Journal of Clinical Laboratory Analysis 23 : 45–50 (2009)

# Silent Myocardial Ischemia Is Associated With Altered Plasma Phospholipids

Hailong Lin,[1] Jing Zhang,[1] and Peng Gao[2]*
[1]Cardiology Division, Dalian Municipal Central Hospital, Dalian, China
[2]National Chromatographic R. & A. Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China

Timely and accurate confirmation of the occurrence of silent myocardial ischemia (SMI) is critical both for prevention and therapy management. Metabolomics assay may offer an alternative for SMI differentiation and altered biomolecule discovery in addition to traditional measures. In this study, plasma samples were obtained from 14 diagnosed SMI subjects and 25 healthy controls and analyzed by liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry in view of metabolomics. Obtained data were subjected to orthogonal signal correction partial least-squares discriminate analysis. Multivariate statistic analysis indicated a clear separation between the two studied groups. Plasma concentration fluctuation of four kinds of phospholipids showed tight relationship with the occurrence of SMI, among which 1-linoleoylglycerophosphocholine (C18:2) was decreased statistically in SMI population ($P = 0.01$). The plasma phospholipids' changes were before enzymatic alteration in SMI, which might be a useful complementary reference to facilitate SMI diagnosis. J. Clin. Lab. Anal. 23 : 45–50, 2009. © 2009 Wiley-Liss, Inc.

**Key words:** myocardial ischemia; metabolomics; phospholipids

## INTRODUCTION

Nowadays, coronary artery disease is a leading cause of morbidity and mortality throughout the modern world. Most of the heart events are preceded or accompanied by myocardial ischemia (MI). Since the 1970s, a so-called silent myocardial ischemia (SMI) was described, which was defined as an objective documentation of MI in the absence of angina or anginal equivalents (1). According to stress test and Holter monitoring data, it was estimated that the frequency of SMI varied from 30 to 43% annually (1). Some individuals, such as those with diabetes, are prone to suffer from SMI (2). Currently, three types of SMI have been recognized according to Cohn classification: Type I: totally asymptomatic patients without prior cardiovascular events. Type II: totally asymptomatic patients with prior myocardial infarction. Type III: patients having symptomatic and also asymptomatic ischemia (3). Because of lack of any indicative sign, type I SMI is more life-threatening than the others and arises to be a troublesome problem for the clinician. Early studies showed that, for type I SMI, the rate of major cardiac events was four to five times higher in the patients with normal exercise electrocardiograms (ECGs) than in those with positive exercise ECGs (4).

Currently, SMI can be detected with an exercise ECG, 24- or 48-hr ambulatory electrocardiography (AECG), or (stress) myocardial scintigraphy. Exercise testing requires a certain level of fitness of the patients, and, in most circumstances, can identify only patients with advanced coronary artery disease who manifest MI (5,6). Myocardial scintigraphy is more expensive, and both scintigraphy and exercise tests are time consuming. In theory, AECG can be applied in virtually any patient, but it is not easily accessible to all the subjects sometimes. For example, people working in a highfield environment are not readily monitored by AECG. Other

Grant sponsor: Scientific Research Program of Dalian Public Health Bureau (2006–2008).

*Correspondence to: Peng Gao, National Chromatographic R. & A. Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China. E-mail: mailgaopeng@126.com

Received 19 May 2008; Accepted 7 November 2008
DOI 10.1002/jcla.20288
Published online in Wiley InterScience (www.interscience.wiley.com).

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techniques, e.g., myocardial single photon emission computed tomography (SPECT) or electron beam computed tomography (EBCT) is by no means of ideal measures for the screening purpose in spite of their noninvasive properties (7). Biochemical analysis can accurately diagnose the occurrence of myocardial infarction, but serum enzymatic changes cannot provide useful hints for subtle insult from MI.

Metabolomics, a science dealing with small molecules, addresses to a combination of data-rich analytical methods together with chemometrics for profiling metabolism and interpreting metabolic fingerprints in complex biological systems (8). It has been widely adopted to evaluate organism response to physiological stressors, disease processes, or drug therapy and toxicity (9–11). Based on proton nuclear magnetic resonance spectra of human sera, patients with stenosis of all three major coronary vessels could be distinguished from subjects with angiographically normal coronary arteries (12). By employing electrospray triple-quadrupole mass spectrometry (MS), Sabatine et al. identified multiple metabolites that either increased or decreased in the sera of patients with exercise stress inducible ischemia (13). In this study, plasma samples from subjects with type I SMI were analyzed by ultra-performance liquid chromatography coupled with quadrupole-time-of-flight (UPLC-Q-TOF) MS. Relevant data were processed through multivariate analysis. Metabolites playing key role in the differentiation between the normal controls and patients were identified and their clinical significance was discussed accordingly. The aims of this study were to prove the value of metabolomics in SMI diagnosis and identify the key altered small molecules that were closely related to the occurrence of SMI.

## MATERIALS AND METHODS

### Study Populations

Our study group consisted of 7 male and 7 female individuals (43–67 years old, median 57.5) with diagnosed type I SMI according to the criteria described elsewhere (14). Twelve-lead ECGs acquired by exercise tests were of at least $\geq$1.0-mm ST-segment depression for males and $\geq$2.0 mm for females within 8 min test. All of the included subjects denied to have suffered from any cardiac disorder or other chronic diseases previously. Patients receiving any medicine administration were excluded. The control group contained 25 randomly selected age- and gender-matched healthy volunteers. No ST-segment and T-wave changes in 10 min exercise test were found for each. Every subject gave written informed consent according to the Guide of Hospital Ethics Committee of Dalian Municipal Central Hospital. Venous blood samples were obtained immediately after stress testing using EDTA as the anticoagulant for 5 min. In order to eliminate any possible interference factor, exercise test was conducted for each individual after 12-hr fasting.

### Plasma Biochemical Assay

Plasma enzymatic activities and some proteins were quantified using Olympus AU 2700 biochemical analyzer (Olympus, Tokyo, Japan). All the reagent kits were from Fuxing-Changzheng Medical Science Co. Ltd. (Shanghai, China). The analyses were performed according to the instructions provided by the manufacturer. The detected items were glutamicoxaloacetic transaminase (GOT), glutamic alanine transaminase (GAT), $\gamma$-glutamyl transpeptidase ($\gamma$GT), alkaline phosphatase (ALK), total cholesterol (TC), triglyeride (TG), high-density lipoprotein-cholesterol (HDL-c), low-density lipoprotein-cholesterol (LDL-c), lactate dehydrogenase (LDH), $\alpha$-hydroxybutyrate dehydrogenase (HBDH), creatine kinase-MB (CK-MB), creatine kinase (CK), and C reaction protein (CRP). The troponin T (TnT) was quantitated by Roche Elecsys 2010 (Diamond Diagnostics, Holliston, MA) using electrochemiluminescence immunoassay with Troponin T STAT kits (Roche Diagnostics GmbH, Germany). Univariate statistical analysis was conducted using MINITAB version 14 (Minitab Inc., State College, PA).

### Metabolomics Analysis

An UPLC-Q-TOF system was employed to perform the metabolomics analysis. Briefly, the blood samples were centrifuged at 3,000*g* for 5 min at 4°C as soon as they were collected. For each sample, 200 µl plasma was added to 800 µl HPLC-grade acetonitrile (Merck, Whithouse Station, NJ), vortexed thoroughly, set at room temperature for 10 min, and centrifuged at 10,000*g* for 15 min at 4°C. Every 900 µl of supernatant was pipetted out, lyophilized, and redissolved in 100 µl acetonitrile/water (4:1, v/v) for subsequent analysis. Chromatographic separation was performed on a 10 cm $\times$ 2.1 mm ACQUITY 1.7 µm $C_{18}$ column (Waters, Milford, MA) using an ACQUITY UPLC system (Waters). The column temperature was set to 35°C. A linear gradient of 2–100% was applied using acetonitrile and 0.1% formic acid (v/v; Merck) as the mobile phase. The total run time was 35 min at a flow rate of 0.35 mL/min. The injected sample volume was 4 µl for each run. MS data were acquired on a Waters Q-TOF micro MS (Waters MS Technologies, Manchester, UK) system in positive electrospray ionization ($ESI^+$) mode. The nebulization gas (nitrogen) was set to 500 L/hr at a temperature of 300°C with 50 L/hr cone gas and 100°C source temperature. Capillary voltage and cone voltage were

set to 3,100 and 35 V, respectively. The MCP detector voltage was set to 2,600 V. The acquisition rate was set to 0.5 s with a 0.1 s interscan delay. Tune page was used to regulate the sample cone voltage. Argon was used as collision gas. The scan range was from 100 to 1,000 mass/charge ($m/z$). Data were collected in the centroid mode. All analyses were acquired using the lock spray to ensure accuracy and reproducibility. Lock mass of leucine–enkephalin was 556.2771 Da for its quasi-molecular ion in $ESI^+$ mode (concentration: 0.8 μg/mL; flow rate: 10 μL/min). The lock spray frequency was set at 20 sec. Commercial standards for metabolite confirmation were purchased from Sigma-Aldrich (Steinheim, Germany) and Avanti Polar Lipids (Alabaster, AL).

The retention time and $m/z$ data pairs for each peak were processed by the Micromass MarkerLynx application manager software (Waters, UK). The ion intensity for each peak was normalized to the sum of all peak intensities in the corresponding chromatogram and then analyzed by orthogonal signal correction partial least-squares discriminate analysis (OPLS-DA) using the SIMCA-P 11.0 software (Umetrics AB, Umea, Sweden).

## RESULTS

### Plasma Biochemical Assay

A total of 14 patients undergoing exercise stress testing with typical ST-segment changes served as the study population. Quantitation of major plasma enzymatic activities and relevant macromolecules indicated that all the parameters having a role to do with cardiac diseases were not significantly different between the SMI and control populations (Table 1).

### Metabolomics Analysis

**TABLE 1. Comparison of Major Serumal Biochemical Parameters in the Two Groups**

| Items | Control group | SMI group | *P* value |
|---|---|---|---|
| GAT (IU/L) | 15.20 | 17.30 | 0.45 |
| LDH (IU/L) | 165.30 | 203.10 | 0.08 |
| HBDH (IU/L) | 126.10 | 140.70 | 0.22 |
| CK-MB (IU/L) | 11.70 | 17.80 | 0.08 |
| CK (IU/L) | 75.50 | 80.90 | 0.12 |
| GOT (IU/L) | 27.70 | 33.20 | 0.54 |
| γGT (IU/L) | 37.33 | 33.7 | 0.45 |
| ALK (IU/L) | 85.00 | 98.6 | 0.29 |
| TC (mmol/L) | 4.73 | 4.92 | 0.22 |
| TG (mmol/L) | 1.03 | 1.09 | 0.77 |
| HDL-c (mmol/L) | 1.24 | 1.37 | 0.24 |
| LDL-c (mmol/L) | 2.31 | 2.81 | 0.13 |
| CRP (mmol/L) | 0.18 | 3.20 | 0.07 |
| TnT (ng/mL) | 0.01 | 0.02 | 0.05 |

Metabolomic analysis indicated that more than 1,700 fragments were detected in each total ion chromatogram (TIC). The average resolved peak number in the TICs was more than 110 using the threshold of signal-to-noise ratio of 10. Figure 1 showed the typical TICs of case and control samples. In order to eliminate the casual discrepancy, the metabolomics data were processed as the following: variables (ion fragments) that emerged in less than 80% of the samples in either group were removed, and the left variables were subjected to orthogonal signal correction processing. Evaluation of the two-dimensional separation pattern of the processed UPLC-MS data by OPLS-DA led to a clear clustering of two distinct populations in the scores plot (Fig. 2A), demonstrating the validity of metabolomics data to the differentiation of SMI and the control groups.

## DISCUSSION

Biosystems that underpin life itself are yielding their secrets as our analytical ability improves. With the advent of many high-throughput and high-sensitivity analytical tools, a new concept has been coined that perturbations arising either as a cause or a consequence of disease may be detected as particular patterns of metabolites or proteins in the biofluid (9,15,16). In the current assay, SMI was provoked by exercise tests in some individuals. TnT, CK-MB, LDH, HBDH, etc., were the commonly used parameters to evaluate myocardial infraction (17), but Table 1 indicated that these clinical parameters were nearly of no value for the differentiation of SMI and normal individuals.

Because the multivariable analysis provides a measure of how correlated changes in variables (say, up- or downregulation of multiple metabolites) can be significant, even if the individual metabolites are not statistically discriminatory by univariate assay, the whole metabolomics data were subjected to orthogonal signal correction PLS-DA. Of note, SMI patients and the normal controls could be distinguished without any ambiguity in the scores plot (Fig. 2A). Correlation loadings plot of Figure 2B displayed the correlation between each variable and the first score component. It was clear that variables (metabolite) 1 and 2 were strongly positively covariant to the normal control group ($|r|>0.8$) and negatively covariant to the SMI population. Conversely, metabolites 3 and 4 showed slight positive relationship to the SMI group with the correlation coefficient of about 0.5 (thus, we would not pay much more attention to discuss them). Because these four metabolites were the furthest variables from the origin, they were the most important metabolites to differentiate the two populations (18). These four variables were all confirmed to be quasi-molecular ions,

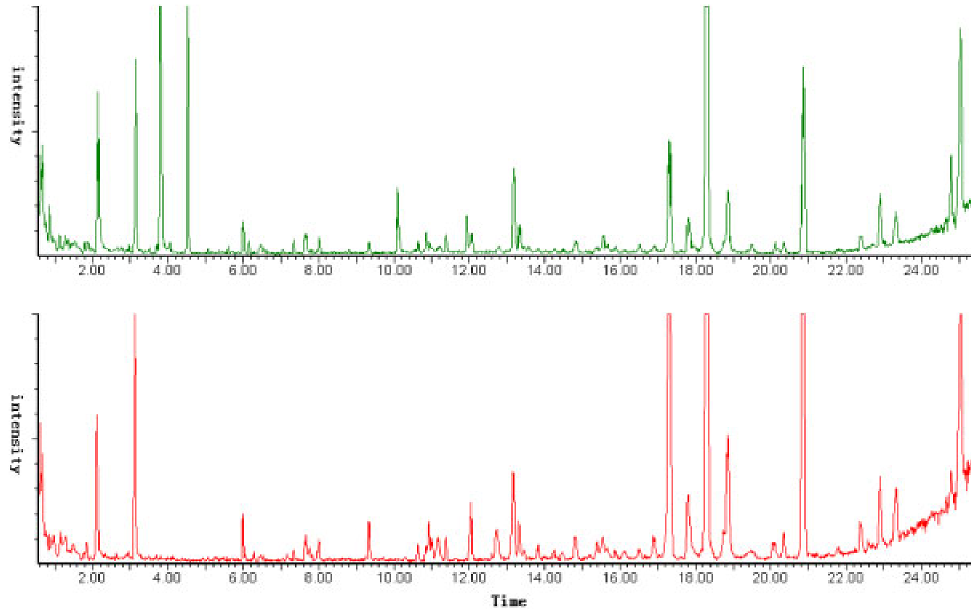


**Fig. 1.** The typical plasma UPLC-Q-TOF mass spectrograms (TICs) of a SMI patient (**top panel**) and a normal control (**bottom panel**) with different metabolite profiles.

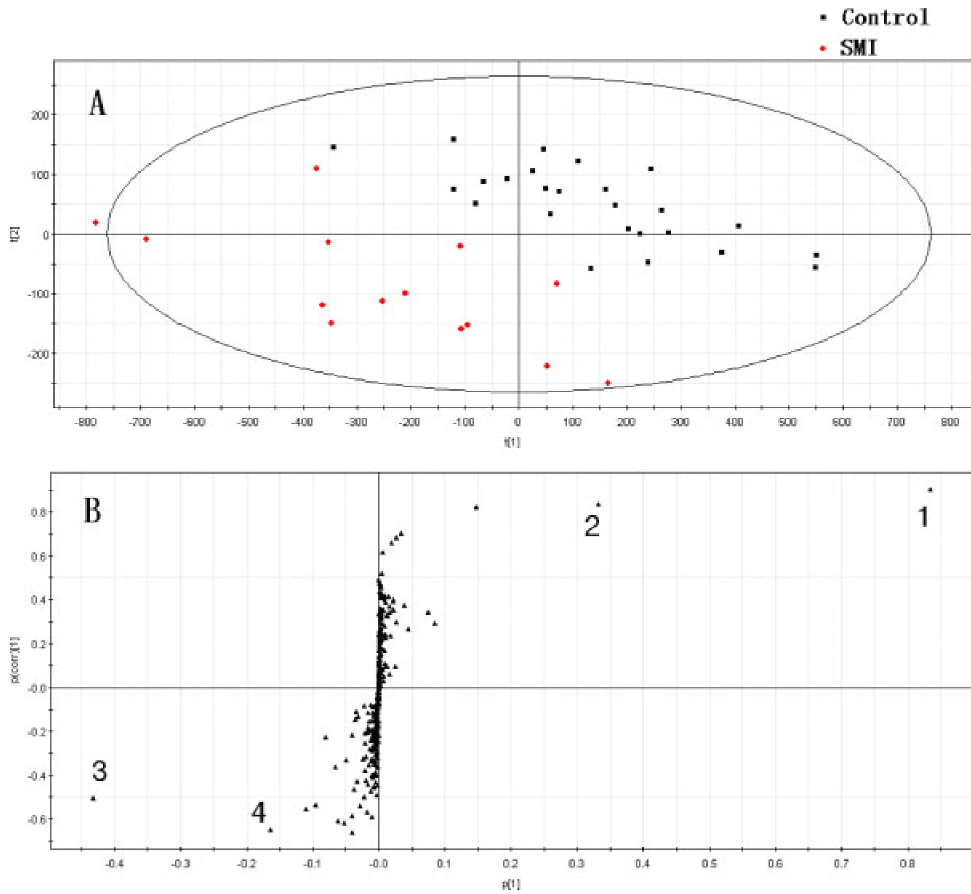


**Fig. 2.** Scores (**A**) and correlation loadings (**B**) plots generated using metabolomics data processed by orthogonal signal correction PLS-DA. 1, 2, 3, and 4 were the most important variables dominating the differentiation model.

and their identities were verified as phospholipids as listed in Table 2 using commercial standards. Exemplarily, Figure 3 showed the identification of metabolite 2 in Figure 2 with an $m/z$ 496, a molecular ion, by MS/MS. Besides many other fragments, the $MS^2$ spectrum showed two characteristic moieties with $m/z$ 184.127 and 313.355. The identification of $m/z$ 496 could also be supported by peaks of $m/z$ 478.337 and 991.710 as $[M+H-H_2O]^+$ and $[2M+H]^+$. Finally, it was identified as 1-palmitoyl-sn-glycero-3-phosphocholine (C16:0) by comparing retention time and $MS^n$ fragments with the commercial standard. The other phospholipids were also identified based on the same strategy. Average signal intensities of the four phospholipids in both groups are depicted in Figure 4. Analysis of variance indicated that only metabolite 1, 1-linoleoylglycerophosphocholine (C18:2), was decreased significantly in the plasma of SMI population ($P = 0.01$).

**TABLE 2. Potential Biomarkers Identified in Plasma of SMI Patient**

| Number | $m/z$[a] | Assignment | tr[b] |
|---|---|---|---|
| 1 | 520.3 | Lysophosphatidylcholine (C18:2)+H | 17.3 |
| 2 | 496.3 | Lysophosphatidylcholine (C16:0)+H | 18.3 |
| 3 | 524.3 | Lysophosphatidylcholine (18:0)+H | 20.9 |
| 4 | 758.6 | Phosphatidylcholine (16:0, 18:2)+H | 25.0 |

[a] $m/z$: mass/charge ratio in positive mode detected by mass spectrometry.
[b] tr: retention time (min).

Phospholipid lysophosphatidylcholine (LPC) is produced by the action of the proinflammatory phospholipase $A_2$ on phosphatidylcholine (PC) and promotes inflammatory effects, including increased endothelial expression of adhesion molecules and growth factors (19,20), monocyte chemotaxis (21), and even has been implicated in the pathogenesis of atherosclerosis (22). Previously, study on isolated hamster hearts under ischemic and hypoxic conditions indicated that a 51% reduction in the biosynthesis of PC was observed in the ischemic heart and a 22% reduction in the biosynthetic rate of PC was also detected in the hypoxic heart. The lower level of circulating PC was thought to be the results of insufficient supply of adenosine triphosphate (ATP) and cytidine triphosphate (CTP) (23). Our results were in agreement with the above-mentioned reports in that at least one kind of downstream product of PC was in lower level in the SMI group (Fig. 4). Studies performed on a rabbit model of acute global MI also demonstrated that inhibition of 1-palmitoyl-lysophosphatidylcholine (C16:0) production could prevent extracellular $K^+$ accumulation, which is the most profound abnormality during acute MI (24). Figure 2B showed that 1-palmitoyl-lysophosphatidylcholine (C16:0) was strongly negatively ($|r| > 0.8$) correlated with SMI patients. This might be a hint that organism could reactively decrease the endogenous generation of 1-palmitoyl-lysophosphatidylcholine (C16:0) to resist the occurrence of MI.

In view of the structure of membrane, phospholipids are the important components in the lipid bilayer.

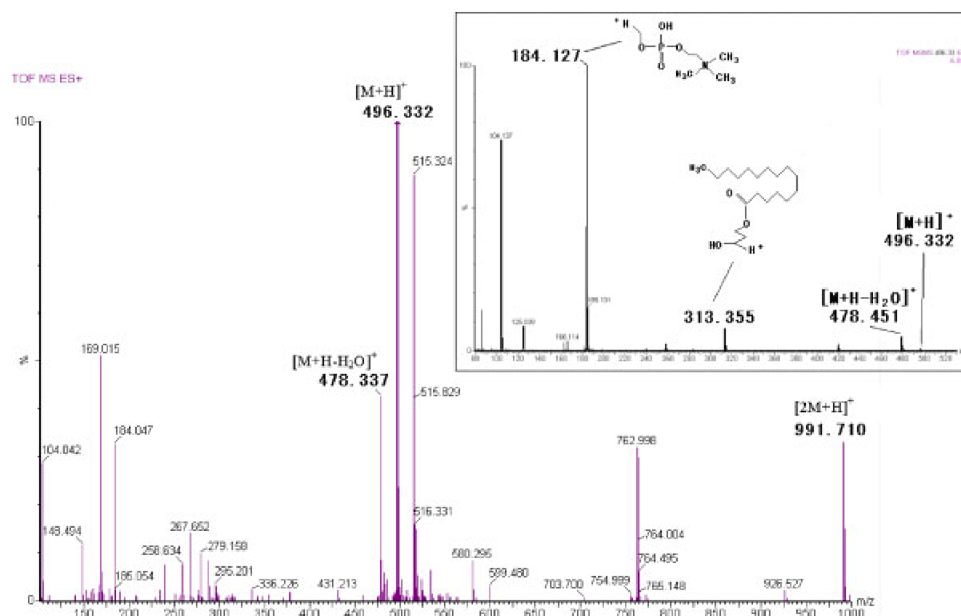


**Fig. 3.** Mass spectrograph of a metabolite showing different levels in the two populations identified through MS/MS (small graph) as 1-palmitoyl-sn-glycero-3-phosphocholine (lysoPC C16:0).

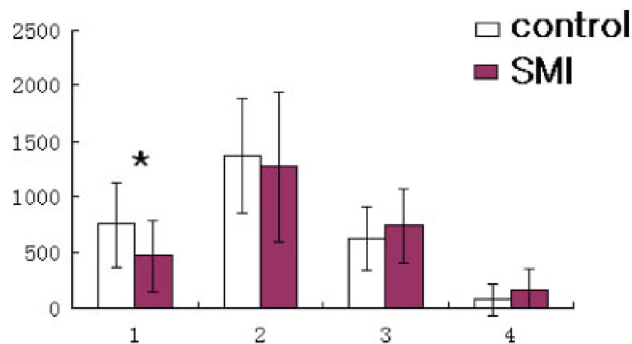


**Fig. 4.** Box plot of the four identified metabolites listed in Table 2 using their absolute intensities detected by mass spectrometry.

Myocardial enzymatic changes lead to cell membrane destruction. The changed phospholipids found in this study implied that, although, SMI had not caused detected cell lysis, the membrane changes stemming from ischemia had taken place. This sign could be utilized as sensitive and early SMI detection in theory and preliminary indicated by our study. Amid the four detected metabolites, only one component was statistically significant by univariate analysis, whereas their covariant relationship was of great value for differentiation purpose via multivariate analysis.

In summary, our findings provided the evidence that SMI was accompanied by plasma phospholipid changes. Compared with traditional diagnostic procedures, metabolomics analysis might be a potential tool to facilitate SMI screening and identification. Hopefully, with proper development and large scale, time serial studies, metabolomics will help clinician to identify new biomarkers and new therapy targets for cardiovascular diseases.

## ACKNOWLEDGMENTS

We thank the friendly help from Clinical Laboratory, Dalian Municipal Central Hospital and the valuable work of the whole nurses in Cardiology Division.

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