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GC-MS based metabolomics strategy to distinguish three types of acute pancreatitis

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ABSTRACT

Acute pancreatitis (AP) is a progressive systemic inflammatory response with high morbidity and high mortality, which is mainly caused by alcohol, bulimia, gallstones and hyperlipidemia. The early diagnosis of different types of AP and further explore potential pathophysiological mechanism of each type of AP is beneficial for optimized treatment strategies and better patient's care. In this study, a metabolomics approach based on gas chromatography-mass spectrometry (GC-MS), and random forests algorithm was established to distinguish biliary acute pancreatitis (BAP), Hyperlipidemia acute pancreatitis (HLAP), and alcoholic acute pancreatitis (AAP), from healthy controls. The classification accuracies for BAP, HLAP, and AAP patients compared with healthy control, were 0.886, 0.906 and 0.857, respectively, by using 5-fold cross-validation method. And some special metabolites for each type of AP were discovered, such as L-Lactic acid, (R)-3-Hydroxybutyric acid, Phosphoric acid, Glycine, Erythronic acid, L-Phenylalanine, D-Galactose, L-Tyrosine, Arachidonic acid, Glycerol 1-hexadecanoate. Furthermore, associations between these metabolites with the metabolism of amino acids, fatty acids were identified. Our studies have illuminated the biomarkers and physiological mechanism of disease in a clinical setting, which suggested that metabolomics is a valuable tool for identifying the molecular mechanisms that are involved in the etiology of BAP, AAP, HLAP and thus novel therapeutic targets.

Introduction

Acute pancreatitis (AP) is a progressive systemic inflammatory response with high morbidity and high mortality, which is mainly caused by alcohol, bulimia, gallstones and hyperlipidemia [1,2]. Clinical researches have showed that biliary acute pancreatitis(BAP), alcoholic acute pancreatitis (AAP) and hyperlipidemia acute pancreatitis (HLAP) account for 43.83%, 17.00%, 6.17% of total incidence respectively [3]. Despite the great advances in critical care medicine over the past 20 years, there are still about 20% AP cases developing into critical illness, characterized by peripancreatic tissues and other distant organs dysfunction and morphologic changes, and then develop into serious secondary local and systemic complications, such as infected pancreatic necrosis (IPN), acute respiratory distress syndrome (ARDS), acute kidney injury (AKI) and sepsis; the mortality rate in this severe form is up to 50% [4–6]. Thus, the early diagnosis of AP and prevention are the most effectively approach in treatment of AP.

Clinically, different types of AP are often treated with different methods. Cholelithiasis is the main cause of AP in China [7]. People with obstructive biliary calculi usually need timely relief of obstruction, including Transabdominal ultrasound, Endoscopic ultrasound (EUS), Magnetic resonance cholangiopancreatography (MRCP), Computerized tomography (CT) or surgical treatment [7–9]. HLAP patients need to reduce triglyceride levels to less than 5.65 mmol/L in a short period of time, limiting the use of lipid emulsions and drugs that may increase blood lipid during HLAP treatment [1,10]. Unfortunately, these methods are generally not suitable for routine laboratory testing in all hospitals, leading to

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missed optimal treatment periods, especially in township hospitals in remote areas. Brief Interventions are often employed to control the progression of AAP patients [11]. Although most AAP patients (96%) are willing to stop or moderate their drinking, it is frustrating to achieve results [12]. Therefore, finding an accurate, rapid, and comprehensive early diagnosis method to distinguish different pathogenesis of AP is beneficial for optimized treatment strategies and better patient care.

Metabolomics is a systematic method for the qualitative and quantitative analysis of all low-molecular-weight metabolites in a certain biological or cell-specific physiological period [13]. Through a set of specific and sensitive biomarkers provided by metabolomics, one can find out the potential relationships between metabolites and physiological and pathological changes to distinguish different biological system states [14–17]. Metabolomics has been applied in human AP biomarker discovery through nuclear magnetic resonance (NMR) spectroscopy or gas-chromatography/mass spectrometry (GC-MS). Sakai et al. first investigated the levels of low-molecular-weight metabolites were altered by the pathogenesis of acute pancreatitis (AP). 93 and 129 metabolites were detected in the serum and pancreatic tissue in the cerulein-induced pancreatitis model, respectively. In the L-arginine-induced AP model, 120 and 133 metabolites were detected in the serum and pancreatic tissue, respectively. They also discovered that the severity of cerulein-induced acute pancreatitis will attenuated by supplementation with glutamic acid and O-phosphoethanolamine [18]. Lusczek et al., proposed a metabolomics approach for comparing urinary metabolic profiles of patients with acute and chronic pancreatitis to healthy controls by proton nuclear magnetic resonance spectra and Principal component analysis (PCA). It proposed that significant increase in urinary adenosine and decrease in urinary citrate may have reflected the patients' inflammatory state and alcohol consumption [19].Tang et al., investigated serum metabolome of lipoprotein lipase (LPL)-deficient heterozygous mice injected with caerulein using GC/MS and orthogonal-projection. Their results suggested that citrate, malic acid and glutamine were significantly decreased in the hyperlipidemia acute pancreatitis (HLAP) subjects than in the healthy control animals [20]. These studies provided a wealth of relevant metabolic material basis for the study of AP, and suggested that GC-MS combined with chemometrics is an effective method to study the development of acute pancreatic metabolic system, provided a good predictive tool for disease diagnosis.

In our previous research, a GC-MS based serum metabolomics method was established to successfully identify potential diagnostic biomarkers for diagnosing different severe degree of AP patients [19]. Thus, the aim of the current study was to establish a serum metabolomics approach to distinguish BAP, AAP and HLAP patients, and random forest algorithm was further used to select some potential diagnostic biomarkers; furthermore, based on these selected important biomarkers, relevant metabolic pathways were constructed for exploring the potential pathogenesis of different types of AP by biological pathway analysis.

Materials and methods 2.1 equipment, chemicals and reagents

Samples analysis was performed on GCMS-QP2010 (Shimadzu Co., Tokyo, Shimadzu). Chromatographic grade chemicals including Pyridine, Methoxyamine hydrochloride, N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), and heptadecanoic acid (internal standard) were purchased from (Sigm-aldrich Co., MO, USA).

Samples collection

All protocols involving the use of participants were reviewed and approved by the ethics committee of People's hospital of Hunan province (Changsha, Hunan, China), and all experiments were performed in accordance with relevant guidelines and regulations, Written informed consent was obtained from all patients (or their next-of-kin) enrolled in this study.

All participants presented at the department of gastroenterology, People's hospital of Hunan province, Changsha, China, from January 30, 2017 to December 24, 2018. All the AP participants took blood on the second day of admission to the hospital before the treatment plan implemented. Volunteers were fasted for 12 h before blood collection to rule out the effects of cigarettes and excessive physical activity. Serum were collected before breakfast in the morning placed in 1.5 mL EP tubes without anticoagulant and stored at −80 °C refrigerator until GC-MS analysis.

The diagnosis of AP needs to meet two of the following characteristics: (1) Abdominal pain consistent with AP, such as intense and persistent upper abdominal pain. (2) Serum amylase and lipase levels at least three times higher than the upper limit of normal. (3) Abdominal imaging examination meets AP imaging changes [21,22]. AP patients were further identified as BAP with biliary system disease and blocks in bile ducts, AAP patients who were reported obviously alcohol consumption (without other etiologies), HLAP patients' only with hyperlipidemia. The patients with possible etiologies (rheumatic disease, neoplastic disease, chronic kidney or liver disease) and ambiguous etiologies were excluded. Healthy control with match ages was selected. Final, 15 health controls (control group), 29 hyperlipidemia acute pancreatitis patient (HLAP group), 20 alcoholic acute pancreatitis (AAP) patient, and 27 biliary acute pancreatitis patient (BAP), diagnosed by physicians of The People's hospital of Hunan Province, were involved in this study (Table S1).

Serum collection and preparation

The following methods will be employed to prepare serum samples: 100 following methods will be employed to prepare °C refrigerator and thawed at 4 °C for 30 min 300 aL of methanol solution (removing the protein in the sample) and 25 μL of heptadecanoic acid (internal standard, 2.1 mg/mL) were added to the sample. After vortex mixing for 15 s, centrifugation (15800 r/min, 4 °C) for 10 min, the supernatant was placed in a derivatization reaction glass tube and dried under high purity nitrogen. The evaporated residue was dispersed in 50 μL of methoxyamine pyridine solution (22.86 mg/mL), vortexed for 15 s, and subjected to an Oximation reaction for 1 h in a 70 °C water bath to protect the carbonyl. In order to increase the volatility of the metabolite, 100 μL of derivatization reagent (BSTFA + 1% TMCS) was added to the sample, vortexed for 15 s, and subjected to a silylation reaction in a 70 °C water bath for 1 h, and vortexed and mixed. It was then centrifuged at 15800 r/min (4 °C) for 10 min. Finally, 1 μL of the supernatant was taken for GC-MS analysis.

100 μL of serum was selected from each of 91 samples, which were mixed together as quality control (QC) samples. Since the QC sample contains most of the data for each group, it was used to verify the stability of the GC-MS system. Before daily analysis, QC samples were analyzed for three times, subsequently injected two internal standard samples, to equilibrate the GC system and validate the stability of equipment in different analysis dates. Then one QC sample was injected interval every five random samples. These QC results were used to monitor the stability of method. All samples above were maintained at 4 °C during analysis.

GC-MS analysis

A DB-5 MS capillary column (30 m × 0.25 mm × 0.25 μm,

Agilent, USA) was used for non-targeted metabolites' study. The temperature program was optimized as follows: initial temperature was held at 70 °C for 4 min, programmed to 110 °C with a rate of 20 °C/min, and rise to 270 °C with a rate of 8 °C/min, then held for 7 min. The total program time was 33 min with 5.0 min solvent cut time. Helium (99.99%) was used as the carrier gas with a flow rate of 1.3 mL/min. The injection volume was 1 μL with split ratio of 30:1. The temperatures of injector, ion source and interface were 280 °C, 200 °C and 275 °C, respectively. The mass spectrometer was operated under electron impact (EI) mode at 70 eV, and a 0.94 kV detector voltage in full-scan mode with 0.2 s/scan (m/z 35–750).

Identification and quantitative analysis

The raw data acquired by the analytical instruments were processed for peak detection and alignment. Based on total ion chromatogram (TIC), MS database NIST 08 (National Institute of Standards and Technology) was used for identifying the structures of common peaks. Full scan mass spectra of these metabolites were further searched and analyzed using biochemical databases including the Human Metabolome Database (HMDB) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The peak areas of metabolites were normalized against the internal standard to semi-quantitatively determine the levels of the metabolites. And a T test was used to compare the differences between BAP, AAP and HLAP with healthy group, it was considered statistically significant when p value was less than 0.05.

Multivariate statistical analysis

Among many machine learning methods, RF has been widely accepted due to some advantages of this trees ensemble technique in dealing with such complex metabolomics data: it not only distinguish different groups (patients/disease group and healthy group), but also can help in finding the significant changed metabolites as potential biomarkers.

RF combines many classification and regression trees to form more accurate classifications. It randomly selects a subset of features to split at each node when growing a tree. Increasing the diversities of trees is an efficient way to increase the classifying and recognition ability of RF method. The detail RF modeling process can be found in Breiman's study [23]. In addition, RF can deal with highly dimensional and correlated datasets without an initial reduction of dimensionality of the data set. Apart from OOB estimation, RF provide two useful tools, the variable importance measure and proximity matrix, which is helpful in data interpretation and visualization.

(i). Proximity Measure

The proximity matrix can be used to identify structure in the data. RF also calculates the proximities between samples. Proximities values can indicate the similarities among all the samples. The proximity between two samples is calculated as the number of times that the two samples end up in the same terminal node of a tree, divided by the number of trees in the forest. After the proximity values are calculated/multidimensional scaling (MDS) plot, whose aim is to visualize the similarity or dissimilarity (calculated as 1-proximity) between samples can be constructed.

(ii) Variable Importance Calculation

RF inherits the ability to estimate feature importance. The variable importance measure can be used to estimate the importance of each metabolite in the model classification. This information can help us to find the potential biomarkers or as a filter to remove non-informative variable. The frequently used type of RF algorithm to measure feature importance is the mean decrease in classification based on permutation. The prediction accuracy of after permutation is subtracted from the prediction accuracy before permutation and averaged of all trees in the forest to give the permutation importance value. In current research, the mean decrease in classification accuracy was accepted to measure variable importance. The importance of each variable calculated as Eq (1)

$$\text{Importance of } j = \text{Accuracy } j_{\text{ normal}} - \text{Accuracy } j_{\text{ permuted}} \quad (1)$$

Pathway analysis

As described in previous studies, MetaboAnalyst 3.0 was used for pathway analysis based on metabolites information [24]. This web analysis tool conducts pathway analysis through pathway enrichment analysis and pathway topological analysis. In this study, “Homo sapiens library” and default ‘Hypergeometric Test’ and ‘Relative-Betweenness Centrality’ algorithms for pathway enrichment analysis and pathway topological analysis, respectively. To identify the most relevant pathways, the impact-value threshold calculated from pathway topology analysis was set to 0.1.

Results

Metabolic profiles of AP patients with different etiologies

Serum metabolites profiles of three different types' AP patients were collected by using a GC-MS based metabolomics approach. The representation TIC chromatograms of each group were presented in Fig. 1. The compositions of four groups' serum metabolites were similar, while the levels of these metabolites were different after normalized by heptadecanoic acid (internal standard). Detailed information about the identified metabolites, including compound name and retention time, was listed in Table 1. As shown in Table 1, 32 endogenous metabolites were identified by reference standards and NIST 08, and were double-checked by HMDB (Human Metabolome Database).

Thirty-four metabolites, which were involved in the metabolic process relating to amino acids, carbohydrates, energy, lipids, were significantly altered in AP patients compared with the control group. Among these metabolites, the serum levels of L-Lysine increased markedly in BAP patients compared with the healthy control, whereas the levels of 17 metabolites (L-Lactic acid, L-Valine, (R)-3-Hydroxybutyric acid, Phosphoric acid, Glycine, N-acetyl-D-glucosamine, D-Galactose, D-Glucose, Mannitol, L-Tyrosine, D-Turanose, Octadecanoic acid, Myo-Inositol, Oleic acid, Cholesterol, Glycerol 1-hexadecanoate) decreased strikingly. Compared with the healthy control group, the levels of 13 metabolites (L-Lactic acid, Glycine, N-acetyl-D-glucosamine, D-Glucose, Mannitol, L-Tyrosine, D-Turanose, Octadecanoic acid, Myo-Inositol, L-Tryptophan, Cholesterol, Glycerol 1-hexadecanoate) decreased strikingly in AAP patients. The serum level of 23 metabolites (L-Lactic acid, Butyric acid, (R)-3-Hydroxybutyric acid, Glycine, L-Serine, L-Threonine, L-Proline, Erythronic acid, L-Glutamine, L-Phenylalanine, Ornithine, L-Tyrosine, Octadecanoic acid, Hexadecanoic acid, L-Tryptophan, Linoleic acid, Oleic acid, Arachidonic acid, Cholesterol, Glycerol 1-hexadecanoate) in HLAP patients decreased observably compared with the healthy control.

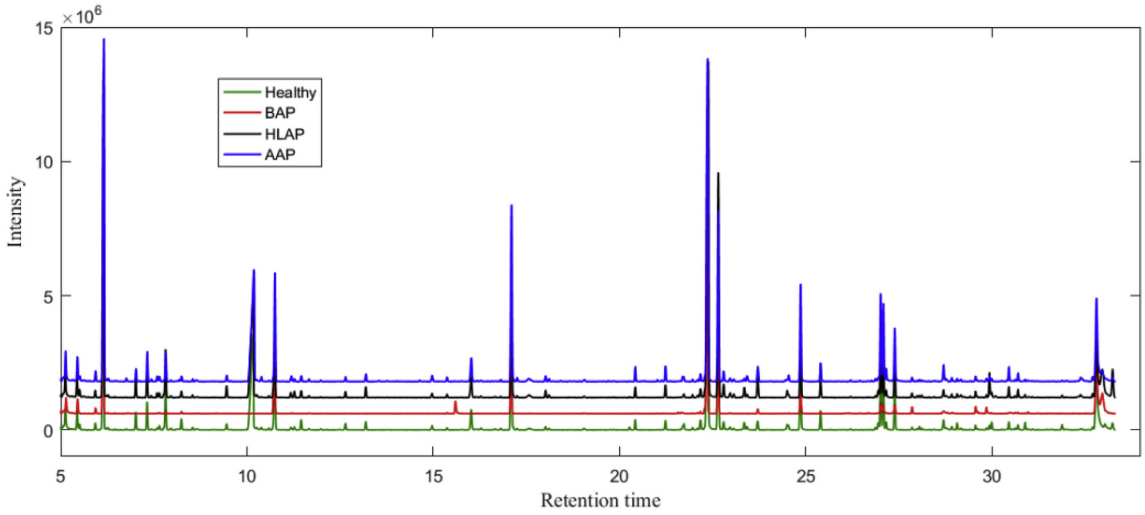

Fig. 1. The TIC plots of different groups.
Note: The total ion chromatograms (TICs) of serum samples from healthy participants (HP) in green, biliary acute pancreatitis (BAP) in red, and hyperlipidemia acute pancreatitis (HLAP) in black, alcoholic acute pancreatitis (AAP) in blue.

Table 1
Qualitative and quantitative analysis of metabolic profiles of BAP, AAP, HLAP and healthy control.

Peak NO.	TR (min)	Endogenou smetabolites	Concentrations(mg/mL)				T			HMDB
			BAP	HLAP	AAP	Control	C−B	C−H	C-A	
1	7.24	L-Lactic acid*	0.5123 ± 0.3851	0.3839 ± 0.1119	0.1979 ± 0.0610	2.4242 ± 5.5006	1	1	1	HMDB00190
2	7.733	L-Valine*	0.0085 ± 0.0072	0.0062 ± 0.0032	0.0038 ± 0.0019	0.0268 ± 0.0611	1	0	0	HMDB00883
3	8.301	Butyric acid*	0.0228 ± 0.0427	0.0465 ± 0.0850	0.0034 ± 0.0013	0.0280 ± 0.0592	0	0	1	HMDB00039
4	8.541	Oxalic acid*	0.1909 ± 0.1037	0.1453 ± 0.0964	0.1053 ± 0.0375	0.1584 ± 0.1004	0	0	1	HMDB02329
5	8.852	(R)-3-Hydroxybutyric acid*	0.0251 ± 0.0324	0.0077 ± 0.0039	0.0072 ± 0.0073	0.0556 ± 0.1211	1	0	1	HMDB00011
6	10.245	Urea	0.2992 ± 0.2068	0.2665 ± 0.1506	0.1741 ± 0.0960	0.2235 ± 0.1650	0	0	0	HMDB00294
7	10.683	Phosphoric acid*	0.1230 ± 0.0604	0.0916 ± 0.0233	0.0850 ± 0.0862	0.2144 ± 0.3289	1	0	0	HMDB02142
8	11.248	Glycine*	0.0082 ± 0.0087	0.0096 ± 0.0093	0.0024 ± 0.0011	0.0194 ± 0.0246	1	1	1	HMDB00123
9	12.203	L-Serine*	0.0069 ± 0.0075	0.0052 ± 0.0066	0.0010 ± 0.0009	0.0064 ± 0.0084	0	0	1	HMDB00187
10	12.512	L-Threonine*	0.0098 ± 0.0074	0.0073 ± 0.0077	0.0020 ± 0.0021	0.0080 ± 0.0083	0	0	1	HMDB00167
11	14.728	L-Proline*	0.0534 ± 0.0212	0.0498 ± 0.0238	0.0336 ± 0.0071	0.0657 ± 0.0434	0	0	1	HMDB00162
12	15.245	Erythronic acid*	0.0083 ± 0.0031	0.0091 ± 0.0038	0.0053 ± 0.0013	0.0090 ± 0.0075	0	0	1	HMDB00613
13	16.213	L-Glutamine*	0.0106 ± 0.0082	0.0075 ± 0.0052	0.0031 ± 0.0016	0.0104 ± 0.0115	0	0	1	HMDB00641
14	16.358	L-Phenylalanine*	0.0087 ± 0.0045	0.0069 ± 0.0046	0.0026 ± 0.0015	0.0099 ± 0.0115	0	0	1	HMDB00159
15	19.076	Ornithine*	0.0228 ± 0.0158	0.0206 ± 0.0180	0.0066 ± 0.0028	0.0196 ± 0.0161	0	0	1	HMDB00214
16	19.554	N-acetyl-D-glucosamine*	0.0440 ± 0.0372	0.0460 ± 0.0273	0.0304 ± 0.0143	0.2257 ± 0.5360	1	1	0	HMDB00215
17	19.972	D-Galactose*	0.0462 ± 0.0829	0.0118 ± 0.0051	0.0137 ± 0.0162	0.1426 ± 0.3713	1	0	0	HMDB00143
18	20.145	D-Glucose*	1.6513 ± 1.1340	1.2711 ± 0.4686	0.8919 ± 0.1534	9.0053 ± 21.1366	1	1	0	HMDB00122
19	20.396	Mannitol*	0.4685 ± 0.4367	0.2943 ± 0.1574	0.1840 ± 0.0451	2.2732 ± 5.5977	1	1	0	HMDB00765
20	20.523	L-Lysine*	0.1297 ± 0.2965	0.0194 ± 0.0317	0.0828 ± 0.1644	0.0456 ± 0.0303	1	0	0	HMDB00182
21	20.647	L-Tyrosine*	0.0375 ± 0.1300	0.0057 ± 0.0041	0.0023 ± 0.0013	0.5352 ± 1.3645	1	1	1	HMDB00158
22	20.935	D-Turanose*	0.0165 ± 0.0165	0.0095 ± 0.0063	0.0056 ± 0.0016	0.2929 ± 0.7977	1	1	0	HMDB11740
23	21.163	D-Arabinose	0.0362 ± 0.0641	0.0149 ± 0.0112	0.0048 ± 0.0059	0.2351 ± 0.8066	0	0	0	HMDB29942
24	21.812	Octadecanoic acid*	0.0183 ± 0.0205	0.0163 ± 0.0200	0.0023 ± 0.0020	0.0590 ± 0.1169	1	1	1	HMDB00827
25	22.165	Hexadecanoic acid*	0.2736 ± 0.1752	0.2454 ± 0.1497	0.0907 ± 0.0221	0.4332 ± 0.6314	0	0	1	HMDB00220
26	22.644	Myo-Inositol*	0.0183 ± 0.0133	0.0131 ± 0.0083	0.0087 ± 0.0029	0.0878 ± 0.2155	1	1	0	HMDB00211
27	24.08	L-Tryptophan*	0.0139 ± 0.0082	0.0150 ± 0.0140	0.0077 ± 0.0110	0.0370 ± 0.0695	0	1	1	HMDB00929
28	24.174	Linoleic acid*	0.0955 ± 0.0469	0.0890 ± 0.0381	0.0601 ± 0.0161	0.1008 ± 0.0677	0	0	1	HMDB00673
29	24.24	Oleic acid*	0.1092 ± 0.0505	0.0747 ± 0.0275	0.0563 ± 0.0217	0.1180 ± 0.1019	1	0	1	HMDB00207
30	25.948	Arachidonic acida*	0.0278 ± 0.0171	0.0221 ± 0.0087	0.0118 ± 0.0050	0.0481 ± 0.0858	0	0	1	HMDB01043
31	26.662	Cholesterol*	0.2111 ± 0.1570	0.1870 ± 0.0925	0.1767 ± 0.1043	0.9179 ± 1.8166	1	1	1	HMDB00067
32	28.629	Glycerol 1-hexadecanoate*	0.1089 ± 0.1069	0.1024 ± 0.0949	0.0074 ± 0.0040	0.6601 ± 1.5332	1	1	1	HMDB31074

Note: 32 data are presented as mean ± SD. T is the *t*-test results between healthy control, BAP, AAP and HLAP; *p* value of <0.05 is considered statistically significant and signed T value is "1", otherwise "0". * Identified by standard substances. C: Controls; B: biliary acute pancreatitis groups; A: alcoholic acute pancreatitis groups; H: Hyperlipidemia acute pancreatitis groups.

Metabolomics-based discrimination model identified the key metabolic disorders of AP patients with different etiologies

Based on these metabolites results, Random Forest (RF) algorithm was employed to distinguish BAP, HLAP, AAP, and healthy groups. Auto-scaled data of 32 metabolites from each group was used as input data. The classification plot of four groups by RF was exhibited in Fig. 2. Four groups' samples were obviously separated; healthy groups were located at the middle top of plot, while the AAP groups were farthest from the healthy groups. Furthermore, a 5-fold

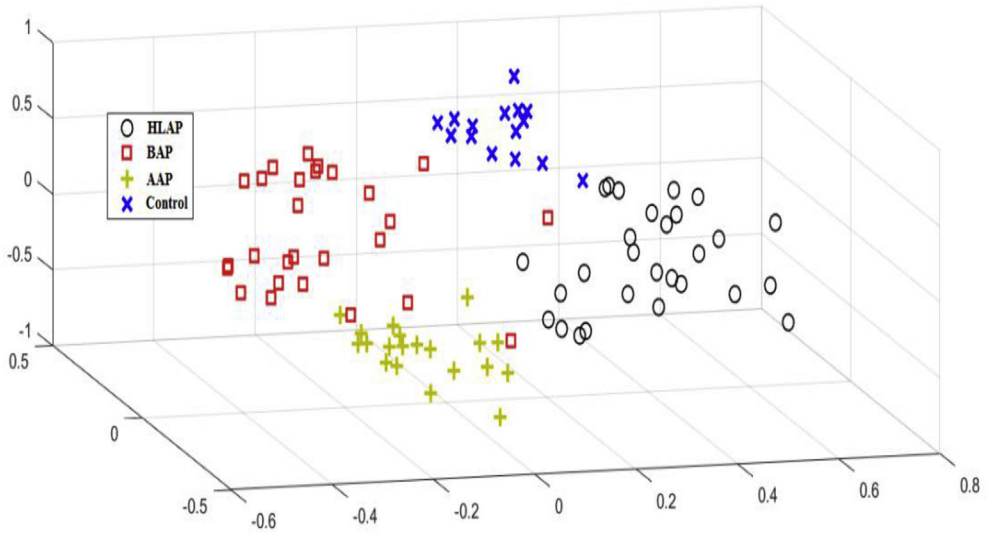

Fig. 2. RF model to discriminate between BAP, AAP, HLAP and healthy control.
Note: Classification importance of RF model of serum samples from healthy participants (HP) in, biliary acute pancreatitis (BAP) in red square, and hyperlipidemia acute pancreatitis (HLAP) in black fork, alcoholic acute pancreatitis (AAP) in yellow cross.

cross validation method was used for evaluating the classification ability of RF. The classification accuracies by using 5-fold cross-validation for BAP group VS healthy, HLAP group VS healthy, and AAP VS healthy, were 0.886, 0.906 and 0.857, respectively.

VIP parameters have been widely used in the screening of biomarkers, which can indirectly reflect the correlation between metabolites and diseases [21,25]. From the graph of classification importance of each variable by RF (Fig. 3), some metabolites showed the consistent trends in distinguish AP patients with healthy, such as L-Tyrosine, Octadecanoic acid, Cholesterol, Glycerol 1-hexadecanoate, and L-Lactic acid. These metabolites showed high contributions in distinguishing AP patients with healthy, with higher variable importance. These results were consistent with the existed researches [26]. While, Glycine and L-Valine showed higher contributions in distinguishing HLAP patients from healthy group; (R)-3-Hydroxybutyric acid and Mannitol showed higher contributions in distinguishing BAP patients and healthy group. These metabolites suggested that different types of AP, might have different

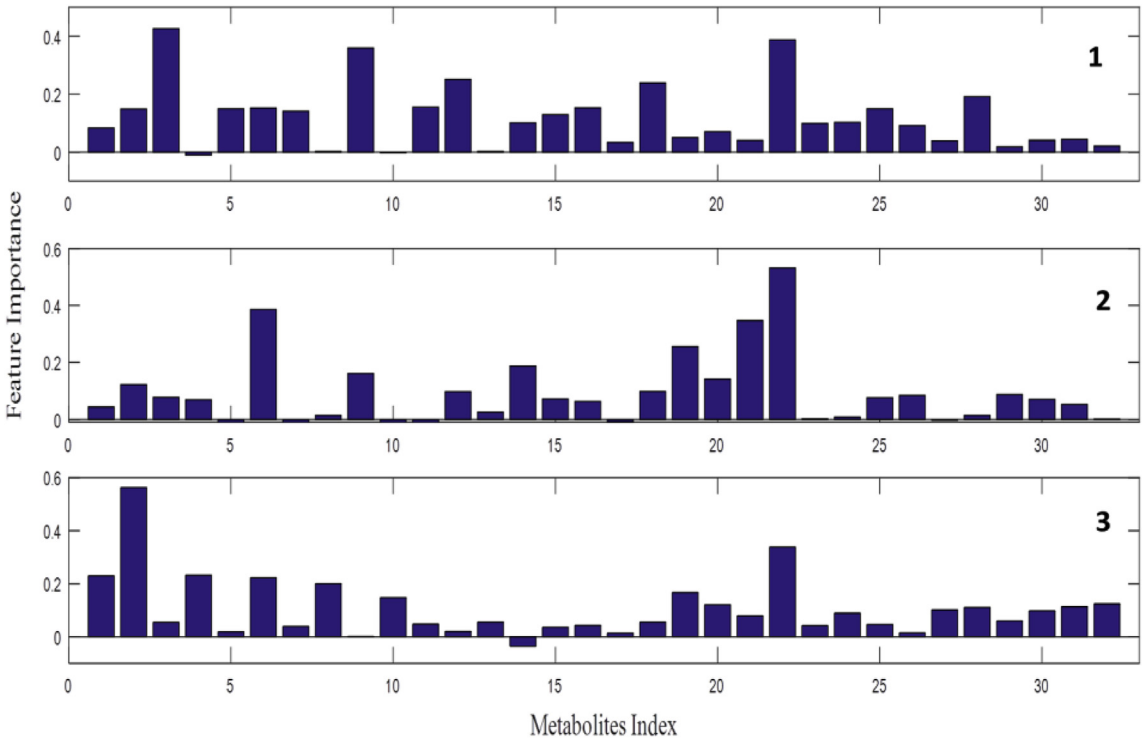

Fig. 3. VIP value of each metabolite to discriminate between BAP, AAP, HLAP and healthy control.
Note: Classification importance of each variable in (1) hyperlipidemia acute pancreatitis (HLAP), (2) biliary acute pancreatitis (BAP), and (3) alcoholic acute pancreatitis (AAP).

affects in human metabolism, resulting in different metabolites' profiles.

Related metabolic pathway analysis for each type of AP

Furthermore, to explore the potential metabolic pathways of three AP types, potential biomarkers with significant differences in each group were introduced into MetaboAnalyst for explore the metabolic pathways of KEGG (Kyoto Gene and Genomic Encyclopedia). The metabolic pathway information of the BAP, HLAP, and AAP groups are displayed in the supporting Tables S2, S3, and S4, respectively. The impacts of BAP, HLAP and AAP metabolic pathways are shown in Fig. 4. Five metabolic pathways of importance (Aminoacyl-tRNA biosynthesis, Thiamine metabolism, Glycolysis or Gluconeogenesis, Propanoate metabolism, and Nitrogen metabolism) were disturbed in the BAP. Five metabolic pathways of importance (Nitrogen metabolism, Aminoacyl-tRNA biosynthesis, Thiamine metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, and Glycolysis or Gluconeogenesis) were disturbed in the HLAP. Five metabolic pathways of importance (Aminoacyl-tRNA biosynthesis, Nitrogen metabolism, Glycine, serine and threonine metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, Fatty acid biosynthesis) were disturbed in the AAP.

Discussion

To our knowledge, this is the first study to identify potential biomarkers and unravel the metabolic mechanisms of AP with different etiologies using serum metabolomics based on GC-MS.

Our data from this study indicated that disease-specific patterns of three types' AP could be identified from their serum metabolomics profile, and further distinguished by using random forest algorithm. Based on the MetaboAnalyst and KEGG database, these metabolites are mainly associated with metabolic pathways, including those of Thiamine metabolism, Glycolysis or Gluconeogenesis, Propanoate metabolism, tyrosine and tryptophan biosynthesis metabolism. These processes were further associated with the metabolism of several amino acids (Glycine, L-lysine, Tyrosine and Phenylalanine), short chain fatty acids. The results were used to identify which changes in the metabolic profiles are involved in the pathophysiological mechanism of different AP.

Metabolites related to amino metabolism

Our finding showed that plasma concentrations of amino acids were altered following AP, which associated with amino acid metabolism (Glycine, serine and threonine metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis). In this study, the level of Glycine ($P < 0.05$) decreased obviously in the BAP group compared with the healthy group. Glycine binds to primary bile acids to form conjugated bile acids and is involved in primary bile acid biosynthesis [27]. Research has shown that abnormal bile acid metabolism causes cholestasis or bile acid flowing back into the pancreatic duct with abnormal bile, which would cause damage to the functions and structures of pancreatic acinar cells and are the key factor for the pathogenesis of almost BAP [28]. These results suggest that modulation of the bile acid biosynthesis pathway by assessing glycine levels may contribute to the diagnosis of BAP.

The level of L-lysine in BAP patients increased significantly compared with the healthy group ($P < 0.05$). Biczó found that L-lysine selectively damages the pancreatic mitochondria, leading to necrosis and apoptosis of acinar cells [29]. In addition, permeability transition pores (mPTP) in the mitochondria of the pancreas have unusual characteristics, including openness independent of inflammatory infiltration and more sensitive to L-lysine [30,31]. High concentrations of pancreatitis often cause severe pancreatitis which is verified in patients with BAP.

Our research testified that the level of Tyrosine and Phenylalanine ($P < 0.05$) decreased significantly in the HLAP group compared with healthy group. It has been also reported that the low levels of L-Tyrosine might contribute to the development of hyperlipidemia [25]. Previous studies has shown that amino acids such as isoleucine, leucine, valine and phenylalanine have been found to be associated with hypertriglyceridemia, which overlaps previous findings on the risk of pancreatitis [25,32]. Tyrosine is an essential amino acid and the raw material for synthesis of epinephrine, which is involved in the regulation of phospholipid metabolism, lipid distribution and transport [33]. Lipid metabolism can cause oxidative stress reaction and acidosis, leading to injury of pancreas, surrounding tissues and hypoxic necrosis, which are considered as an important induce of AP [34]. AP could lead to abnormal lipid metabolism under the regulation of nerves and body fluids [35]. Phenylalanine is one of the essential amino acids in human body, which can be oxidized into tyrosine by hydroxylase catalysis, and

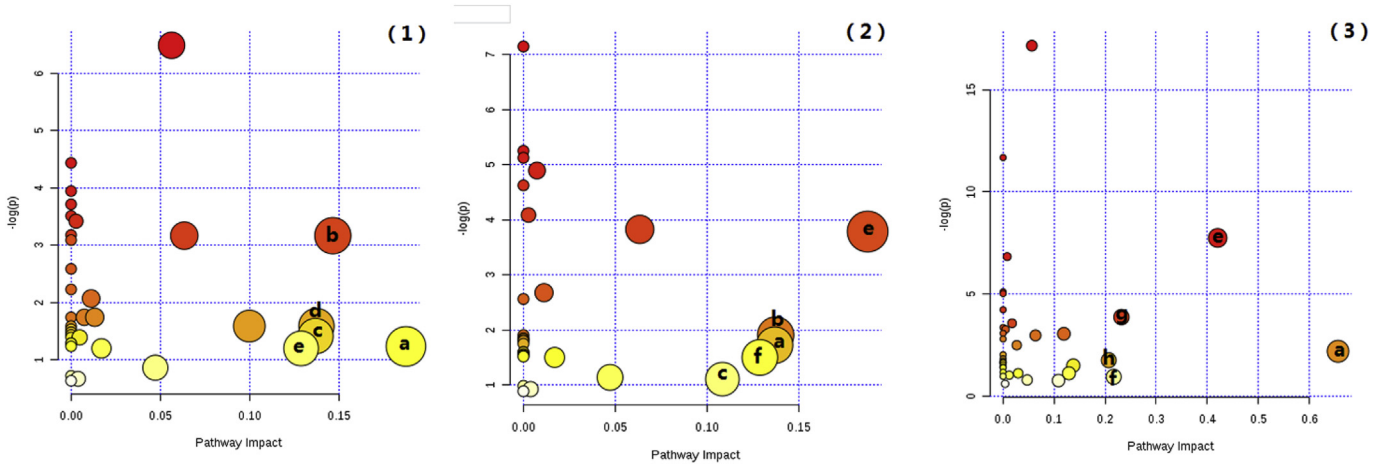

Fig. 4. Summary of metabolic pathways analysis.
Note: As shown, five important metabolic pathways in the serum of patients with (1)BAP, (2) HLAP and (3)AAP were identified: (a) Aminoacyl-tRNA biosynthesis, (b) Thiamine metabolism, (c) Glycolysis or Gluconeogenesis, (d) Propanoate metabolism, (e) Nitrogen metabolism, (f) Phenylalanine, tyrosine and tryptophan biosynthesis, (g) Glycine, serine and threonine metabolism, and (h) Fatty acid biosynthesis.

together with tyrosine to synthesize important neurotransmitters and hormones, participated in the body's sugar metabolism and fat metabolism [36]. These results may implicate that abnormal reduction of tyrosine and phenylalanine should be one of the possible properties of the HLAP.

Metabolites related to fatty acid metabolism

Additionally, our observations indicated that fatty acid metabolism also associated with the dysregulation of short chain fatty acids including Arachidonic acid (AA), linoleic acid, and 3-hydroxybutyric acid. Fatty acids, as energy sources and membrane components, have biological activities that act to influence cell and tissue metabolism (including lipid metabolism), function, and responsiveness to hormonal and other signals [37]. Previous studies have showed the association between abnormal changes in fatty acids (such as AA and linoleic acid) and the deterioration of AP. Domschke and Krystyna found the levels of serum free fatty acid were significantly higher than those in healthy people [42,43]. Nakamura et al., have analyzed the fatty acid composition in AAP patients, they found the linoleic acid and AA levels in pancreatitis patients also variant with status of illness [44].

Furthermore, Arachidonic acid (AA), one of the ω-6 polyunsaturated fatty acids, is involved in the formation of inflammatory signals such as prostaglandin E2, prostaglandin I2, and human Leukotriene C4 [38–41]. Excessive alcohol intake will increase total hydrogen oxidation and epoxidation of AA, resulting in depletion of AA. The level of AA ($P < 0.05$) descended significantly in the AAP group compared with those of BAP, HLAP and HP group, in this study. This result may implicate that AA is the key endogenous metabolite that distinguishes AAP from the other three groups.

3-hydroxybutyric acid is a ketone body synthesized from acetate and acetyl-CoA. Change of 3-hydroxybutyrate is generally considered to be associated with disorders of dyslipidemia [45]. The occurrence of hyperlipidemia is often accompanied by a decrease in 3-hydroxybutyrate, which may be caused by the accumulation of ketone bodies and the conversion of acetoacetate to acetone. In our study, the level of 3-hydroxybutyrate decreased obviously in the HLAP and AAP groups compared with that in the HLAP group ($P < 0.05$).

Although we have examined the possible role of varying metabolites in three APs, there are some limitations in the present study. First, Changes in serum metabolism in the same individuals with BAP, AAP or HLAP have not been considered. Non-targeted metabolomics methods should be used in further studies to accurately reveal the pathophysiology of different types of AP accurately, especially for the AAP patients, the metabolic of alcohol should be taken into account. In addition, only one hospital's sample source limits the expansion of our sample size. Carrying out larger-scale sample collection and analysis will be of great significance for future research to find as many suitable biomarkers as possible to differentiate BAP, AAP and HLAP patients. Finally, due to the limitations of the detection equipment, we only employed serum metabolism based on GC-MS technology to test our samples. More metabolomics techniques such as nuclear magnetic resonance (NMR) and high performance liquid chromatography-mass spectrometry (HPLC-MS) are needed to confirm and enrich this study.

Conclusion

Early diagnosis and distinguish biliary acute pancreatitis (BAP), alcoholic acute pancreatitis (AAP) and hyperlipidemia acute pancreatitis (HLAP) is still difficult by disease phenotypes. While, metabolomics is an appropriate approach for rapidly and accurately distinguish different types of AP, and explore associated metabolism pathways. Our study had established a classification model based on different types of AP metabolites profile and random forest algorithm, and different types of AP were accurately identified. These results indicated that disease-specific patterns can be identified by coupling serum metabolomics profiles with pattern recognition. A range of metabolites that represent the metabolic regulations of different types of AP were discovered, and relevant pathways were further explored. The current results suggested that metabolomics is a valuable tool for identifying the molecular mechanisms that are involved in the etiology of different types of AP.

Conflicts of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2019.05.456.

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