty acids by mitochondria and excessive production of ketone bodies.[36,42]

Glutamate belongs to the main components in tumor growth and progression; its concentration plays a key role in cancer cell invasion and migration.[31] Glutamate has been implicated in tumorigenesis through activation of AMPA receptors. Herner et al.[43] showed that activation of the glutamate-mediated AMPA receptor increases invasion and migration of cancer cells via activation of the classical MAPK pathway. An increased glutamate level was observed in PC patients accompanied by downregulation of GluR subunits 1, 2, and 4. Increased tissue glutamate can directly activate AMPA receptors on PanIN and/or cancer cells to switch on invasive and migratory programs, probably via the activation of the K-ras/MAPK cascade.[43,44]

In general, tumor cells upregulate the production of biosynthetic intermediates to build new cells, while ATP levels and energy production grow.[45] Glucose, amino acids, and fatty acids are used as fuel sources, increasing the uptake of these metabolic substrates to meet raised demands for biosynthesis and energy production to support rapid cell proliferation in the cancer tissue.[31] This may explain decreased levels of several amino acids in the plasma of PC patients. Moreover, low levels of amino acids may reflect chronic insufficient dietary intake and abnormal glucose metabolism, which may lead to ammonia detoxification problems.

## Predictions of Possible Early-Stage PC in RODM Patients

The discrimination of PC and T2DM patients based on alterations of plasma metabolite concentrations can be utilized to construct a prediction model for the identification of persons at high risk of PC development. The aim is to identify either misdiagnosed pancreatogenic diabetes or early-stage PC within this group. Patients with diabetes diagnosed within the last 4 years are at significantly higher risk of developing PC compared to individuals who have had diabetes for a longer period.[8] Similarly, elderly patients with RODM are at about 8-times higher risk than the common population.[2] Thus, RODM patients are a crucial cohort for PC screening, where it is important to determine the patients for further screening, especially with EUS or ERCP.


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The prediction models were constructed based on an OPLS-DA discrimination of PC and T2DM cohorts, where RODM samples were repeatedly assigned. Overall, 100 OPLS-DA prediction models were constructed. The concentration data were used in 35 models, while binning was performed in 15 models. Two types of normalization were employed in each model, resulting in 100 overall models (for details, see Materials and Methods). The models varied by the number of samples used. The samples involved in a given model were selected using a random number generator. The number of PC samples ranged between 20 and 40, while T2DM ranged between 15 and 31 samples. To identify the at-risk individuals within the RODM group, only models with an overall accuracy of above 85% were considered. Finally, 50 selected models were used for RODM classification. The prediction model based on a concentration profile of 58 metabolites of 40 PC and 31 T2DM patients with classification of 59 RODM samples is presented in Figure 7. In this particular model, 13

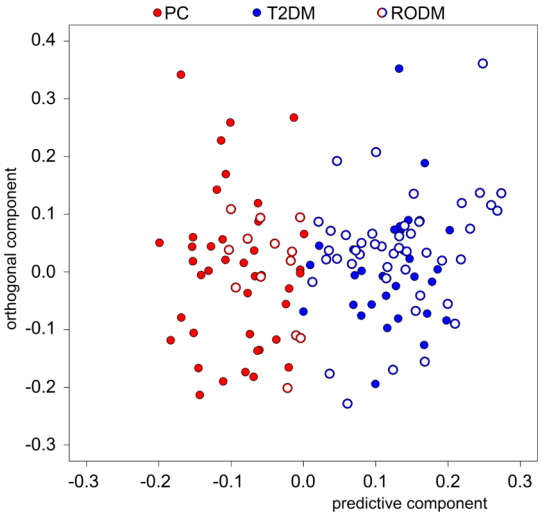



**Figure 7.** OPLS-DA predictive model based on 58 concentrations normalized to total area. Discrimination contains 40 PC samples (red dots), 31 T2DM samples (blue dots), and 59 RODM classified samples shown as hollow circles; red for samples classified to the PC group or blue in case of classification to the T2DM group. Acc. 88.2%, Sen. 86.6%, and Spe. 90.0% was obtained for the original PC and T2DM discrimination.

RODM samples were classified as PC. Altogether, 6 of 59 RODM samples were assessed as PC in more than 80% of 50 selected models (Table 1). These patients especially are at high risk for future development of PC and deserve special attention.

**Table 1. Samples Classified At-Risk (%) in 50 Prediction Models with Overall Accuracy above 85% after Monte Carlo Validation**

| sample | classification % | year of birth | sample collection | DM diagnosis |
|---|---|---|---|---|
| RODM 003 | 86 | 1951 | 12/2016 | 12/2015 |
| RODM 008 | 88 | 1944 | 02/2017 | 01/2016 |
| RODM 011 | 84 | 1956 | 03/2017 | 02/2017 |
| RODM 015 | 88 | 1950 | 04/2017 | 04/2017 |
| RODM 036 | 82 | 1961 | 07/2017 | 08/2015 |
| RODM 071 | 97 | 1950 | 03/2019 | 03/2019 |

The obtained list of identified at-risk individuals was passed on to the physicians to examine their current health condition. Four patients were observed with significant changes in pancreas pathology, which can be retrospectively attributed to early-stage PC or other risky lesions present at the date of sample collection. Patient RODM 011 was diagnosed with PC. Patients RODM 008, RODM 015, and RODM 036 were diagnosed with chronic pancreatitis with multiple cystic lesions detected. Chronic pancreatitis is considered by some authors as an early manifestation of PC and not only a risk factor.[46,47] A normal pancreas was found in patients RODM 003 and RODM 071 using CT, MRI, and EUS imaging methods; nevertheless, a careful follow-up will take place. Detailed results of the clinical examination can be found in the Supporting Information.

NMR metabolomics is obviously sensitive to different kinds of pancreatic pathology and can help in the preselection of patients for early surgical treatment, which is the main aim of modern pancreatology.

## CONCLUSIONS

PC patients were successfully distinguished from healthy controls and long-term diabetic patients based on $^{1}$H NMR metabolomic analysis of blood plasma. Significantly elevated levels of 3-hydroxybutyrate, mannose, and glutamate accompanied by a decreased content of creatine, alanine, valine, proline, and lysine were found in PC patients. All these metabolites were already associated with PC in the past. This metabolic panel was further successfully tested for the discrimination of PC samples from HC and diabetes mellitus patients. Additionally, the levels of tryptophan and propionate moved from deficiency to surfeit when shifting from diabetes mellitus to PC, indicating that PC induces significant alterations of metabolic pathways involving these two metabolites. Taking a prerequisite that diabetes mellitus can be regarded as a first manifestation of PC, a prediction model based on OPLS-DA of PC and T2DM was built for the identification of at-risk individuals for future development of PC. The subsequent screening was performed on recently diagnosed diabetes mellitus patients. Six of 59 RODM samples were assessed as PC in more than 80% of 50 selected prediction models. As these patients are at high risk for future development of PC, their current health condition was re-examined, and a fair correlation to the prediction was found. The health conditions can be retrospectively attributed to misdiagnosed pancreatogenic diabetes or to early-stage PC at the time of sample collection.

Hopefully, the results presented here convincingly highlight the exciting potential of the evolving field of metabolomics and may accelerate its transfer into clinical practice for the diagnosis of fatal diseases with nonspecific symptoms.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.0c00990.

Biochemical characteristics of the samples; acute and chronic medication of PC and T2DM patients; statistically significant metabolites; confusion matrix for simultaneous discrimination of PC, T2DM, and HC; metabolic pathway analysis of the group discrimination; PCA based on concentration data; OPLS-DA based on


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binning data; box plots of dysregulated metabolites; ROC curves; and CT, MRI, and EUC of at-risk RODM patients (PDF)

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### Author Contributions

J.S., V.S., and B.B.—study concept and design; L.M., Š.H., and J.S.—acquisition of data; L.M., Š.H., and J.S.—analysis and interpretation of data; L.M.—drafting of the manuscript; L.H., V.S., and B.B.—critical revision of the manuscript for important intellectual content; L.M. and Š.H.—statistical analysis; V.S. and B.B.—obtained funding; L.H. and V.S.—technical or material support; J.S.—study supervision.

### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

AMPA, Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; AUC, area under the curve; CA 19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CPMG, Carr-Purcell-Meiboom-Gill; CT, computed tomography; $D_2O$, deuterated water; ERC, Pendoscopic retrograde cholandiopancreatography; EUS, endoscopic ultrasound; EUS-FNA, endoscopic ultrasound-fine needle aspiration (biopsy); FPG, fasting plasma glucose; GC-MS, gas chromatography-mass spectrometry; GluR, glutamate receptor; Hb1c, glycated haemoglobin; HC, healthy controls; HILIC−LC−MS, hydrophilic-liquid chromatography-mass spectrometry; HPLC−ESI−MS, high performance liquid chromatography-electrospray ionization-mass spectrometry; HSA, human serum albumin; IPMN, intraductal papillary mucinous neoplasm; $K_3$EDTA, tripotassium ethylenediaminetetraacetic acid; K-ras, Kirsten rat sarcoma (gene); MAPK, mitogen-activated protein kinase; MRI, magnetic resonance imaging; NMR, nuclear magnetic resonance; OPLS-DA, orthogonal partial least squares-discriminant analysis; PanIN, pancreatic intraepithelial neoplasia; PC, pancreatic cancer (patients); PCA, principal component analysis; PQN, probabilistic quotient normalization; ROC, receiver operating characteristic; RODM, recent-onset diabetes mellitus (patients); RP-LC-MS, reverse phase-liquid chromatography-mass spectrometry; T2DM, type 2 diabetes mellitus (patients); T3cDM, pancreatogenic diabetes (patients); TSP, sodium salt of trimethylsilyl-[2,2,3,3-$d_4$]-propionic acid

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