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Brief communication (Original)

Novel biomarkers of hyperlipidemic acute pancreatitis: metabolomic identification

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Background: Recognition of hypertriglyceridemia is critical for the diagnosis of hyperlipidemic pancreatitis (HLP) and the selection and evaluation of therapy.
Objective: Investigate metabolic profiling technologies for identifying novel biomarkers and pathways activated in HLP.
Methods: Blood and urine samples were obtained from 24 patients and 39 healthy people. A gas chromatography and mass spectrometry was employed to study the metabolic profile in HLP and healthy groups. Functional pathway trend analysis using multivariate statistical analysis was performed.
Results: HLP patients could be precisely distinguished from the healthy controls. In the patient, levels of aconitate, citrate, hippurate, p-hydroxyphenylacetate and p-hydroxyphenylpopionic acid were decreased, while levels of tryptophan, tyrosine, tyramine,16-hexadecanoic acid, and 18-octadecanoic acid were increased. The change of energy metabolism-related mechanisms, fatty acid metabolism, gut microbiota metabolism, and metabolism of tyrosine could be used to distinguish HLP patients.
***Conclusions*:** Novel biomarkers could be identified by application of metabolomics. Metabolic profiling was useful for studies of pathogenesis of HLP.

Keywords: Blood, hyperlipidemic pancreatitis, metabolism, urine

Hypertriglyceridemia may lead to acute pancreatitis. It is may be seen as an epiphenomenon of pancreatitis. The role of hypertriglyceridemia in the pathogenesis of acute pancreatitis is still uncertain [1]. A serum triglyceride (TG) level of more than 1,000 to 2,000 mg/dL in patients with type I, IV, or V hyperlipidemia is an identifiable risk factor.

Recognition of hypertriglyceridemia is critical for the diagnosis of HLP and the selection and evaluation of responses to therapeutic interventions. Hypertriglyceridemia may be primary in the origin or secondary to other clinical conditions and the use of oral contraceptives.

Recent system biology aims at integration of all pertinent components such as genes, proteins, and metabolites. All intricate relationship into a holistic biological network may provide comprehensive thought for understanding the behavior of biological system [2, 3]. Metabonomic profiling is a practical approach to capture the system response to perturbations by measuring variations in small molecule/endpoint metabolic product [4]. Consequently, the strategy of integrated transcriptomic and metabonomic profiling turns out to be a critical step toward good understanding of complex biological systems.

In this study, we investigated metabolic profiling technologies for identifying novel biomarkers and pathways activated in HLP.

Patients and methods

The study was approved by the Ethics Committee of Tongji University School of Medicine. Twenty-four HLP patients were recruited from Shanghai 10th People's Hospital between 2007 and 2009, and 39 healthy volunteers with age and sex-matched were selected as controls. Serum and urine samples were taken for metabolic profiling.

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The protocol for this study was in accordance with diagnostic criteria for hypertriglyceridemia and acute pancreatitis (AP) by the Chinese Medical Association [5] as follows: clinical features of AP, and the hypertriglyceridemia level is ≥11.3 mmol/L within 24 to 48 hours of the onset of AP, or 5.6 to 11.3mmol/L in the absence of other etiologic factors.

The basic data included sex, age, body mass index (BMI), alcohol and tobacco use, and diabetes mellitus. Physical examination and laboratory tests were performed within 24 hours of admission. Triglyceride (TG) level at time of admission, total cholesterol (TC), C-reactive protein (CRP), and serum lipase liver enzymes were measured with abdominal computed tomographic scanning. The severity of the patient condition were classified according to the Ranson score, Glasgow score, Apache II score, CRP level, and Balthazar CT stage score [6,7]. Urine and serum samples were collected from fasting subjects. Serum samples were stored at -80°C until use. All urine samples were centrifuged at 6000 g for 10 minutes at room temperature, and the supernatants were stored at -80°C for metabonomic analysis.

Regarding Metabonomic profiling of serum and urine samples, gas chromatography (GC) and mass spectrometry (MS)-based metabonomic profiling was performed on serum and urine samples using the method by Qiu *et al* [8, 9].

Data analysis

GC/MS data were converted to NetCDF format via DataBridge (Perkin-Elmer Inc, Waltham Massachusetts, USA). A pretreatment was conducted as previously described [10]. The mean-centered and autoscaled data were input into the SIMCA-P 11.5 Software (Umetrics, Umea, Sweden) for multivariate statistical analysis. Principal component analysis (PCA) was used to obtain an overview of variations among the different groups. Orthogonal projections to latent structures discriminant analysis (OPLSDA) were utilized to establish a prediction model to identify the differential metabolites accountable for the disease.

Results

Twenty-four patients with mean age of 42 years were referred to our investigation of HLP. Diabetes mellitus and alcohol intake were found in 47.8% and 42.7% of the patients. There was no difference of sex and age between two groups. Average BMI was 28 kg/m^2 of the patients. The severity of the disease (APACHE II score) was 37.5 %. The information of patients is shown in **Table 1**.

Analysis of GC/MS spectra

GC/MS spectra of serum and urine samples showed significant difference between healthy and HLP. Using PCA as a data reduction tool, the healthy and HLP were readily separated in spectra from serum, or urine samples. These data were subsequently interrogated using OPLSDA model, as shown in **Figure 1**.

Metabolic profiles

We identified 20 differential metabolites in serum and urine samples between the HLP and healthy groups, most of which were fatty acids, glucogenic amino acids, and ketogenic amino acids. Serum samples showed relative increases in hexadecanoic acid, eicosanoic acid and octadecanoic acid, and decreases in glycine, alanine, citrate, and fumaric acid in HLP. Urine samples of patients showed relatively high concentrations of proline, leucine, tyramine, phenylalanine, tyrosine, histidine, octadecanoic acid, and hexadecanotic acid. In addition, glycine, citrate p-hydroxyphenylacectate, and hippurate were in lower concentrations in HLP. All metabolites were significantly different between healthy and HLP groups (**Table 2)**.

Table 1. Patient information

	HLP	Healthy
Number of subjects	24	39
Age (mean; range)	42.0 (19-59)	39.2 (22-57)
Sex (male, female)	11/13	17/22
Body mass index	22.1-30	16.8-25.5

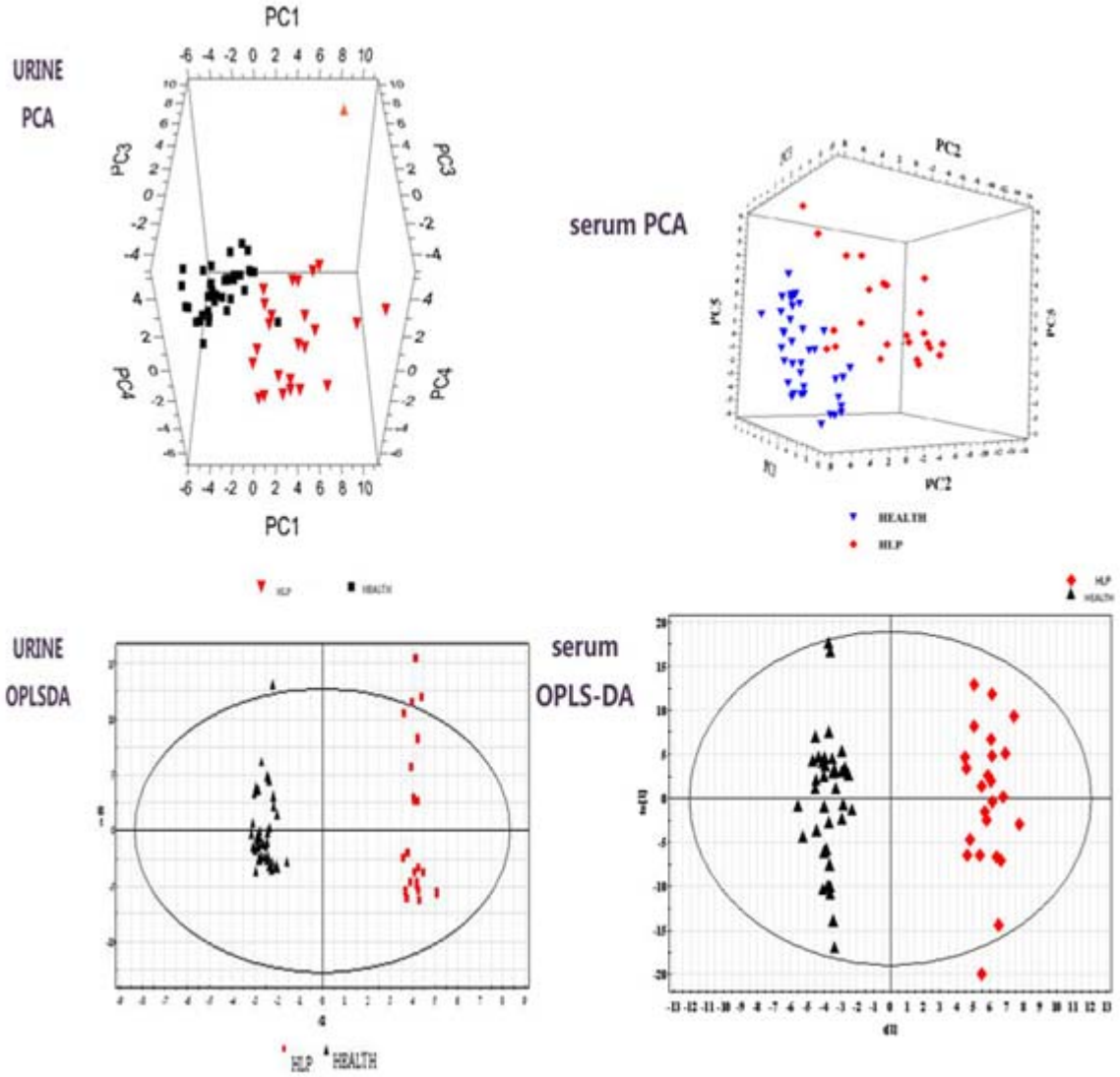

Figure 1. The scores plot of principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) derived from GC/MS spectra of serum and urine, indicating discrimination between health and hypertriglyceridemia acute pancreatitis. Red triangle or rectangle represents HLP, while black or blue triangle or rectangle represents health.

Table 2. Differential metabolites between healthy and HLP groups using OPLSDA on SIMCA-P software

Differential metabolites	RT/min	Change	Derivatization
Serum			
Glycine	5.59	↓	TMS
Alanine	7.4	↓	TMS
Citrate	10.25	↓	TMS
Fumaric acid	15.8	↓	TMS
Eicosanoic acid	16.31	↓	TMS
Octadecanoic acid	29.53	↓	TMS
Hexadecanoic acid	20.81	↓	TMS

Table 2. Differential metabolites between healthy and HLP groups using OPLSDA on SIMCA-P software (continuous)

Differential metabolites	RT/min	Change	Derivatization
Urine			
Proline	26.38	↓	ECF
Leucine	12.91	↓	ECF
Tyramine	12.06	↓	ECF
Phenylalanine	30.32	↓	ECF
Tyrosine	20.62	↓	ECF
Histidine	32.44	↓	ECF
Glycine	8.71	↓	ECF
Citrate	16.34	↓	ECF
Aconitate	15.63	↓	ECF
Octadecanoic acid	28.7	↓	ECF
Hexadecanotic acid	24.22	↓	ECF
P-hydroxyphenylacectate	19.16	↓	ECF
Hippurate	19.29	↓	ECF

RT/min indicates retention time (min) on gaschromatograph. ECF and TMS represent ethyl chloroformate and bis (trimethylsilyl)-trifluoroacetamide) (BSTFA) derivatization methods for urine or serum samples prior to GC/MS analysis.

Discussion

In this study, metabonomic technologies have been successfully used to investigate the systemic metabolic response to hypertriglyceridemia-induced AP. Our results demonstrated the impact of hypertriglyceridemia on body systems as indicated by the up- or down-regulated levels of the low-molecular-weight metabolites in patients groups, compared to the control group. It was shown that several pathways, involving the tricarboxylic acid (TCA) cycle (citrate, aconitate), tyrosine metabolism (tyrosine phenylalanine, tyramine), and gut microbiota metabolicactivity (P-hydroxyphenylacectate, hippurate) were affected.

In our study, hippurate and P-hydroxyphenylacectate were the most notable urinary metabolites differences in two groups. They were the phenolic metabolites of gut flora. Such variations could be attributed to the initial differences in gut microbiota. Gut flora-related differential metabolites detected in healthy and HLP patients were the character of hypertriglyceridemia. In general, gut barrier failure in severe AP, as an example of critical illness, does not involve the gut barrier and an increase in permeability (experimentally and clinically) [11-14], leading to translocation of enteric bacteria. In our patients with SAP, the colony forming units of Gram-negative bacterium and Gram-positive Cocci significantly increased compared with the healthy persons, while bifidobacterum, lactobacillum, and bacteroides decreased. This may lead to flora imbalance of gut and increase of endotoxin. Therefore, it is possible to deduce different gut microbiota structures and activities between healthy and HLP patients.

It is likely that marked differences might exist in the energy metabolism-related metabolites between patients and healthy HLP persons. Following the hyperlipidemic pancreatitis, these compounds significantly decreased compared to the healthy control groups. This suggests that hyperlipidemic pancreatitis may inhibit the activity of above compounds, ultimately repressing energy metabolism. Since the metabolic enzyme of tricarboxylic acid cycle exists mainly in mitochondria, the disorder of TCA cycle means disturbances in the mitochondria function. It has been shown that mitochondria play an important role in the development and progression of cancer [15], neurological disorders [16], and cardiovascular disease [17]. Therefore, future research must address the functional aspects of mitochondria in hyperlipidemic pancreatitis development and therapeutic response.

Compared to healthy controls, the altered serum and urine metabolites were observed in HLP subjects, including the significantly increased long-chain fatty acid, polyunsaturated fatty acid, and hexadecanoic

acid. This suggests a hypercatabolic state in HLP patients. Havel [18] proposed a mechanism that hydrolysis of TG in and around the pancreas by pancreatic lipase seeping out of the acinar cell leads to accumulation of free fatty acids in high concentrations, which has been well accepted.

In conclusion, novel candidates for HLP biomarkers were identified by utilizing a gas chromatography and mass spectrometry (GC/MS) approach. Many metabolites were differentially expressed in healthy and HLP groups, involving tricarboxylic acid cycle, gut microbiotametabolic activity, fatty acid metabolism-related mechanisms, and tyrosine metabolism. This finding may provide important clues to the mechanism of HLP in humans.

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The first author (Yan Zhao) and the second author (Wei Jia) contributed equally to this work.

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