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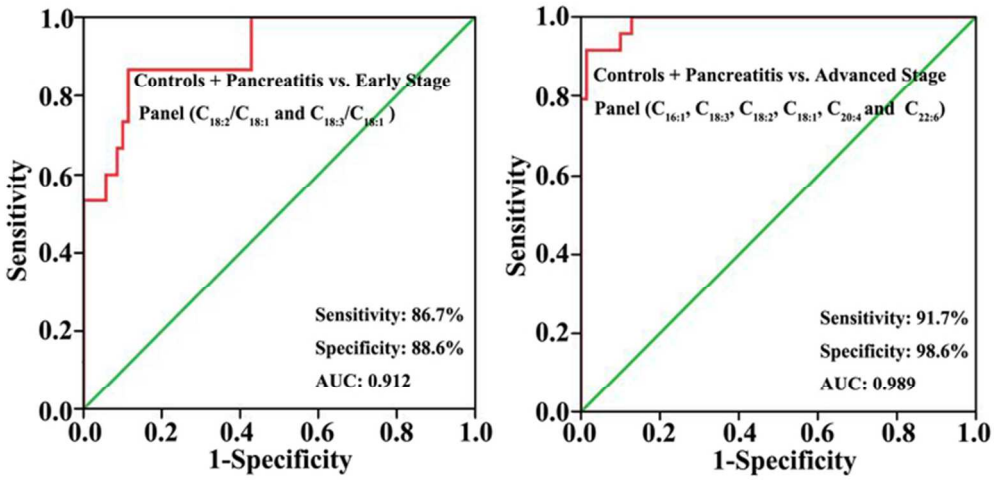

Simultaneous qualitative and quantitative determination of serum unsaturated fatty acids as early-stage cancer indicators using chip-based nanoESI-FTICR MS
32x16mm (600 x 600 DPI)

High-throughput and high-sensitivity quantitative analysis of serum unsaturated fatty acids by chip-based nanoelectrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry: early stage diagnostic biomarkers of pancreatic cancer

Yaping Zhang[a], Ling Qiu[b], Yanmin Wang[c], Xuzhen Qin[b], Zhili Li[a,*]

[a]Department of Biophysics and structural biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & School of Basic Medicine, Peking Union Medical College, Beijing, PR China

[b]Department of Clinical Laboratory, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, PR China

[c]Department of Clinical Laboratory, Heze Municipal Hospital, Shandong, PR China

***Corresponding author:** Zhili Li, Department of Biophysics and structural biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & School of Basic Medicine, Peking Union Medical College, 5 Dongdan San Tiao, Beijing 100005, PR China. Phone: +86-10-69156479; E-mail: lizhili@ibms.pumc.edu.cn

Abbreviations: CBDInanoESI, chip-based direct-infusion nanoelectrospray; FTICR MS, Fourier transform ion cyclotron resonance mass spectrometry; MS, mass spectrometry; PC, pancreatic cancer; FFAs, free fatty acids; LOD, limit of detection; RSD, relative standard deviation; ROC, area under the receiver operating characteristic; AUC, area under receiver operating characteristic curve; PUFA,

polyunsaturated fatty acid; MUFA, monosaturated fatty acid.

Abstract

In this study, Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) coupled with chip-based direct-infusion nanoelectrospray ionization source (CBDInanoESI) in a negative ion mode is first employed to evaluate the effect of serum and its corresponding supernatant matrixes on the recoveries of serum free fatty acids (FFAs) based on spike-and-recovery experimental strategy by adding analytes along with analog internal standard (IS). The recoveries between serum (69.8-115.6%) and the supernatant (73.6-99.0%) matrixes are almost identical. Multiple point internal standard calibration curves between the concentration ratios of individual fatty acids to ISs, ($C_{17:1}$ as IS of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, or $C_{18:1}$ or $C_{21:0}$ as IS of $C_{20:4}$ or $C_{22:6}$) versus their corresponding intensity ratios were constructed for $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$, respectively, with correlation coefficients of great than 0.99, lower limits of detection between 0.3 and 1.8 nM, and intra- and inter-day precision (relative standard deviations < 18%), along with the linear dynamic range of three orders of magnitude. Sequentially, this advanced analytical platform was applied to perform simultaneous quantitative and qualitative analysis of multiple targets, e.g. serum supernatant unsaturated FFAs from 361 participants including 95 patients with PC, 61 patients with pancreatitis and 205 healthy controls. Experimental results indicated that the levels of $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, $C_{20:4}$ and $C_{22:6}$, as well as the level ratios of $C_{18:2}/C_{18:1}$ and $C_{18:3}/C_{18:1}$ of the PC patients were significantly decreased compared with those of healthy controls and the patients with pancreatitis ($p<0.01$). It is worth noting that the ratio of $C_{18:2}/C_{18:1}$, polyunsaturated fatty acid (PUFA) ($C_{18:2}$, $C_{18:3}$, $C_{20:4}$, and $C_{22:6}$),

panel a ($C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{20:4}$ and $C_{22:6}$) and panel b ($C_{18:2}/C_{18:1}$ or $C_{18:3}/C_{18:1}$) performed excellent diagnostic ability, with area under the receiver operating characteristic curve of ≥0.869, sensitivity of ≥ 85.7% and specificity of ≥ 86.7% for differentiating the early stage PC from non-cancer subjects, which are greatly higher than those of clinically used serum biomarker CA 19-9. More importantly, this platform can also provide a fast and easy way to quantify the levels of FFAs in less than 30 seconds per sample.

Keywords: chip-based nanoelectrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry; unsaturated fatty acids; serum; pancreatic cancer; pancreatitis; early stage diagnosis.

Introduction

Pancreatic cancer (PC) is one of the most deadly human malignancies. In 2012, about 44,920 cases were diagnosed with PC and an estimated 37,390 patients died of the disease in the United States.[1] The patients with PC have a very low survival rate (less than 5% within 5 years) because of the limitation in early diagnosis.[2] An overall view of the present diagnostic techniques, computer tomography and magnetic resonance imaging are more common and reliable techniques for the detection of PC. However, the computer tomography has poor diagnostic rate for smaller lesion and does not facilitate to show the relationship between the tumor and surrounding structures.[3] Magnetic resonance imaging can display septa within a lesion with higher sensitivity than the computer tomography,[4] but it is difficult for patients with cardiac pacemaker or some metallic foreign-body. At present, most serum tumor marker assays are commonly used to detect cancers, which usually appear at advanced stages of cancers.[5, 6] Recent study pointed out that circulating tumor cells could be used to early diagnosis of PC because they could disseminate into peripheral blood in the preinvasive and early stages of PC,[7] but a large volume of blood sample will be need due to a low probability event in blood. Hence, it is necessary to construct a noninvasive, simple, sensitivity, convenient method to detect early stage PC with a small volume of blood.

Previous studies have indicated that changes in the levels of unsaturated free fatty acids (FFAs) were closely associated with malignancy.[8-10] Unsaturated FFAs involve in energy generation through β-oxidation to satisfy a large energy requirement during cellular proliferation. Also they have displayed controversial effect on

inflammation,[11] and specifically, arachidonic acid is the vital substrate for diverse inflammatory molecules.[12] However, there are few reports concerning the relationship between serum unsaturated FFAs levels and pancreatic diseases.

Metabolomics, defined as comprehensive study of all low-molecular-weight metabolites in organism, has been successfully used to disease diagnosis and biomarkers screening.[13-15] Metabolome analysis usually employs liquid chromatography, gas chromatography, or capillary electrophoresis coupled to mass spectrometry (MS)[16-20] and nuclear magnetic resonance analysis.[21] These analytical methods, especially for the former analytical techniques, involve complicated, time-consuming sample preparation, longer analytical time per sample and high cost of analysis per sample, and also face a challenge of experimental reproducibility as well as the stability and precision of MS for a large sample size. Recently, a simple, rapid and high-throughput technique was employed to screen the biomarkers in lung cancer,[13] colorectal cancer,[22] and diabetes[23] using direct-infusion electrospray ionization (or matrix-assisted laser desorption/ionization)-Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS), with similar sensitivity of liquid chromatography or gas chromatography coupled to MS. But some carry-over and ionization suppression still existed. Rapidfire-based instrument has been used to the selective detection of enzyme substrates or products in high-throughput, [24-26] but the system is still not adequate for large scale high-throughput analysis in a reasonable time frame,[27] and for multiple targets analysis.

In the present study, in order to overcome the shortcoming mentioned above, chip-based direct-infusion nanoelectrospray ionization source (CBDInanoESI)

coupled to FTICR MS is employed to quantify the levels of serum FFAs with high throughput, high sensitivity, high resolution, and high mass accuracy compared to the conventional liquid chromatography, gas chromatography or capillary electrophoresis coupled to MS, as well as Rapidfire system and paper spray source coupled with mass spectrometry. The linearity, stability, precision and recovery test were also performed to evaluate feasibility of this platform. Comparison of fatty acids (FAs) spike-and-recovery between serum and the corresponding matrixes was also performed. This platform was further used to quantify the levels of the supernatant FFAs from 361 serum samples, including 95 patients with PC, 61 with pancreatitis and 205 healthy controls. The experimental results indicated that the FFAs panels, such as a combination of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{20:4}$ and $C_{22:6}$, with the AUC of 0.879, the sensitivity of 86.7% and the specificity of 90.0%, have excellent diagnostic accuracy to differentiate early-stage PC from the patients with pancreatitis plus normal controls.

Materials and methods

Chemicals and Reagents

Palmitoleic acid ($C_{16:1}$), heptadecenoic acid ($C_{17:1}$), linolenic acid ($C_{18:3}$), linoleic acid ($C_{18:2}$), oleic acid ($C_{18:1}$), stearic acid ($C_{18:0}$), arachidonic acid ($C_{20:4}$), heneicosanoic acid ($C_{21:0}$), and docosahexaenoic acid ($C_{22:6}$) and ammonium acetate (all with purity of more than 99%, except $C_{22:6}$ with purity of > 98%) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Palmitic acid ($C_{16:0}$, purity of > 99%)

was purchased from J&K (J&K Scientific Ltd, China). HPLC-grade methanol, ethanol and acetonitrile were supplied by Fisher Scientific (Pittsburg, PA, USA). The ultrapure water was purified by a Milli-Q system (Millipore, USA).

Participants

In this study, there are 156 patients, including 95 patients with PC and 61 patients with pancreatitis, from Peking Union Medical College Hospital (Beijing, China). The PC stages was based on the Union for International Cancer Control (UICC) tumor-node metastasis (TNM) classification, which consist of 15 patients with early stages (stage I or II) and 24 patients with advanced stages (stage III or IV). Evaluations of hematochemical parameters were performed in Peking Union Medical College Hospital, and clinical records were reviewed to ensure that these individuals were in correct body status. 205 serum samples for healthy controls were collected at Heze Municipal Hospital (Shandong, China), with no clinically relevant abnormalities. The characteristics of all subjects are summarized in **Table 1**. All samples used in this study are the remaining sera after clinical laboratory examination. All participants gave informed consents. This study was approved by the Ethics Review Board at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences.

Preparation of standard stock solutions

$C_{17:1}$ and $C_{21:0}$ were used as internal standards (ISs). Their mixture stock solutions were prepared in ethanol at the concentrations of 83.3 mM and 33.3 mM, respectively, and further diluted to proper concentrations prior to use.

The primary standard solutions of $C_{16:1}$ (2052 μM), $C_{18:3}$ (490 μM), $C_{18:2}$ (3413 μM), $C_{18:1}$ (5015 μM), $C_{20:4}$ (1097 μM) and $C_{22:6}$ (337 μM) were prepared in ethanol, respectively. Then equal volume of these 6 solutions was amalgamated into a solution followed by 500-fold dilution as the standard mixture stock solution at the final concentrations of 684.0 nM for $C_{16:1}$, 163.3 nM for $C_{18:3}$, 1137.7 nM for $C_{18:2}$, 1671.7 nM for $C_{18:1}$, 365.7 nM for $C_{20:4}$ and 112.3 nM for $C_{22:6}$) and further diluted by methanol/acetonitrile/5 mM ammonium acetate in water (42/28/30, v/v/v) to proper concentrations for use.

Sample Preparation

Serum sample was thawed at 4 °C, and then 50 μL of each sample was transferred into a 1.5 mL tube followed by the addition of 950 μL of methanol/acetonitrile (3/2, v/v) to precipitate serum proteins. The resulting mixture was vortexed for 30 s and then stored at -20 °C overnight. After the mixture was centrifuged at 19000 g for 30 min at 4 °C, the supernatant was transferred into a new 1.5 mL tube. 20 μL of the above supernatant was mixed with 1 μL of the IS solution (83.3 μM $C_{17:1}$ and 33.3 μM $C_{21:0}$) in a 2 mL tube followed by the addition of 500 μL of hexane and 500 μL of water, and then the resulting mixture was vortexed for 30 s. After centrifugation at 1500 g for 10 min, the hexane layer was transferred into a glass vial and air-dried, and then 1 mL of methanol/acetonitrile/5 mM ammonium acetate (42/28/30, v/v/v) was added into the glass vial to redissolve the sample for analysis.

Mass Spectrometry

All the experiments were performed using a 9.4 T Apex-ultra™ hybrid Qh-FTICR MS (Bruker Daltonics, Billerica, MA, USA) coupled with an automated chip-based nanoelectrospray NanoMate system (Advion BioSciences, Ithaca, NY, USA) in the negative ion mode. The NanoMate system includes a cooling unit to keep the sample tray at 4℃, which helps reduce sample evaporation and maintain analytes stability. Briefly, 0.1 μL of the redissolved sample was absorbed into the pipet tip followed by 0.5 μL of air, and then delivered to the backplane of the microchip. The microchip contains a 20 ×20 array of nozzles. Nanoelectrospray ionization was initiated from the nozzle by applying a voltage of -1.8 kV and a head pressure of 0.7 psi at a flow rate of about 100 nL/min. Instrument calibration was performed using FAs mixture including $C_{15:0}$ (Molecular weight = 242.22458 Da), $C_{17:0}$ (270.25588 Da) and $C_{21:0}$ (326.31848 Da). Mass spectrum of each sample was accumulated for 10 full scans at the *m/z* range of 150-400 in broadband mode with time-domain size of 1 Mb. The resolution of the instrument is 200,000 at *m/z* 400. Both capillary and spray shield voltages were 0 V. The drying gas temperature was 150 °C with a flow rate of 4.0 L/min. The time of flight, source accumulation and ion accumulation time were 0.0007 s, 0.08 s and 0.4 s, respectively.

Data handling

The original MS data were acquired using ApexControl 3.0.0 (Bruker Daltonics) in expert mode and dealt with DataAnalysis 4.0 (Bruker Daltonics). The deconvolution results were extracted and then transferred to Microsoft Excel. The identification of the FFAs were confirmed by comparing with accurate molecular weight (mass error ≤

0.00025 Da) and observed isotope abundance distribution (relative intensity deviation of observed to theoretical values is less than 2%). If FFAs signals were absent, the baseline strength in each spectrum was adapted as their values for the following statistical analysis. It should be noted that the concentration ratios of $C_{18:2}/C_{18:1,}$ $C_{18:3}/C_{18:2}$ and $C_{18:3}/C_{18:1}$ were also termed as three new variables, respectively, in the following statistical analysis.

Statistical analysis

Statistical analysis was performed using SPSS (version 16.0, Chicago, IL, USA). The results are presented as mean ± standard deviation (SD). The PC patients were randomly assigned to the training or validation set. The healthy controls were enrolled as age- and sex-matched with the PC patients and were casually assigned to the training or validation set. All patients with pancreatitis were involved in the validation set. The variables were analyzed by Mann–Whitney U test. Receiver operating characteristic (**ROC**) analysis was performed to calculate the area under the **ROC** curve (**AUC**), specificity and sensitivity. For the validation set, the diagnostic model was assessed based on the different sample subset compared to the training set, and the specificity and sensitivity were calculated at the cut-off values obtained in the training set. In addition, we also evaluated the effect of age and gender of the participants on the levels of the FFAs using Mann-Whitney U test. Data, which are not normally distributed, were logarithmically transformed to obtain normal distribution before statistic analysis. Continuous variables were analyzed by one-way ANOVA with LSD test. In all cases, *p* values less than 0.05 (95%, confidence interval)

were considered to be statistically significant.

Method validation

The reliability of CBIDnanoESI(-)-FTICR MS for the FFAs analysis was validated through its linearity, limit of detection (LOD), stability, precision and spike-and-recovery.

Calibration curves

To generate calibration curves of each of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$ at mixture conditions, their standard mixture stock solution were diluted to five different concentrations (e.g. 2, 10, 20, 50 and 80-fold), respectively, resulting in six standard mixture solutions: the first mixture of 684.0 nM $C_{16:1}$, 163.3 nM $C_{18:3}$, 1137.7 nM $C_{18:2}$, 1671.7 nM $C_{18:1}$, 365.7 nM $C_{20:4}$ and 112.3 nM $C_{22:6}$); the second mixture of 342.0 nM $C_{16:1}$, 81.7 nM $C_{18:3}$, 568.8 nM $C_{18:2}$, 835.8 nM $C_{18:1}$, 182.8 nM $C_{20:4}$ and 56.2 nM $C_{22:6}$; the third mixture of 68.4 nM $C_{16:1}$, 16.3 nM $C_{18:3}$, 113.8 nM $C_{18:2}$, 167.2 nM $C_{18:1}$, 36.6 nM $C_{20:4}$ and 11.2 nM $C_{22:6}$; the fourth mixture of 34.2 nM $C_{16:1}$, 8.2 nM $C_{18:3}$, 56.9 nM $C_{18:2}$, 83.6 nM $C_{18:1}$, 18.3 nM $C_{20:4}$ and 5.6 nM $C_{22:6}$; the fifth mixture of 13.7 nM $C_{16:1}$, 3.3 nM $C_{18:3}$, 22.8 nM $C_{18:2}$, 33.4 nM $C_{18:1}$, 7.3 nM $C_{20:4}$ and 2.3 nM $C_{22:6}$); and the sixth mixture of 8.6 nM $C_{16:1}$, 2.0 nM $C_{18:3}$, 14.2 nM $C_{18:2}$, 20.9 nM $C_{18:1}$, 4.6 nM $C_{20:4}$ and 1.4 nM $C_{22:6}$. Then 1 μL of the IS solution was added into the above six standard mixture solutions with the final concentrations (83.3 nM for $C_{17:1}$ and 33.3 nM for $C_{21:0}$), respectively. Each of the resulting mixture solutions was analyzed three times, and the results were expressed as mean ± SD. $C_{17:1}$ is an IS for

quantifying the amounts of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, or $C_{18:1}$, and $C_{21:0}$ is an IS of $C_{20:4}$ or $C_{22:6}$. Multiple point internal standard calibration curves between the concentration rates of individual fatty acids to internal standards (ISs, $C_{17:1}$ or $C_{21:0}$) versus their corresponding intensity ratios were constructed for $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$, respectively. LOD is the lowest concentration of analytes having a ratio of signal to noise =3.

Stability

The third standard mixture solution mentioned above was used as a quality control (QC) sample. QC sample was analyzed once every 10 test samples. The relative standard deviation (**RSD**) were calculated based on the intensity ratios of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, or $C_{18:1}$ to $C_{17:1}$ and $C_{20:4}$ or $C_{22:6}$ to $C_{21:0}$, respectively, which were selected to investigate the experimental stability and reproducibility.

Precision

Three different serum samples from different individuals (control, pancreatitis and cancer) were used to test experimental precision. Each of three samples was analyzed four times on the same day for intraday precision and three times on the consecutive three days for interday precision.

Comparison of FAs spike-and-recovery in both serum and the corresponding supernatant matrixes

To assess the effect of different matrixes on the efficiency and recovery of FAs extraction, the spike-and-recovery experiment was performed based on serum matrix

(named as set one) and the corresponding supernatant matrix (named as set two). Briefly, a serum sample was prepared by mixing 10 healthy controls sera. For set one, 10 μL of the resulting serum mixture was spiked with 50 μL of the mixture of 171.0 μM $C_{16:1}$, 40.8 μM $C_{18:3}$, 284.4 μM $C_{18:2}$, 417.9 μM $C_{18:1}$, 91.4 μM $C_{20:4}$ and 28.1 μM $C_{22:6}$ or the mixture of 17.1 μM $C_{16:1}$, 4.1 μM $C_{18:3}$, 28.4 μM $C_{18:2}$, 41.8 μM $C_{18:1}$, 9.1 μM $C_{20:4}$ and 2.8 μM $C_{22:6}$, followed by the addition of 1 μL of the IS solution (83.3 mM for $C_{17:1}$ and 33.3 mM for $C_{21:0}$). For set two, 10 μL of the serum mixture was precipitated by the addition of 990 μL of methanol/acetonitrile (3/2, v/v). The resulting mixture was vortexed for 30 s and then stored at -20 °C overnight. After the mixture was centrifuged at 19000 g for 30 min at 4 °C, 20 μL of the supernatant was spiked with 1 μL of the mixture of 171.0 μM $C_{16:1}$, 40.8 μM $C_{18:3}$, 284.4 μM $C_{18:2}$, 417.9 μM $C_{18:1}$, 91.4 μM $C_{20:4}$ and 28.1 μM $C_{22:6}$ or the mixture of 17.1 μM $C_{16:1}$, 4.1 μM $C_{18:3}$, 28.4 μM $C_{18:2}$, 41.8 μM $C_{18:1}$, 9.1 μM $C_{20:4}$ and 2.8 μM $C_{22:6}$, followed by the addition of 1 μL of the IS solution (83.3 μM for $C_{17:1}$ and 33.3 μM for $C_{21:0}$). The FAs extraction for two sets was performed as described in the section of sample preparation. The percent recovery of the added FAs was calculated based on the following equation (1).

$$\%R = \frac{Concentration\ of\ FA\ in\ spiked\ sample - concentration\ of\ FA\ in\ unspiked\ sample}{Concentration\ of\ added\ FA} \times 100\% \quad (1)$$

Results and discussion

Method performance

As shown in **Table 2**, CBDInanoESI-FTICR MS in the negative ion mode can

simultaneously generate excellent multiple point internal standard calibration curves for $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$ using $C_{17:1}$ as IS for the former four FAs and $C_{21:0}$ as IS for the latter two FAs, with correlation coefficient of great than 0.99 and the LODs between 0.3 nM ($C_{16:1}$) and 1.8 nM ($C_{20:4}$), as well as with excellent linear dynamic range of three orders of magnitude for FTICR MS. Our results indicate that this platform can provide excellent LODs compared to MALDI-FTICR MS with the LODs of 0.2 μM for FAs [23]. The data from the QC experiments show that the reproducibility is less than 19%, and intraday and interday precision for three different samples is less than 18%, which are similar to previous studies.[28, 29] This platform can quantify the FFAs levels down to about 1 nM approximately 50-fold less compared to the amount of sample detected by liquid chromatography/MS[29-31] and gas chromatography/MS,[32] which are similar to previous studies.[33-35] Compared with liquid chromatography or gas chromatography coupled to MS, the obvious advantage of CBIDnanoESI-MS is simple, high sensitivity, high throughput, low sample-consuming (less than 1 μL) and no carrier over, as well as without the need of optimization of separation conditions for FFAs or metabolites in chromatography. Our results also indicate that the combination of nanoMate with FTICR MS can simultaneously perform qualitative and quantitative analysis for multiple targets, as well as with high resolution, high sensitivity, high throughput and high mass accuracy. This platform offers the possibility for increased throughput for studies, in as little as 30 seconds per sample, supporting a large sample size for biomarker screening and identification, as well as drug development.

Comparison of recovery of FAs in serum and the corresponding supernatant matrixes

Cancer blood samples play essential roles in screening potential diagnostic, prognostic and predictive biomarkers for early-stage cancer and understanding of cancer development. Due to personality differences, it is necessary to use the same serum sample to obtain as much information as possible about disease-related molecules so that we can personally understand molecular mechanisms and describe basic pathological processes. In this study, in order to use less volume of serum or the corresponding supernatant to obtain as much information as possible about disease-related metabolites, we compared the FAs recoveries in both serum and the corresponding supernatant matrixes. For the FAs spike-and-recovery experiments, two known concentrations of the mixture of FAs mixed with IS were added to the serum and the corresponding supernatant, respectively. As shown in **Table 2**, the values of the mean percent recovery for each of FAs ($C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$, and $C_{22:6}$) in both serum and the corresponding supernatant matrixes at two different concentration levels are similar. At the low concentration level (R1), the mean percent recoveries ranged from 69.8% to 115.6% for these six analytes in serum matrix (set one), with the SD ranged from 10.5% to 15.4%, and from 74.3% to 99.0% for these six analytes in supernatant matrix (set two), with the SD from 6.3% to 15.4%. At the high concentration level (R2), the mean percent recoveries ranged from 73.7% to 99.7% for these six analytes in serum matrix (set one), with the SD ranged from 8.8% to 13.7%, and from 75.1% to 94.2% for these six analytes in supernatant matrix (set two),

with the SD from 7.6% to 14.3%. The simulated blank experiment showed similar recoveries (ranged from 67.5% to 118.0% for the mixture of 22.8 μM $C_{16:1}$, 5.5 μM $C_{18:3}$, 37.9 μM $C_{18:2}$, 55.7 μM $C_{18:1}$, 12.2 μM $C_{20:4,}$ and 3.7 μM $C_{22:6}$ in bovine albumin at four different concentrations of the albumin (30, 40, 50, and 60 g/L)) to those in real serum and the supernatant. These results indicate that the supernatant can be replaced the corresponding serum to quantify the levels of serum FFAs and that the mixture of methanol/acetonitrile (3/2, v/v) as a serum protein precipitating solution can efficiently remove serum proteins.

Qualitative and quantitative determination of FFAs in human serum

Representative mass spectra of the FFAs from three types of the participants mentioned above are shown in **Fig. 1**. The FFAs detected in this study were identified based on their observed accurate molecular masses with a mass error of less than 0.00025 Da between the observed and theoretical mass and reliable isotope distributions with the RSD of less than 2% between the observed and theoretical intensity for the isotope distributions (**Supplementary Table S1**). The levels of six FFAs were calculated based on their respective calibration curves listed in **Table 2** and their corresponding serum concentration levels are shown in **Fig. 2**. Our data show that this platform can simultaneously perform qualitative and quantitive analysis for multiple targets, with high resolution and high mass accuracy.

Effect of gender and age of the participants on the levels of the FFAs

Comparison of the FFAs levels between females (n=84, 58.6 ± 11.9 years) and males

(n=96, 59.3 ± 10.2 years) for healthy controls using Mann-Whitney U test show no statistic significance (**p>0.07, Supplementary Table S2**), whereas the effect of age on the levels of the FFAs for healthy controls between four different age groups (group 1, 34 - 45 years (n = 35); group 2, 46 - 55 years (n = 40); groups 3, 56 - 65 years (n = 65) and group 4, 66 - 81 years (n = 65)) is observed (p<0.05, **Supplementary Table S3**). For the PC patients, statistic analysis indicate that no gender-specific difference in six FFAs levels between females (n=40, 60.4 ± 11.0 years) and males (n=45, 58.8 ± 10.5 years) was found (p> 0.07, **Supplementary Table S4**) and that the difference in the levels of the FFAs between four different age groups (group 1, 35-45 years (n = 17); group 2, 46-55 years (n = 19); groups 3, 56-65 years (n = 30) and group 4, 66-78 years (n = 29)) was also not observed (**p>0.07, Supplementary Table S5**). For the patients with pancreatitis, the effect of gender on the levels of six FFAs between female (n=29, age 55.5 ± 11.7) and males (n=32, age 55.8 ± 10.6) is same to that of the PC patients (p>0.3, **Supplementary Table S6**) and the impact of age on the FFAs levels between four different age groups (group 1, 35 - 45 years (n = 10); group 2, 46 - 55 years (n = 24); groups 3, 56 - 65 years (n = 10) and group 4, 66 – 79 years (n = 17)) show no statistic significance (p>0.05, **Supplementary Table S7**). Results indicate that the patients with PC or pancreatitis may have different FFAs metabolic mechanisms compared to healthy controls, who show different FFAs levels at the different ages, implying that FFAs metabolism may be closely associated with the development of PC and pancreatitis.

Association of changes in the levels of FFAs with physiological status

In the training set study, the changes in the levels of six FFAs between healthy controls and the PC patients were compared using the Mann-Whitney U test. Changes in the levels of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{20:4}$, and $C_{22:6}$, as well as in the ratios of $C_{18:2}/C_{18:1}$ and $C_{18:3}/C_{18:1}$, of the PC patients were significantly decreased compared to healthy controls ($p<0.001$, except $C_{20:4}$ with $P<0.01$, **Fig. 2**), which consist with those in tumor tissue of pancreatic cancer [36]. The ROC analyses of the above mentioned 7 variables, PUFA, panel a (a combination of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{20:4}$ and $C_{22:6}$) and panel b (a combination of $C_{18:2}/C_{18:1}$ and $C_{18:3}/C_{18:1}$) were performed. Their AUC, cut-off values, sensitivity and specificity are listed in **Table 3**. It should be noted that $C_{16:1}$, $C_{18:2}/C_{18:1}$, panel a and panel b could provide high diagnostic ability, with the AUC values of 0.907, 0.907, 0.933 and 0.908, respectively, along with the sensitivity of >82% and the specificity of >82, except $C_{18:2}/C_{18:1}$ with the low specificity of 75%. Representative ROC curve for panel a is shown in **Fig. 3a**. The diagnostic ability of these variables was further assessed in the following independent validation study with a large sample size, along with the benign disease (pancreatitis).

In the validation set, as shown in **Fig. 2**, the levels of $C_{16:1}$ or $C_{18:1}$ of normal controls were significantly increased or decreased compared to pancreatitis patients ($p<0.001$ for $C_{16:1}$; $p<0.05$ for $C_{18:1}$). The levels of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$ of the PC patients were remarkably decreased compared to healthy controls ($p<0.001$, except $C_{18:1}$ with $p<0.05$). It is worth noting that the levels of $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$, and $C_{22:6}$ of the PC patients also were remarkably decreased compared to the patients with pancreatitis ($p<0.001$), whereas no statistical significance for $C_{16:1}$

was found between the patients with PC and pancreatitis. The ratios of $C_{18:3}/C_{18:1}$ and $C_{18:2}/C_{18:1}$ levels also exhibited the obvious difference between the PC patients, the patients with pancreatitis and healthy controls ($p<0.01$). The diagnostic ability, sensitivity and specificity of the above variables based on the cut-off value obtained from the training study were calculated and listed in **Table 3.**

As shown **in Table 3,** the $C_{16:1}$, $C_{18:2}/C_{18:1}$, panel a and panel b were confirmed to have high diagnostic accuracy in the validation set, with the AUC of >0.84, the sensitivity of >80% and the specificity of >76%. Representative ROC curve of panel a is shown in **Fig. 3b**. It should be pointed out that $C_{16:1}$ or MUFA had high diagnostic accuracy to differentiate normal subjects from pancreatitis patients, with the AUC of >0.81, the sensitivity of >85% and the specificity of 62%. Representative ROC curve of MUFA is shown in **Fig. 3c**. It is interesting to note that PUFA or panel c (a combination of $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$) had high diagnostic ability to differentiate the patients with pancreatitis from with PC, with the AUC of ≥ 0.90, the sensitivity of >73% and the specificity of >90%. Representative ROC curve of panel c between pancreatitis patients and the PC patients is shown in **Fig. 3d**.

As shown in **Fig. 2**, the levels of six FFAs from 361 subjects were gradually decreased from healthy controls, patients with pancreatitis to the patients with PC in the validation study. This phenomenon is consistent with previous study[37], which pointed out that changes in serum FFA levels are associated with some pathophysiological mechanisms responsible for disturbed FA metabolism in PC. The levels of $C_{16:1}$ of pancreatitis were significantly decreased or no statistical significance

compared to healthy controls ($p<0.001$) or PC, whereas the levels of $C_{18:1}$ of pancreatitis were obviously increased compared to healthy controls and PC ($p<0.01$). The levels of $C_{18:2}$, $C_{18:3}$, $C_{20:4}$ or $C_{22:6}$ of pancreatitis patients were remarkably increased compared to PC ($p<0.001$), but no difference in their levels were observed between healthy controls and pancreatitis. These data strongly imply that different metabolic mechanisms of unsaturated FA between healthy controls and pancreatitis and between pancreatitis and PC might exist.

Association of changes in the levels of FFAs with PC stages

As shown in **Fig. 2**, the levels of $C_{18:3}$, $C_{18:2}$, $C_{20:4}$ and $C_{22:6}$, as well as the ratio of $C_{18:3}/C_{18:1}$ of the early stage or advanced stage of PC patients were significantly decreased compared to healthy controls plus pancreatitis patients (named as non-cancer participants) ($p<0.001$). The ROC analysis indicated that each of $C_{20:4}$, $C_{18:2}/C_{18:1}$, PUFA, panel a and panel b exhibited excellent diagnostic ability to differentiate early stage of PC from non-cancer participants, with the AUC of >0.86, the sensitivity of >85% and the specificity of ≥80% (**Table 4**). Representative ROC curve of panel b with the AUC of 0.912, the sensitivity of 86.7% and the specificity of 88.6% is shown in **Fig. 3e**.

Compared to the advanced stage of PC with non-cancer participants, it is found that $C_{20:4}$, $C_{22:6}$, PUFA or panel d (a combination of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$) had excellent diagnostic performance to differentiate advanced stage PC patients from non-cancer subjects, with the AUC of >0.94, the sensitivity of > 88% and the specificity of >70% (**Table 4**). Representative ROC curve of panel d with the

AUC of 0.989, the sensitivity of 91.7% and the specificity of 98.6% is shown in **Fig. 3f**.

Results in **Table 4** reveal that almost variables, especially for panel a and b, have excellent diagnostic ability to differentiate early-stage PC from non-cancer participants, with the AUC of > 0.87, the sensitivity of > 86% and the specificity of >88%, which is better than biomarker CA 19-9, with the sensitivity of 79 % and the specificity of 82%[38, 39]. Our data indicate that metabolite-based serum tumor marker panels can provide an important improvement in the diagnostic ability to distinguish between pancreatic cancer and pancreatitis, which is a high suspicion of malignancy. As shown in **Fig. 2**, no statistical difference in the levels of six FFAs between early-stage and advanced-stage PC may further confirm that metabolic mechanism of FA of PC is different from that of pancreatitis. Our data suggest that a better understanding of FFAs dyregulation in pancreatic cancer may lead to early-stage diagnosis of pancreatic cancer.

Conclusions

Although the FA metabolism is very important for disease detection, straightforward quantitative methods for rapid quantitative determination of FFAs are still missing. In this study, the spike-and-recovery experimental results show that the FFAs extraction efficacy and recovery between serum and the corresponding supernatant matrixes are almost identical, indicating that the levels of FFAs in the supernatant can represent their amounts in serum. The quantitative data on the levels of the FFAs of 361 participants including healthy controls, patients with pancreatitis and PC obtained

using CBDInanoESI-FTICR MS suggest that this platform has ability to provide high sensitivity and high throughput analysis of the FFAs levels, with high resolution and high mass accuracy. The ratio of $C_{18:2}/C_{18:1}$, PUFA, biomarker panel a or panel b has excellent diagnostic accuracy for differentiating early-stage PC from non-cancer participants. Our results strongly indicate that the serum FFAs profile has great clinical potential in early diagnosis of PC and its progression monitoring, and also reveal that the different FFA metabolism mechanisms between PC and pancreatitis may exist.

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Reference

1 R. Siegel, D. Naishadham, A. Jemal *CA Cancer J Clin* 2013, ***63***, 11-30.

2 J. Klapman, M. P. Malafa *Cancer Control* 2008, ***15***, 280-287.

3 G. M. Israel, N. Hindman, M. A. Bosniak *Radiology* 2004, ***231***, 365-371.

4 J. H. Lee, J. K. Kim, T. H. Kim, M. S. Park, J. S. Yu, J. Y. Choi, J. H. Kim, Y. B.

Kim, K. W. Kim *Br J Radiol* 2012, ***85***, 571-576.

5 M. Goggins *Clinical Cancer Research* 2011, ***17***, 635-637.

6 K. Satake, T. Takeuchi *Pancreas* 1994, ***9***, 720-724.

7 C. Ren, H. Chen, C. Han, G. Jin, D. Wang, D. Tang *Med Hypotheses* 2013, ***80***, 833-836.

8 Q. Huang, Y. Tan, P. Yin, G. Ye, P. Gao, X. Lu, H. Wang, G. Xu *Cancer Res* 2013, ***73***, 4992-5002.

9 W. Lv, T. Yang *Clin Biochem* 2012, ***45***, 127-133.

10 D. K. Nomura, J. Z. Long, S. Niessen, H. S. Hoover, S. W. Ng, B. F. Cravatt *Cell* 2010, ***140***, 49-61.

11 S. Serini, E. Piccioni, N. Merendino, G. Calviello *Apoptosis* 2009, ***14***, 135-152.

12 P. C. Calder *Biochimie* 2009, ***91***, 791-795.

13 Y. Guo, X. Wang, L. Qiu, X. Qin, H. Liu, Y. Wang, F. Li, G. Chen, G. Song, S. Guo, Z. Li *Clin Chim Acta* 2012, ***414***, 135-141.

14 W. R. Wikoff, J. A. Gangoiti, B. A. Barshop, G. Siuzdak *Clin Chem* 2007, ***53***, 2169-2176.

15 Y. Qiu, G. Cai, M. Su, T. Chen, X. Zheng, Y. Xu, Y. Ni, A. Zhao, L. X. Xu, S. Cai, W. Jia *J Proteome Res* 2009, ***8***, 4844-4850.

16 W. Dai, Q. Huang, P. Yin, J. Li, J. Zhou, H. Kong, C. Zhao, X. Lu, G. Xu *Anal Chem* 2012, ***84***, 10245-10251.

17 J. Y. Kim, J. Y. Park, O. Y. Kim, B. M. Ham, H. J. Kim, D. Y. Kwon, Y. Jang, J. H. Lee *J Proteome Res* 2010, ***9***, 4368-4375.

18 T. Kobayashi, S. Nishiumi, A. Ikeda, T. Yoshie, A. Sakai, A. Matsubara, Y. Izumi, H. Tsumura, M. Tsuda, H. Nishisaki, N. Hayashi, S. Kawano, Y. Fujiwara, H. Minami, T. Takenawa, T. Azuma, M. Yoshida *Cancer Epidemiol Biomarkers Prev* 2013, ***22***, 571-579.

19 R. Ramautar, G. W. Somsen, G. J. de Jong *Electrophoresis* 2013, ***34***, 86-98.

20 B. Tan, Y. Qiu, X. Zou, T. Chen, G. Xie, Y. Cheng, T. Dong, L. Zhao, B. Feng, X. Hu, L. X. Xu, A. Zhao, M. Zhang, G. Cai, S. Cai, Z. Zhou, M. Zheng, Y. Zhang, W. Jia *J Proteome Res* 2013, ***12***, 3000-3009.

21 C. M. Rocha, J. Carrola, A. S. Barros, A. M. Gil, B. J. Goodfellow, I. M. Carreira, J. Bernardo, A. Gomes, V. Sousa, L. Carvalho, I. F. Duarte *J Proteome Res* 2011, ***10***, 4314-4324.

22 F. Li, X. Qin, H. Chen, L. Qiu, Y. Guo, H. Liu, G. Chen, G. Song, X. Wang, S. Guo, B. Wang, Z. Li *Rapid Commun Mass Spectrom* 2013, ***27***, 24-34.

23 Y. Zhang, Y. Wang, S. Guo, Y. Guo, H. Liu, Z. Li *Anal Chim Acta* 2013, ***794***, 82-89.

24 T. G. Holt, B. K. Choi, N. S. Geoghagen, K. K. Jensen, Q. Luo, W. A. LaMarr, G. M. Makara, L. Malkowitz, C. C. Ozbal, Y. Xiong, C. Dufresne, M. J. Luo *Assay Drug Dev Technol* 2009, ***7***, 495-506.

25 M. K. Highkin, M. P. Yates, O. V. Nemirovskiy, W. A. Lamarr, G. E. Munie, J. W. Rains, J. L. Masferrer, M. M. Nagiec *J Biomol Screen* 2011, ***16***, 272-277.

26 X. Wu, J. Wang, L. Tan, J. Bui, E. Gjerstad, K. McMillan, W. Zhang *J Biomol Screen* 2012, ***17***, 761-772.

27 M. Leveridge, R. Buxton, A. Argyrou, P. Francis, B. Leavens, A. West, M. Rees, P. Hardwicke, A. Bridges, S. Ratcliffe, C. W. Chung *J Biomol Screen* 2013.

28 W. B. Dunn, D. Broadhurst, P. Begley, E. Zelena, S. Francis-McIntyre, N. Anderson, M. Brown, J. D. Knowles, A. Halsall, J. N. Haselden, A. W. Nicholls, I. D. Wilson, D. B. Kell, R. Goodacre *Nat Protoc* 2011, ***6***, 1060-1083.

29 C. Hellmuth, M. Weber, B. Koletzko, W. Peissner *Anal Chem* 2012, ***84***, 1483-1490.

30 X. M. Persson, A. U. Blachnio-Zabielska, M. D. Jensen *J Lipid Res* 2010, ***51***, 2761-2765.

31 J. J. Kamphorst, J. Fan, W. Lu, E. White, J. D. Rabinowitz *Anal Chem* 2011, ***83***, 9114-9122.

32 L. Liu, Y. Li, C. Guan, K. Li, C. Wang, R. Feng, C. Sun *J Chromatogr B Analyt Technol Biomed Life Sci* 2010, ***878***, 2817-2825.

33 E. R. Wickremsinhe, B. L. Ackermann, A. K. Chaudhary *Rapid Commun Mass Spectrom* 2005, ***19***, 47-56.

34 L. A. Leuthold, C. Grivet, M. Allen, M. Baumert, G. Hopfgartner *Rapid Commun*

Mass Spectrom 2004, ***18***, 1995-2000.

35 J. M. Dethy, B. L. Ackermann, C. Delatour, J. D. Henion, G. A. Schultz *Anal Chem* 2003, ***75***, 805-811.

36 G. Zhang, P. He, H. Tan, A. Budhu, J. Gaedcke, B. M. Ghadimi, T. Ried, H. G. Yfantis, D. H. Lee, A. Maitra, N. Hanna, H. R. Alexander, S. P. Hussain *Clin Cancer Res* 2013.

37 J. Macasek, M. Vecka, A. Zak, M. Urbanek, T. Krechler, L. Petruzelka, B. Stankova, M. Zeman *Nutr Cancer* 2012, ***64***, 946-955.

38 K. S. Goonetilleke, A. K. Siriwardena *Eur J Surg Oncol* 2007, ***33***, 266-270.

39 V. Molina, L. Visa, C. Conill, S. Navarro, J. M. Escudero, J. M. Auge, X. Filella, M. A. Lopez-Boado, J. Ferrer, L. Fernandez-Cruz, R. Molina *Tumour Biol* 2012, ***33***, 799-807.

Table 1. Characteristics of the participants for the training and validation study

	Training Set		Validation Set		
	Controls (n=60)	**PC (n=28)**	**Controls (n=145)**	**Pancreatitis (n=61)**	**PC (n=67)**
Male/Female	26/34	11/17	87/58	32/29	38/29
Age (years)					
Mean ± SD	60.5±15.2	60.1±17.0	56.7±13.0	55.6±14.2	55.9±13.0
Range	45-79	47-78	34-81	35-79	35-76
Stage (male/female)					
Ⅰ & Ⅱ(Early Stage)		1/3			7/4
Ⅲ & Ⅳ(Advanced Stage)		2/5			13/4

SD: standard deviation; PC: pancreatic cancer.

Table 2. The calibration equation, linearity, limits of detection, stability, precision and recovery of FAs

FAs	Linearity (n=3)			LOD	QC	Intraday precision (%)			Interday precision (%)			Recovery (%, n=3)	
	FA (nM)	Equation	R^2	(nM)	(%)	S1	S2	S3	S1	S2	S3	R1(set1/set2)	R2(set1/set2)
$C_{16:1}$	8.5-684.0	Y=0.495(±0.013)X+0.096(±0.013)	0.993	0.3	<19	<7	<2	<9	<7	<6	<10	93.4/91.9	94.7/88.0
$C_{18:3}$	2.0-163.5	Y=0.740(±0.005)X-0.024(±0.001)	0.998	0.8	<17	<6	<7	<11	<18	<13	<16	108.9/82.3	92.3/94.2
$C_{18:2}$	14.2-1138.0	Y=0.849(±0.008)X-0.120(±0.011)	0.999	1.1	<17	<5	<9	<7	<11	<5	<4	115.6/99.0	99.7/89.7
$C_{18:1}$	20.9-1672.0	Y=0.850(±0.012)X+0.329(±0.046)	0.998	0.8	<13	<9	<12	<7	<10	<7	<10	107.0/83.3	96.9/92.4
$C_{20:4}$	4.6-366.0	Y=1.689(±0.097)X+0.550(±0.049)	0.991	1.8	<19	<16	<18	<8	<18	<13	<12	69.8/74.8	74.6/79.4
$C_{22:6}$	1.4-112.3	Y=1.292(±0.068)X+0.033(±0.046)	0.995	1.4	<8	<13	<9	<13	<14	<15	<16	73.6/74.3	73.7/75.1

X: Concentration ratio of individual FAs to IS (the concentrations of ISs are 83.3 nM $C_{17:1}$ and 33.3 nM $C_{21:0}$); Y: respective corresponding intensity ratio of FAs to IS

S1: control sample; S2: pancreatitis sample; S3: cancer sample; R1: FAs mixture of 17.1 nM $C_{16:1}$, 4.1 nM $C_{18:3}$, 28.4 nM $C_{18:2}$, 41.8 nM $C_{18:1}$, 9.1 nM $C_{20:4}$ and 2.8 nM $C_{22:6}$; R2: FA mixture of 171.0 nM $C_{16:1}$, 40.8 nM $C_{18:3}$, 284.4 nM $C_{18:2}$, 417.9 nM $C_{18:1}$, 91.4 nM $C_{20:4}$ and 28.1 nM $C_{22:6}$.

Table 3. The AUC, cut-off values, sensitivity and specificity of the significantly expressed FFAs between controls and patients

FFAs	Training set				Validation set											
	Controls vs. PC				Controls vs. PC			Controls vs. Pancreatitis				Pancreatitis vs. PC				
	AUC (95% CI)	Sens (%)	Spec (%)	cut-off (μM)	AUC (95% CI)	Sens (%)	Spec (%)	AUC (95% CI)	Sens (%)	Spec (%)	cut-off (μM)	AUC (95% CI)	Sens (%)	Spec (%)	cut-off	
$C_{16:1}$	0.907(.840-.974)	86.7	82.1	29.9	0.843(.780-.906)	80.7	76.1	0.814(.746-.882)	88.3	62.3	26.1					
$C_{18:3}$	0.795(.692-.901)	88.3	67.9	13.6	0.885(.829-.940)	95.9	67.2					0.853(.784-.922)	85.2	77.6	15.8	
$C_{18:2}$	0.782(.661-.902)	83.3	71.4	219.5	0.835(.772-.898)	68.3	79.1					0.795(.718-.871)	83.6	65.7	177.4	
$C_{18:1}$								0.632(.547-.717)	82.0	44.1	259.3	0.701(.611-.791)	55.7	79.1	376.1	
$C_{20:4}$	0.717(.590-.845)	88.3	50.0	72.8	0.842(.778-.907)	86.2	68.7					0.815(.741-.889)	91.8	62.7	52.8	
$C_{22:6}$	0.790(.685-.895)	65.0	82.1	25.9	0.873(.818-.929)	68.3	86.6					0.897(.840-.953)	95.1	71.6	18.8	
$C_{18:2}/C_{18:1}$	0.907(.827-.987)	96.7	75.0	0.7	0.860(.800-.921)	86.2	79.1	0.764(.692-.835)	71.7	72.1	0.8	0.697(.605-.788)	73.8	59.7	0.6	
$C_{18:3}/C_{18:1}$	0.788(.679-.896)	86.7	60.7	0.1	0.738(.656-.821)	81.4	62.7	0.629(.546-.712)	43.4	80.3	0.1	0.653(.557-.750)	72.1	62.7	0.08	
MUFA								0.864(.801-.926)	85.2	80.0	0.3					
PUFA	0.806(.709-.902)	96.4	55.0	0.2	0.911(.861-.961)	85.1	73.1					0.900(.845-.955)	73.1	96.7	0.7	
Panel a	0.933(.879-.986)	89.3	85.0	0.3	0.935(.896-.973)	86.6	85.5									
Panel b	0.908(.829-.988)	82.1	88.3	0.3	0.880(.832-.928)	83.6	81.4	0.779(.709-.848)	68.9	78.6	0.3	0.680(.587-.773)	43.3	90.2	0.5	
Panel c												0.907(.855-.959)	77.6	90.2	0.7	

Note: Sens: Sensitivity; Spec: Specificity; MUFA: $C_{16:1}$ and $C_{18:1}$; PUFA: $C_{18:2}$, $C_{18:3}$, $C_{20:4}$ and $C_{22:6}$; Panel a: $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{20:4}$ and $C_{22:6}$; Panel b: $C_{18:2}/C_{18:1}$ and $C_{18:3}/C_{18:1}$; Panel c: $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$.

Table 4. AUC, cut-off values, sensitivity and specificity of the significantly expressed FFAs between non-cancer-participants and different stage patients

FFAs	Controls + Pancreatitis vs. Early stage				Controls + Pancreatitis vs. Advanced stage		
	AUC (95% CI)	Sens (%)	Spec (%)	Cut-off	AUC (95% CI)	Sens (%)	Spec (%)
$C_{16:1}$	0.681(.538-.824)	62.9	66.7	37.5	0.820(.724-.917)	62.9	80.2
$C_{18:3}$	0.782(.606-.958)	88.6	73.3	14.1	0.892(.804-.980)	88.6	79.2
$C_{18:2}$	0.843(.689-.997)	95.7	73.3	158.9	0.855(.755-.956)	95.7	62.5
$C_{18:1}$				239.4	0.646(.510-.783)	74.3	58.3
$C_{20:4}$	0.877(.739-1.016)	97.1	80.0	52.1	0.958(.922-.993)	95.7	70.8
$C_{22:6}$	0.865(.733-.996)	90.0	73.3	18.5	0.947(.900-.994)	88.6	91.7
$C_{18:2}/C_{18:1}$	0.915(.839-.991)	85.7	86.7	0.7	0.851(.751-.951)	85.7	75.0
$C_{18:3}/C_{18:1}$	0.720(.573-.867)	52.9	86.7	0.1	0.702(.569-.835)	84.3	54.2
MUFA				0.4	0.825(.730-.920)	66.7	84.3
PUFA	0.869(.716-1.022)	86.7	90.0	0.2	0.974(.943-1.006)	91.7	90.0
Panel a	0.879(.744-1.014)	86.7	90.0	0.3			
Panel b	0.912(.834-.991)	86.7	88.6	0.3	0.867(.784-.951)	79.2	82.9
Panel d				0.7	0.989(.973-1.004)	91.7	98.6

Note: MUFA: $C_{16:1}$ and $C_{18:1}$; PUFA: $C_{18:2}$, $C_{18:3}$, $C_{20:4}$ and $C_{22:6}$; Panel a: $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{20:4}$ and $C_{22:6}$; Panel b: $C_{18:2}/C_{18:1}$ and $C_{18:3}/C_{18:1}$; Panel d: $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$.

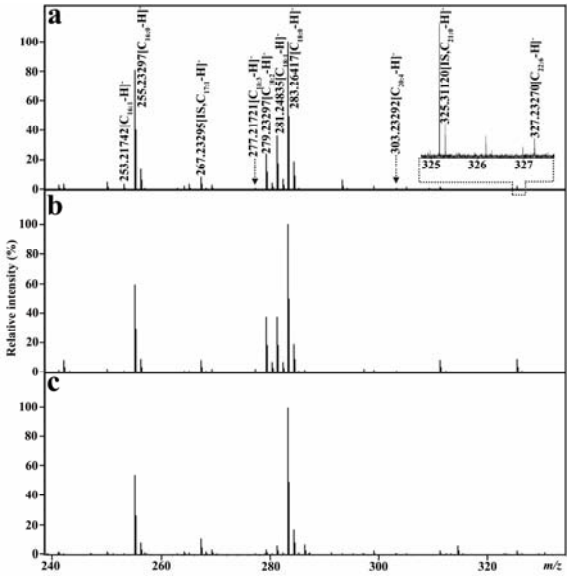

Fig. 1. Representative mass spectra of serum FFAs from the healthy controls (a), patients with pancreatitis (b), and patients with pancreatic cancer (c) by chip-based direct-infusion nanoESI-FTICR MS under the negative ion mode.

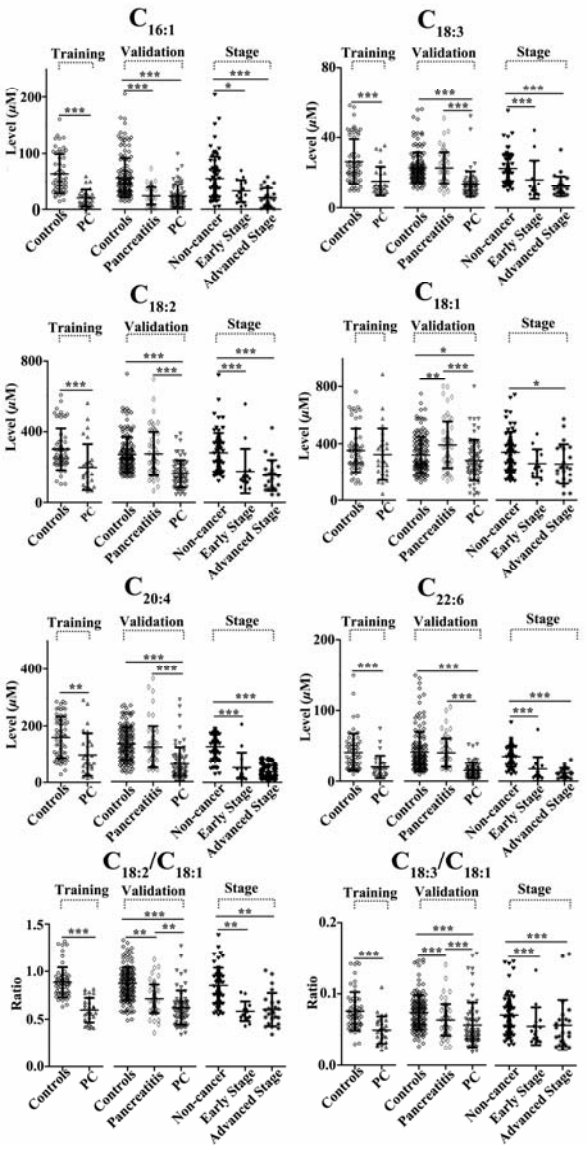

Fig. 2. Scatter plots of the levels of serum FFAs ($C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$, and $C_{22:6}$) and the level ratios of $C_{18:2}/C_{18:1}$ and $C_{18:2}/C_{18:1}$ in the training set, validation set and PC stages.

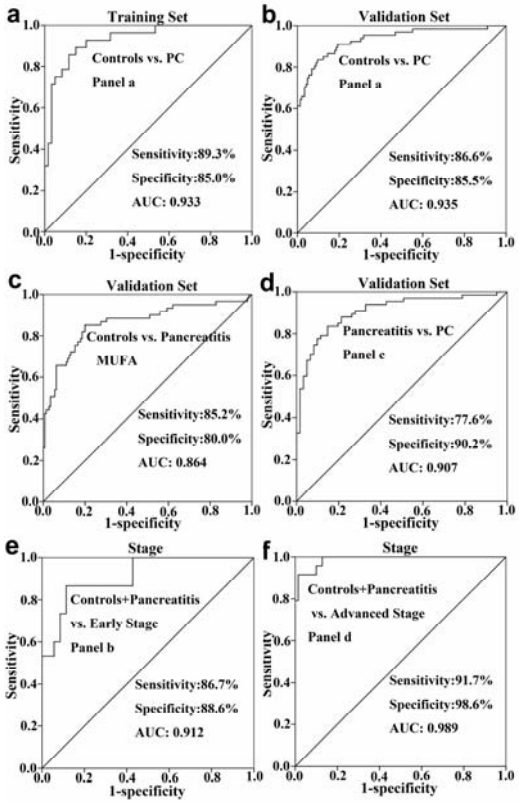

Fig. 3. Representative ROC curves analysis of serum FFAs. (a) panel a (a combination of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{20:4}$ and $C_{22:6}$) between controls vs. PC in the training set; (b) panel a between controls vs. PC in the validation set; (c) MUFA (a combination of $C_{16:1}$ and $C_{18:1}$) between controls vs. pancreatitis in the validation set; (d) panel c (a combination of $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$) between pancreatitis and PC in the validation set; (e) Panel b (a combination of $C_{18:2}/C_{18:1}$ and $C_{18:3}/C_{18:1}$) between controls plus pancreatitis and early-stage PC; and (f) Panel d (a combination of $C_{16:1}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, $C_{20:4}$ and $C_{22:6}$) between controls plus pancreatitis and advanced stage PC.