Metabolomic profile for the early detection of coronary artery disease by using UPLC-QTOF/MS

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Abbreviations

CAD, coronary artery disease; AMI, acute myocardial infarction; UPLC-QTOF/MS, ultra-performance liquid chromatography coupled to time of flight mass spectrometry; AUROC, area under the curve; Cer, ceramide; IDA, information-dependent acquisition; QC, quality control; PC, glycerophosphatidyl choline; LysoPC, Lysoglycerophosphatidyl choline; PE, glycerophosphatidyl ethanolamine; LysoPE, Lysophosphatidyl ethanolamine; PG, glycerophosphatidyl glycerol; PI, glycerophosphatidyl inositol; SM, sphingomyelin; TG, triacylglycerol; PA, glycerophosphate; DHEAS, dehydroepiandrosterone sulfate; PCA, principal component analysis; OPLS-DA, orthogonal partial least-squares discriminant analysis.

Abstract

Traditional risk factors cannot promote prediction capacity for the patients with coronary artery disease (CAD), who usually do not show apparent symptoms until they develop acute myocardial infarction (AMI). As such, novel predictive diagnostic strategies are essential to accurately define patients at risk of acute coronary syndrome. In this study, non-targeted metabolomic profiling using ultra-performance liquid chromatography coupled to time of flight mass spectrometry (UPLC-QTOF/MS) was performed in combination with multivariate statistical model to analyze the serum samples of patients with stable angina (n = 38), acute myocardial infarction (AMI) (n = 34) and healthy age- and gender-matched controls (n = 71). Results showed a clear distinction in metabolomic profiles between stable angina and AMI when using OPLS-DA with both positive and negative models. Internal cross-validation methods were used to confirm model validity with an area under the curve (AUROC) = 0.983. We identified various classes of altered metabolites including phospholipids, fatty acids, sphingolipids, glycerolipids and steroids. We then demonstrated the differential roles of these metabolites using multivariate statistical model. Phospholipids previously associated with CAD were shown to have lower predictive capacity to discriminate AMI patients from stable angina patients. Interestingly, ceramides, bile acid and steroids hormone such as Cer(t18:0/16:0), Cer(d18:0/12:0), dehydroepiandrosterone sulfate (VIP scores of 1.99, 1.97, 1.64, respectively), were found to be associated with the progression of CAD. These results suggest that metabolomic approaches may facilitate the development of more stringent and predictive patient criteria in the diagnosis and treatment of CAD.

Keywords: Coronary artery disease; metabolomics; UPLC-QTOF/MS; Human serum; Stable angina; Acute myocardial infarction

1. Introduction

Coronary artery disease (CAD) is the leading cause of morbidity and mortality worldwide, and is becoming increasingly prevalent in China [1,2]. The mortality associated with CAD has dramatically risen in Chinese populations relative to all other diseases [3], which therefore contributes significantly to the overall increase in health care costs. CAD is the result of complex metabolic dysfunctions that arise from various environmental and genetic influences. Many anabolic and catabolic pathways involve lipid, amino acid, bile steroid and carbohydrate metabolism, all of which are commonly disrupted in typical CAD events. Dysfunctional metabolism associated with inflammation and oxidative stress often results in the development of atherosclerosis and ultimately stable angina pectoris, a symptom that caused by the partial obstruction of coronary arteries [4]. Complete thrombotic occlusion of a coronary artery causes myocardial necrosis and is termed acute ST-elevation myocardial infarction also known as acute myocardial infarction (AMI) [5]. Proper diagnosis of this condition prior to the progression into AMI is crucial to preventing associated morbidity and mortality of this disease.

Efficient diagnosis of CAD is often not available as the clinical presentation of the disease often overlaps with that of patients with life-threatening acute coronary syndromes. Moreover, AMI and stable angina are not always present at the time of initial patient examination [6]. Thus, it is important to identify high-risk factors for diagnosis and intervention at various stages of CAD, which could ultimately result in early CAD prevention and personalized therapy for treating this disease [7]. However, the Chinese population has resorted to a so-called ‘epidemiological transition’. This transition is related to the acceleration in urbanization, industrialization, and the westernized lifestyle – especially related to dietary options in China [8,9]. With this

transition, the traditional CAD risk factors, such as smoking, obesity, hypertension, dyslipidemia, and hyperglycemia, have not been able to increase the predictive value for the disease [10].

Metabolomics is established method used to identify biomarkers for CAD, and results have demonstrated both feasibility and flexibility across physiological, interventional, and epidemiological human studies [11]. Three core technologies have prevailed as a common platform for metabolite profiling: nuclear magnetic resonance spectroscopy (NMR) [12], gas chromatography coupled to mass spectrometry (GC-MS) [13] and liquid chromatography coupled to mass spectrometry (LC-MS). Recent advances in LC-MS have strongly influenced the evolution of metabolomics. Compared to other techniques, LC-MS has an increased sensitivity as well as range of metabolites [11]. Ultra-performance liquid chromatography coupled to time of flight mass spectrometry (UPLC-QTOF/MS) with information-dependent acquisition (IDA) has benefits in deconvolution and resolution of coeluting metabolites. Furthermore, an automated external calibration system for mass accuracy with an injector system provides robust and reproducible response intensity. These advanced methodologies make it possible to identify and quantify hundreds of molecular species from human samples [14-16].

Applications of metabolic profiling in CAD have been developed using established animal models [17,18]. Human tissue has also been used with UPLC-QTOF/MS for the purpose of studying the effects of atherosclerosis [19]. However, studies using metabolomics as a potential diagnostic criterion for AMI and stable angina in human samples are limited, especially in China. Although a recent report applied LC-QTOF/MS for the characterization of AMI and stable angina in human serum samples [20], but they did not provide a predictive value of their model.

In this work, non-targeted metabolomic profiling combined with multivariate statistical techniques was used to study the changes in serum to characterize the

metabolic progression from stable angina to AMI. We used UPLC-QTOF/MS to identify misregulated metabolites. OPLS-DA analysis revealed that these altered metabolites could be classified into two patient groups. We focus on developing rapid and feasible metabolomics approaches to predict and distinguish various stages of CAD. The understanding and characterization of these normal and altered pathways will ultimately help to establish a specific metabolomic signature associated with varying stages of disease.

2. Materials and methods

2.1 Study design

A total of 72 patients with significant CAD were included in the study (Supplementary Table S1) that was obtained from Hangzhou First People's Hospital, Hangzhou, China. The selected cohort was composed of 38 and 34 individuals diagnosed with stable angina and AMI, respectively. The average age of the cohort was stable angina: 68.8 ± 9.3 and AMI: 65.1 ± 12.1 . 62.0% and 63.2% of stable angina and AMI were male. Controls ($n = 71$) were healthy, age- and gender-matched volunteers with no declared history of CAD. All samples were obtained after overnight fasting and were collected by venipuncture or through a tube that contains a clot activator and a gel for serum separation. Samples were stored at room temperature until processing. Samples were processed within 6 hours of collection and frozen at $-80\text{ }^{\circ}\text{C}$ until the time of analysis. The study was approved by Hangzhou First People's Hospital following the Helsinki Declaration.

2.2 Metabolite extraction

100 μl of serum was precipitated by adding 300 μl of precooled acetonitrile and vortexing for 1 min. Precipitated protein was then removed by centrifugation (15,000

g, 30 min) at 4 °C. To avoid operational inaccuracy, every sample was prepared in triplicate, and 100 µl supernatant was subsequently combined into microcentrifuge tube, and stored at −40 °C until further analysis.

2.3 UPLC–QTOF MS/MS analysis

Liquid chromatography was performed using a Nexera UHPLC-CBM20Alite system (Shimadzu, Tokyo, Japan) with a pump (LC30AD), a column oven (CTO30A) and an autosampler (SIL30AC). Mobile phase A was 0.1% formic acid aqueous and mobile phase B was acetonitrile. The gradient was 0 min, 4% B; 3 min, 5% B; 3.5 min, 15.2% B; 11 min, 16% B; 15.5 min, 23% B; 21 min, 65% B; 26 min, 100% B, 30 min, 100% B, then back to 4% B in 0.1 min and equilibrated for 5 min for the next injection. The column oven and the flow rate were set at 40 °C and 0.3 ml/min, respectively. Samples (2 µl) were injected onto a Waters 100 mm× 2.1 mm ACQUITY BEH C18 1.7 µm reversed phase column (Waters, Ireland).

Mass spectrometry was performed with a quadrupole-TOF MS (TripleTOF 5600+, AB Sciex, Concord, Canada) operated in positive and negative mode with a DuoSpray ion source. All analyses were performed at the high sensitivity mode for both TOF MS and product ion scan. The automated calibration device system (CDS) was set to perform an external calibration for each sample. For ESI positive ion mode: source temperature, 600 °C; curtain gas (CUR), 25; both GS1 and GS2 at 50; ion-spray voltage floating (ISVF), 4.5 kV; collision energies (CE), 30 eV. Data acquisition was performed using the information-dependent acquisition mode: TOF as survey scan (accumulation time 250 ms) and MS-MS as dependent scan with a CE, 40 eV; collision energy spread (CES), 15 eV in 8 MS-MS dependent experiments (accumulation time 90 ms). For all experiments, the TOF mass range was m/z 100-1200. For ESI negative ion mode: source temperature, 600 °C; CUR, 25; both GS1 and GS2 at 50; ISVF, 4.5 kV; CE, 30 eV; IDA mode: TOF as survey scan

(accumulation time 250 ms) and MS-MS as dependent scan with a CE, -40 eV; CES, 15 eV in 8 MS-MS dependent experiments (accumulation time 90 ms). For all experiments, the TOF mass range was m/z 100-1000. MS data acquisition was performed using Analyst TF (version 1.5.1, AB Sciex).

Randomized samples were injected twice to obtain chromatograms in both ionization modes. A standard QC strategy [19] was used for the UPLC-MS analysis. The QC sample was prepared by mixing equal volumes (100 μ l) from each of the 143 samples (including samples from patients and control) and was injected before and after the experimental samples to assess instrument stability and analyte reproducibility. The ESI source was cleaned every day to avoid carryover of samples. A stability study was performed by repetitive injections of samples to avoid variability associated with disturbances in metabolic profile.

2.4 Data extraction

Raw LC-MS data were imported in Peak View (version 1.1, AB Sciex) and Marker View (version 12.1, AB Sciex). The tolerance window for both parameters was set at 0.5 min and 10 ppm for elution time and mass accuracy, respectively. Saturated peaks were removed prior to total sum area based on module generated using Marker View. Normalized intensities were then calculated for each sample in each dataset (positive and negative mode).

2.5 Statistical analysis

Descriptive statistics compared the clinical and characteristics between different groups. Welch's two-sample t tests were used for continuous variables and exact methods for categorical ones. All tests were 2-sided and statistical significance was declared for values of $P < 0.05$.

Unsupervised (principal component analysis, PCA) and supervised (orthogonal partial least-squares discriminant analysis, OPLS-DA) multivariate pattern recognition techniques were applied to pre-processed metabolite concentration data to discriminate between sample features of healthy controls, stable angina and AMI patient samples using SIMCA (Version 13.0.2, Umetrics). For every samples overview, PCA reduced the dimensionality of measured variables to load the features on scores plot. By applying the integrated orthogonal signal correction filter, OPLS-DA provides clear class discrimination and avoids overfitting. It allows for a direct comparison of the variance between degree of disease status (stable angina or AMI; y variable) and metabolite concentrations (x variables).

The accuracy for classification and regression was assessed by means of double cross-validation and permutation testing [21,22]. The original data set was split into a training set (90%) and a test set (10%) prior to any step of statistical analysis. No samples in the test set were used for parameter selection. A receiver operating characteristics (ROC) curve was generated to define the predictive accuracy of the OPLS-DA model from 7-fold cross validation (predictive Y variables, in the SIMCA software). Area under the ROC curve (AUROC) was calculated by using SPSS statistical software (version 22.0, IBM, Armonk, NY, USA). The variable influence on projection (VIP) parameter was generated for a weighted, quantitative measure of discriminatory power of the metabolites. Represented by a unitless number, the higher the value, the greater is the discriminatory power of the metabolite. VIP scores >1 were considered the most class discriminating of those metabolites [23].

2.6 Structural assignment of candidate biomarkers

For metabolite structure assignment, the sample chromatographic peaks for each sample were first matched to metabolites from online MS databases (Metlin[24] HMDB [25] and Lipidmaps [26]). Metabolite identification was performed by

matching accurate m/z and isotopic pattern with a mass accuracy less than 10 ppm. The robustness of the identification was confirmed by matching the masses of the fragments from the MS/MS spectra for each metabolite.

3. Results

3.1 Optimization of UPLC-QTOF/MS analysis

Serum metabolites from CAD patients were analyzed with a global approach using a non-targeted method of analysis. Optimization of the chromatographic separation protocol was important due to: 1) highly abundant features in chromatographic peaks that could affect the detection of smaller molecules. 2) The high abundance of lipids present in the serum of CAD patients [27]. 3) Ion suppression induced by the coelution of lipids of the same class, interclass separation is important as it can help in the identification of specific lipid subtypes based on structural properties. Three different elution gradients (21, 30, 35 min) were chosen for optimization, and every test was repeated 5 times. Supplementary Figure S1 shows base peak chromatograms and IDA dot charts from the analysis of serum pools using the three chromatographic methods using in positive ionization mode. Comparing 21 min and 30 min tests, abundant constituents were separated within the initial 12 min, which significantly increased the appearance of features (7735 to 10539). In the 35 min test, lipids with low polarity were separated within the last 15 min, which released more information regarding the subclass of lipids. The performance of the gradient program to adequately re-equilibrate the system was also assessed. This process was set to 5 min to accommodate adequate stability prior to each injection, and to ensure total analysis not exceeding 35 min. Additionally, longer chromatographic method did not improve the final resolution in any ionization modes (data not shown). Finally, the 35 min gradient method was selected for analysis of

samples from the complete cohort of individuals.

3.2 Quality control and analytical reproducibility

For LC-MS analyses, an efficient and reproducible method is crucial in order to obtain reliable metabolic profile data. The use of QC samples is generally preferred to assess reproducibility instrument performance and stability in metabolomic studies [20, 28]. However, thousands of features could be obtained in a single sample. It is essential to use a multivariate approach in order to properly interpret this type of data. PCA scores plots can be used as statistical strategy for evaluating analytical reproducibility and stability. In addition, peaks' retention time (RT) and area reproducibility was evaluated using the 13 QC samples interspersed throughout the run to calculate the coefficient of variation (CV%). The QC samples were localized in the center of the PCA scores plot (Figure 1). Compared to the distribution of the other samples, this result highlights the reproducibility and instrument stability throughout the whole process.

Each analysis took 35 min to be completed, which means that the complete study was finished within 72 h. The most abundant peaks per RT window in the QC samples were used to evaluate in short term (consecutive run) and long term (within 72 h). The results are shown in Supplementary Figure S2. The peaks of these QC samples demonstrated an RT and area CV% values less than 6. The high retention time and analytical reproducibility of the detected peaks demonstrate that the presented methodologies provide the robustness and precision required for a thorough metabolic profiling study.

3.3 Multivariate analysis for discrimination of CAD diagnostics

Before further analysis, two steps were executed: 1) Drug and drug metabolites

were excluded, such as aspirin and its metabolite salicylic acid, and iohexol which is used as intravascular contrast media in CAD patients. Other common medicine for cardiovascular disease such as calcium channel blockers, aspirin, statins, beta-blockers, diuretics, ACE-I or ARB and clopidogrel were also excluded. Since this work focuses predominantly on endogenous compounds, the drug and drug metabolites were not included in database for statistical model. 2) Only those molecular features that experience a p value of less than 0.05 (t -test) and Log_2 fold change $> \pm 1$ were considered between pairs of groups. Also, the features not detected in at least 75% of samples in one condition (stable angina or AMI) were excluded. The database was created and contained information about the molecular weight, formula, and retention time of each endogenous compound. Parameters were set at 10 ppm for mass accuracy and 0.5 min for retention time to minimize errors from quantitation of isomers.

This database was analyzed using both unsupervised (PCA) and supervised (OPLS-DA) multivariate pattern recognition. Group was classified based on disease status (healthy control, stable angina, AMI) at first observation shown on the PCA scores plot (Supplementary Figure S3). A clear separation between samples from healthy controls ($n=71$) and CAD patients ($n=72$, including patients with stable angina and AMI) was observed. The samples from stable angina ($n=38$) and AMI ($n=34$) also formed distinct groups. Thus, we have identified distinct metabotypes of various disease states.

Supervised OPLS-DA was further applied to develop a statistical model with the capability to discriminate between the two groups diagnosed: stable angina and AMI (Figure 2). It helps us to optimally extract candidate metabolites responsible for differentiating between two disease groups. OPLS-DA model parameters for explained variation (R^2) and cross-validated predictive ability (Q^2) were desirable: stable angina vs AMI: $R^2 = 0.583$, $Q^2 = 0.52$; $R^2 = 0.318$, $Q^2 = 0.226$, in positive and negative models, respectively. The trend of most altered candidate metabolites

according to the VIP scores and fold change is reflected in Figure 4. It is apparent that simultaneous perturbations in ceramides, bile acid and steroids hormone metabolic pathways are responsible for the observed class separation.

3.4 Characterization of altering metabolite structure

Manual match validation of mass accuracy and MS/MS spectral was further performed to confirm the identification of metabolites. Several classes of metabolites were identified in the cohort of CAD patients, including phospholipids, fatty acids, sphingolipids, glycerolipids and steroids. The preference of the MS/MS spectra of family dependent product ions or neutral losses for each metabolite feature is strongly associated with the class of metabolites. Characteristic MS/MS spectra of a number of representative fragments have been previously described [29].

In particular, phospholipids are the most common family of metabolites detected in human serum. The MS/MS spectra of polar head group and alkanoyl chains of glycerophospholipids are most useful to identify the glycerophospholipid subclass [27,29]. Glycerophosphatidyl cholines (PC) are optimally detected in both positive and negative ionization mode. Phosphocholines ($C_5H_{15}NO_4P^+$, m/z 184.0721) represent the primary fragment observed from PC by ESI-MS/MS with positive ionization mode, and neutral loss of m/z 79.9651 was also easily observed in spectra which represent the phosphate group of the PC structure. Glycerophosphatidyl ethanolamines (PE) with their positive and negative ions were observed in high abundance that corresponded to $[M+H]^+$ and $[M-H]^-$. The polar head group (phosphoethanolamine) of PE was well observed: m/z 141.0207 and 140.0101 in positive and negative ionization modes, respectively. Glycerophospholipids (PI) contain a phosphodiester of the six-carbon sugar inositol as a polar head group. Unique fragment such as m/z 241.0116 and neutral loss of m/z 180.0639 in negative ionization modes that represent the headgroup was also observed in our study. In

addition, the loss of the *sn*-1 and *sn*-2 fatty-acyl substituents as neutral carboxylic acids and ketene were also yielded in MS/MS spectra. These results correspond to those from a study by Hsu et, al.[30].

The electrospray ionization of sphingomyelin (SM) closely follows the trend of related PC. They have the same fragmentation pattern in positive ionization with the product ion m/z 184.0721, which corresponds to the phosphorylcholine moiety. However, collision-induced decomposition of the fatty-acyl group (ceramide) could provide us useful information to define SM. Steroids are usually present in serum mainly as the sulfate form [31]. Thus, it is relatively easy to observe specific ions at m/z 96.9600 and neutral loss of m/z 79.9575 in negative ionization mode, which corresponds to the sulfate moiety.

Low molecular weight molecules such as uric acid as well as some free fatty acids such as oleic acid and palmitic acid were identified using standard MS/MS spectra found in Metlin (<https://metlin.scripps.edu/index.php>). A few representative MS/MS spectra used for structural elucidation along with parent and fragment ion structures are presented in Supplementary Figure S4. All selected metabolite information about RT, molecular formula (adduct), mass error, precursor ion, and characteristic product ions were listed in Supplementary Table S2.

3.5 Development and validation of a prediction model

As described above, many candidate metabolites were selected for the initial statistical modeling analysis. Each metabolite offered different contribution to the prediction model. For further analysis, permutation testing and cross-validation were performed to establish internal validation. Given the small sample size, permutation testing (1000 times) performed better with random and repeated assignment of class labels. To validate the model against overfitting, a 7-fold cross-validation step was employed: 7 models were built with exactly one seventh of the data excluded from

each model and each sample excluded a single time. This procedure was repeated in a precise manner to ensure that each sample had been excluded one time [32]. Although these validation methods resulted in the omission of a portion of the data, the power of prediction was enhanced. The overall predictive ability of the metabolite profiling method was evaluated by using Y-predcv values. Model sensitivity and specificity comparing CAD and controls, are shown with an ROC curve (Figure 3). The calculated AUROC of 0.938 is indicative of strong predictive power for stable angina patients versus AMI patients.

4. Discussion

Metabolomic analysis of serum from CAD patients was executed with a global approach based on non-targeted UPLC-QTOF/MS. CAD events were known as metabolism syndrome and always accompanied with many comorbidities, such as diabetes, non-alcohol fatty liver disease, hyperglycemia and dyslipidemia. Given the complexity of these disorders, non-targeted metabolites profiling was option for exploring CAD-associated disease. Many previous reports have utilized this technique to identify markers for diagnostic and prognostic purposes. However, in the majority of these studies have focused mostly on lipids profiling [27,33] or polar metabolite analysis [34-35]. Due to the large number of misregulated metabolites with a vast range of chemical structures and properties present in serum of CAD patients, a global unbiased metabolomic analysis is essential in developing a chemical profile of this disease.

In this work, 124 altered metabolites in the dataset were identified based on the algorithm of permutation testing and cross-validation. The MS/MS spectra of these metabolites were identified with high confidence based on existing spectra in online libraries and published literature. We also compared the level of these metabolites

between CAD patients and healthy control individuals, as well as between patients with stable angina and AMI. The results demonstrate that multivariate models using certain classes of metabolites can accurately distinguish stable angina from AMI patients.

Lipids were the most abundant metabolites found in human serum. Lipids play a key role in a number of processes such as platelet-activation [36], cell signalling, and formation of the cytoskeleton [37]. Dysfunctional lipid metabolism is often associated with inflammation, oxidative stress and inducing macrophage apoptosis, which greatly increases the overall risk for atherosclerotic CAD [4]. Age-dependent mitochondrial dysfunction is closely correlated with abnormal mitochondrial fatty acid oxidation which in turn increases CAD risk [38]. Identified lipids in this study include subclasses of glycerophospholipids, sphingolipids, glycerolipids and fatty acids.

The glycerophospholipids was one of the most prominent significantly altered lipids among different groups. PE and PC are two major subclasses of glycerophospholipids. PE is a glycerophospholipid in which a phosphoryl ethanolamine moiety occupies a glycerol substitution site. As is the case with diacylglycerols, PE can have many different combinations of fatty acids (including palmitic acid and arachidonic acid) of varying lengths and saturation attached at the C-1 and C-2 positions [39]. It is the source of saturated and unsaturated fatty acid via the action of phospholipase. In our dataset (Supplementary Figure S5 and Supplementary Table S2), most of PE and lysophosphatidyl ethanolamine (LPE) species showed a strong negative association with CAD (versus control), but most species demonstrated no significant difference between AMI and stable angina patients. The decreased concentration of PE species in serum of CAD patients may be attributed to higher oxidative stress and catabolism of polyunsaturated phospholipids into tissues, as previously described [19,40,41]. However, most identified PEs do not correlate with the progression of CAD.

Lysoglycerophosphatidyl cholines (LPCs) displayed a similar trend with PEs (Supplementary Figure S6). LPCs are generated through the action of phospholipase A2 or derived from the polar surface of lipoproteins. Many previous studies have reported that LPCs regulate important physiological and pathophysiological processes. The contribution of LPCs in patients with CAD has implications for cellular signalling and inflammation in the development of different pathologies [42,43]. Oxidized LDL, which contains a high content of LPCs, has been shown to promote atherogenesis by inducing apoptosis [44]. LPCs also can be detected in atherosclerotic lesions, which help to explain the relatively lower levels of LPCs in circulation. Most LPCs were slightly lower in the stable angina group compared to the AMI group, however this difference was not statistically significant (Fold change < 1.5, VIP scores < 1). This effect has been previously reported by Fernández [45] and Santiago [27].

Most PCs showed a negative association with CAD compared to healthy controls. However, some PC species such as PC(20:5/16:0) and PC(20:4/20:5) combined with long-chain polyunsaturated fatty acids, which were decreased in the stable angina group compared to the AMI group (Supplementary Figure S6). PCs and LPCs play an important role in arachidonic acid metabolism, which is associated with the biosynthesis of the prostaglandins and other eicosanoids that are involved in the activation of monocytes and macrophages [46]. Meikle [4,33] et al. confirmed that plaque instability was the result of dysfunction of arachidonic acid metabolism. Further evidence for this is the similar trend of free fatty acids that were detected in serum (Figure 4 and Supplementary Table S2).

The AMI phenotype is likely more heterogeneous in etiology and difficult to predict in general. What is more, dyslipidemia appears paradoxically protective against cardiovascular events, because those patients are treated with medication but also because of unmeasured confounders [47]. Our results suggest that the glycerophospholipid species plays differential roles of in the onset and progression of

CAD, and the variation of myocardial fatty acid uptake capacity and abnormal glycerophospholipids' metabolism are likely important reasons for the significant differences between two patients groups.

The opposite trend is shown by ceramides, the concentration of which are higher in patients with stable angina compared to patients with AMI (Figure 4 and Figure 5). Levels of ceramides were slightly higher in healthy control with fold change less than 1.2. Ceramides also known as N-acylsphingosine are one of the byproducts of the hydrolysis of sphingomyelin by the enzyme sphingomyelinase. It is an important signal transduction metabolite in the subcellular fractions of human epidermis and circulating blood cells. The pathway of ceramide signalling activated in response to myocardial ischemia/ infarction [48]. Mello et. al. [49] reported that ceramides play a role in immune-related inflammatory responses in patients with CAD via the cytokine, IL-6. The fold change of most ceramide species detected in patients with stable angina were more than twice that of patients with AMI, as well as VIP scores > 1.5 (Figure 4 and Figure 5). The minor ceramide release to the circulation and/or increased rate of its phosphorylation could be the cause of this result. This result is consistent with Luan's study [50]. They reported decreased serum sphingolipids base levels in heart failure patients compared with CAD patients. Heart failure is the end product of myocardial damage, which is usually followed by AMI [51]. This suggests that the ceramides pathway is important in the progression of CAD, which is known for its involvement in atherosclerosis [52]. Interestingly, compared to other ceramide species, Cer(d18:1/24:0) show lowest level in stable angina group due to its unsaturated alkanoyl chain.

Bile acids are physiological detergents that facilitate excretion, absorption, and transport of fats and sterols in the intestine and liver (enterohepatic circulation). Bile acids are also steroidal amphipathic molecules derived from the catabolism of cholesterol. Thus, bile acids and associated receptors regulate lipid, glucose, and energy metabolism, which are usually used as detergents for the treatment

dyslipidemia and nonalcoholic fatty liver disease. Charach et al. [53] previously observed that CAD patients have significantly decreased bile acid excretion levels than healthy control. Additionally, Santiago [20] reported that patients with AMI presented the lowest level of bile acids. These results correspond to results from our study (Figure 4 and Supplementary Table S2). Bile acids clear cholesterol from the plasma and the intracellular compartment by activating the 7- α -hydroxylase and high density lipoprotein (HDL) transportation [54]. In the progression of CAD, inhibition of bile acids excretion results in the accumulation of cholesterol and low density lipoprotein (LDL). HDL-based therapies have been shown to fail in clinical trials [4]. We hypothesize that this is partly due to suppression of bile acids in the enterohepatic circulation of patients with acute CAD events.

CAD is a systemic and multifactorial disease that results from a variety of causes such as diet and composition of the gut microbiome [55]. Previous studies [56] have found that diet-related risk factors of CAD are significantly differentiated between populations of Chinese and Western countries. Several species of bile acid and sulfated steroids were observed in our study. All of them were susceptible to dietary and diurnal rhythm. These diet-related factors have been hypothesized to be important for the analysis of the progression of CAD in Chinese populations with starch-based diets. Sulfated steroid species demonstrated a high discrimination capability between stable angina and AMI groups (Figure 4, Figure 5 and Supplementary Table S2), such as dehydroepiandrosterone sulfate (DHEAS). DHEAS is a sulfated form of adrenal hormone, which has been shown to exert direct effects on insulin sensitivity and lipid metabolism [57]. It alleviates vascular inflammation or oxidative stress by a different mechanism, such as the activation of a G-protein-coupled receptor or peroxisome proliferator-activated receptor [58]. Measurement of DHEAS is preferable to DHEA, as levels are more stable. Tivesten et al. [31] demonstrated that decreased DHEA and DHEAS predict an increased risk of CAD, but not cerebrovascular disease. This study used a large population-based cohort analysis, and quelled the previous controversy

about the association between DHEAS levels and CAD [59-61]. However, the potential mechanisms of higher circulating levels of DHEAS being protective against CAD were inconclusive. In our study, the lowest level of DHEAS and other sulfated steroids were observed in the stable angina group, suggesting that these metabolites will be useful in the diagnosis and treatment of CAD.

Limitations of this study include small sample size and lack of an external, independent cohort of patients for model validation. In addition, homogeneity of enrolled patients was not prerequisite for this study. Potential drug interactions and associated comorbidities may have a confounding effect on our analysis. We also lack the ability to determine temporal relationships and causality of disease. Further studies that exclusively factor in specific comorbidities such as diabetes are needed to further refine our preliminary findings.

5. Conclusions

A dataset with 124 selected metabolites has been characterized, and achieved superior discrimination of disease status via cross-validated multivariate models, which specifically correlate with status via cross-validated multivariate models. These metabolites including phospholipids, fatty acids, sphingolipids, glycerolipids and steroids display differential expression in the onset and progression of CAD. Most analyzed phospholipids and fatty acids showed a strong negative association with CAD (compared to control), but demonstrated no significant capability to discriminate between patients with stable angina and AMI. Conversely, the relative level of ceramides was significantly decreased in the AMI patient group (compared to stable angina), and slightly higher in healthy controls (compared to CAD). Both bile acids and steroids hormone showed significant correlation with the onset and progression of CAD. Particularly, DHEAS was demonstrated to be one of the most perturbed metabolites as it was able to discriminate between healthy controls and CAD patients, as well as patients with stable angina and AMI (VIP score of 1.53 and

1.64, respectively). Taken together, the finding of discriminatory metabolites in serum preceding stable angina and AMI offers insight into disease pathogenesis.

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Author contributions

The study was conceived by Jingkui Tian, Jinyu Huang, Lin Zhang. The metabonomic profiling was by Xiaobao Xu, Beibei Gao. Qijie Guan and Dandan Zhang provided statistical and analytical support. Xianhua Ye, Liang Zhou, Guoxin Tong, Hong Li were responsible for sample collection. All authors reviewed and approved the manuscript.

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Figure captions

Figure 1. Scores plots of principal components analysis (PCA) models generated from the UPLC-MS analyses of human serum, demonstrating the biological samples and the quality control samples (QC). A: Positive electrospray ionization mode (ESI⁺) and B: negative electrospray ionization mode (ESI⁻).

Figure 2. Scores plot of the OPLS-DA model built to discriminate between patients diagnosed with stable angina and those diagnosed with acute myocardial infarction (AMI). A: Positive electrospray ionization mode (ESI⁺) and B: negative electrospray ionization mode (ESI⁻).

Figure 3. ROC curve analysis using cross-validated predicted-Y values of OPLS-DA model discriminating stable angina patients from AMI (A), as well as healthy control from CAD patients. ROC curve for all groups is represented by their area under curve (AUC). AUROC = 0.938 and 0.975 indicating strong predictive ability.

Figure 4. Statistically significant metabolite changes between healthy control and CAD patients (A, B), as well as stable angina patients and AMI patients (C, D), which demonstrate differential expression in the onset and progression of CAD. Bar plots represent, A: VIP scores obtained from multivariate statistical model differentiating healthy control and CAD patients. B: fold change of these metabolites obtained from healthy control compared to CAD patients. C: VIP scores obtained from multivariate statistical model differentiating stable angina patients and AMI patients. D: fold change of these metabolites obtained from stable angina patients compared to AMI patients. PC: glycerophosphatidyl choline; LysoPC: Lysoglycerophosphatidyl choline; Cer:ceramide; PE: glycerophosphatidyl ethanolamine.

Figure 5. Means and 95% least significance difference (LSD) intervals for each group of patients for the metabolites: PC(16:0/20:3), PC(16:0/20:5), dehydroepiandrosterone sulfate, Cer(d18:0/14:0), Cer(d18:1/24:0) and glycochenodeoxycholate sulfate. p-value of ANOVA test for those metabolites, and the CAD as categorical factors are also shown. PC: glycerophosphatidyl choline; Cer: ceramide.