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Plasma Phospholipids and Sphingolipids Identify Stent Restenosis After Percutaneous Coronary Intervention

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ABSTRACT

OBJECTIVES The aim of this study was to evaluate the diagnostic utility of plasma metabolomic biomarkers for in-stent restenosis (ISR).

BACKGROUND ISR remains an issue for patients after percutaneous coronary intervention. Identification of biomarkers to predict ISR could be invaluable for patient care.

METHODS Next-generation metabolomic profiling was performed in the discovery phase from the plasma of 400 patients undergoing percutaneous coronary intervention. In the validation phase, targeted analysis was conducted using stable isotope dilution–multiple reaction monitoring mass spectrometry in another independent group of 500 participants.

RESULTS A set of 6 plasma metabolites was discovered and validated for the diagnosis of ISR as early as 1 month after percutaneous coronary intervention. This biomarker panel classified patients with ISR and control subjects with sensitivity of 91% and specificity of 90% in the discovery phase. The diagnostic accuracy in the independent validation phase was 90% (95% confidence interval: 87% to 100%). The defined 6 metabolites all belong to sphingolipid and phospholipid metabolism, including phosphatidylcholine diacyl C36:0, phosphatidylcholine diacyl C34:2, phosphatidylinositol diacyl C36:4, phosphatidic acid C34:1, ceramide, and sphingomyelin diacyl 18:1/20:1. These biomarkers play essential roles in cell signaling that regulates the proliferation and migration of vascular smooth muscle cells.

CONCLUSIONS Next-generation metabolomics demonstrates powerful diagnostic value in estimating ISR-related metabolic disturbance. The defined plasma biomarkers provide better early diagnostic value compared with conventional imaging techniques. (J Am Coll Cardiol Intv 2017;■:■–■) © 2017 by the American College of Cardiology Foundation.

With the rapid advancement of interventional cardiology over the past decade, percutaneous coronary intervention (PCI) has become the treatment of choice for atherosclerotic coronary artery disease revascularization. The major limitation of PCI is in-stent restenosis (ISR), which is characterized by intimal hyperplasia, smooth muscle cell proliferation, and vascular renarrowing (1).

The rate of ISR with bare-metal stents ranges from 20% to 30% (2) and with drug-eluting stents from 5% to 10% (3). Noninvasive blood biomarkers for the

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ABBREVIATIONS AND ACRONYMS

ISR = in-stent restenosis

IVUS = intravascular ultrasound

MRM = multiple reaction monitoring

PCI = percutaneous coronary intervention

QCA = quantitative coronary analysis

S1P = sphingosine-1-phosphate

VIP = variance in projection

VSMC = vascular smooth muscle cell

identification of restenosis-prone patients could potentially alter decision making regarding treating patients with multiple stents or offering more prolonged antiplatelet therapy to patients at higher risk for ISR.

Although the molecular etiology of ISR remains unclear, previous studies have identified several associations between changes in cellular metabolism and the occurrence of ISR (4,5). This suggests a potential clinical application of an ISR-specific metabolome for diagnostic purposes. Metabolomics identifies a large number of metabolites in the biological systems and their changes associated with pathophysiological conditions. A few metabolic biomarkers have been identified for heart failure, myocardial infarction, and incident coronary heart disease (6–8).

However, data regarding metabolic biomarkers of ISR are limited. Previously, on the basis of analysis of serum samples using gas chromatography-mass spectrometry, Hasokawa et al. (9) showed that among 83 metabolites analyzed, 8 were significantly different between minor and major restenosis groups, including amino acids and sugars. However, the study targeted only a limited number of metabolites, and no validation step for the selected markers was conducted. With the development of analytic techniques, next-generation metabolomics based on liquid chromatography coupled with high-resolution mass spectrometry is more robust and sensitive, allowing the accurate detection of hundreds of metabolites simultaneously, which facilitates the discovery of novel biomarkers for early diagnosis and prevention of coronary heart diseases.

In the present study, we performed comprehensive metabolomic profiling of plasma samples from patients who had undergone PCI and developed ISR using our next-generation metabolomics platform and identified a panel of plasma metabolite markers with potential for the early prediction of ISR. In addition, we performed an independent blinded cross-validation using a separate group of 500 patients with PCI. To improve the clinical applicability of the selected biomarkers, we also quantified the absolute concentrations of these metabolites in the plasma of both control subjects and patients with ISR using stable isotope dilution-multiple reaction monitoring (MRM) mass spectrometry.

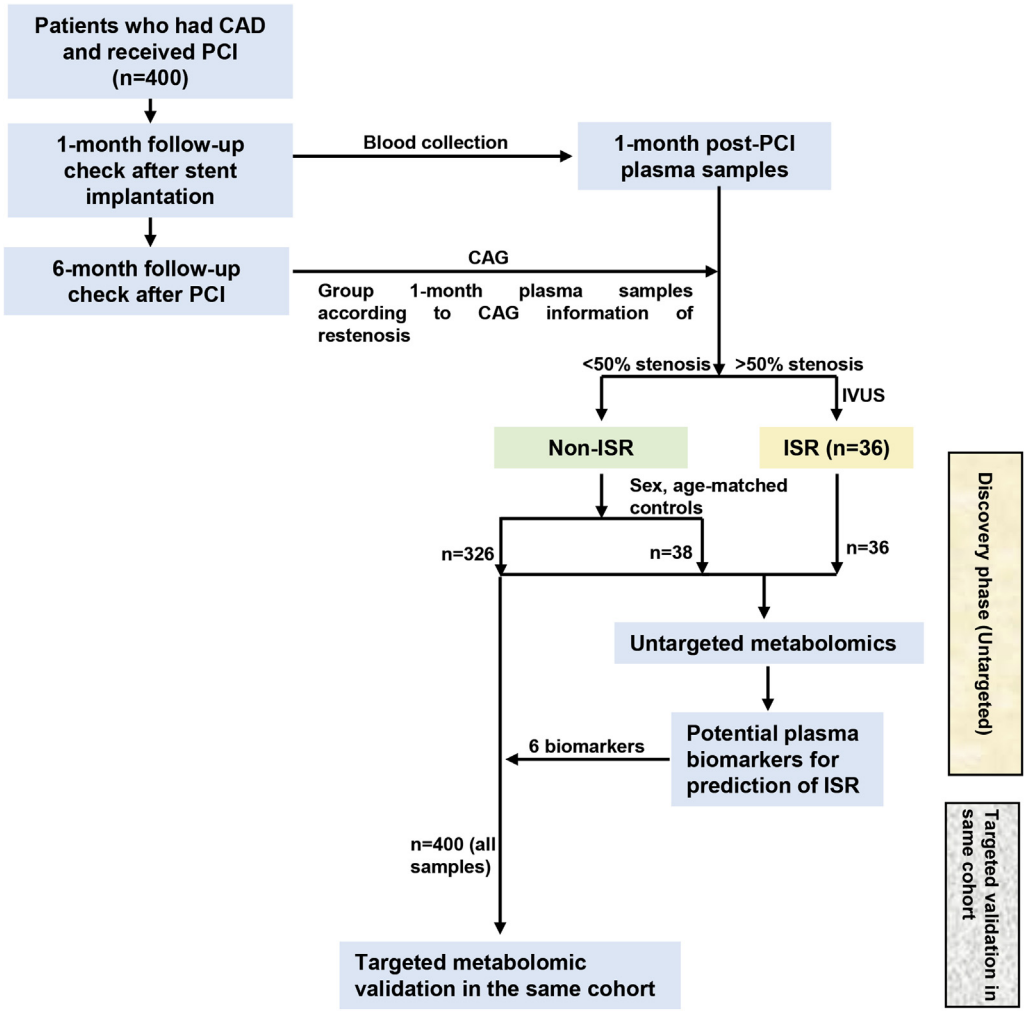
METHODS

PATIENTS AND STUDY DESIGN. The study protocol was approved by the Institutional Review Board of the Beijing Anzhen Hospital ethics committee (AZHEC2012-0516). Verbal and written consent was obtained from all subjects. The discovery phase included patients admitted for PCI to Beijing Anzhen Hospital between January 2013 and November 2013, and each patient received 1 second-generation rapamycin-eluting stent (Firebird 2, Microport, Shanghai, China). Patients were scheduled for 1- and 6-month follow-up after stent implantation. At 1 month, blood samples were drawn from each patient into lithium-heparin tubes, and plasma was obtained by centrifugation for 10 min at 1,000 g using a refrigerated centrifuge. The plasma samples were then transferred to clean tubes and stored at −80°C for further use. At 6-month follow-up, coronary angiography was performed using standard Judkins techniques. Quantitative coronary analysis (QCA; Pie Medical Imaging, Maastricht, the Netherlands) was performed by 2 experienced interventional cardiologists to evaluate restenosis, defined as angiographic stenosis ≥50%. Intraobserver variability and interobserver variability of QCA were determined. Intravascular ultrasound (IVUS; Philips Volcano, San Diego, California) was applied to verify the accuracy of the angiographic classification of ISR. Correlation analysis of QCA measurements and IVUS calculation was conducted. IVUS was also performed to guide the selection of matched control subjects. Patients without restenotic lesions were used as the control group. Participants were excluded for the presence of other major disorders, including active inflammation, renal diseases, cancer, diabetes, and liver cirrhosis. Among 400 patients enrolled in the discovery stage, 36 patients with ISR and 38 age- and sex-matched patients without ISR were used for untargeted metabolomic analysis to search for biomarkers. The remaining 326 patients were used for targeted analysis. The study flow is depicted in **Figure 1**, and baseline patient characteristics for untargeted analysis are listed in **Table 1**. Detailed procedures are described in the Online Appendix.

The validation phase of the study was composed of another independent set of patients admitted to Anzhen Hospital, affiliated with Capital Medical University, for PCI between May 2014 and June 2015. The inclusion criteria and study protocols were identical to those of the discovery phase. A total of 500 patients were enrolled in the validation stage. The experimental design flow for the validation cohort is depicted in Online Figure 1, and patient characteristics are described in Online Table 1.

UNTARGETED METABOLOMIC PROFILING. Details of untargeted metabolomic analysis are provided in the Online Appendix. Briefly, metabolites in the plasma

FIGURE 1 Study Flow Diagram for the Discovery of Biomarkers



CAD = coronary artery disease; CAG = coronary angiography; ISR = in-stent restenosis; IVUS = intravascular ultrasound; PCI = percutaneous coronary intervention.

were extracted using extraction buffer containing 25% acetonitrile, 40% methanol, and 35% H_2O (1:7 v/v, plasma/extraction buffer). The separation of the metabolites was achieved on a high-strength silica T3 column (1.7 μm, 2.1 × 100 mm; Waters Corporation, Milford, Massachusetts) using an ACQUITY H-class ultraperformance liquid chromatography system (Waters Corporation). The column eluent was analyzed using a high-resolution quadrupole time-of-flight instrument (Xevo G2 QTOF, Waters Corporation) operating in either negative or positive electrospray ionization mode. Raw mass spectrometric data were processed using MassLynx version 4.1 and MarkerLynx software (Waters Corporation). Metabolite identification was based on accurate mass (within 10 ppm) and tandem mass spectrometric data and validated using standards. Multivariate analysis and biomarker analysis were performed using MetaboAnalyst version 3.0 (McGill University, Montreal, Quebec, Canada).

TARGETED BIOMARKER ANALYSIS USING LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY. To verify the results of untargeted metabolomic profiling, the selected biomarkers from untargeted analysis were simultaneously detected and quantified in all plasma samples using liquid chromatography-tandem mass spectrometry. The targeted assay was

TABLE 1 Patients Characteristics for Untargeted Metabolomics in the Discovery Phase

	ISR (n = 36)	Non-ISR (n = 38)	p Value	Cohen's Difference (ISR vs. Non-ISR)
Age (yrs)	59.4 ± 9.4	62.9 ± 10.1	0.127	−0.359
BMI (kg/m^2)	23.2 ± 0.7	24.3 ± 0.3	0.203	−2.04
Smoking	5 (13.9%)	6 (15.6%)	1.000	−1
Hypertension	3 (8.3%)	3 (7.8%)	0.712	0
LDL-C (mg/dl)	98.2 ± 3.3	97.7 ± 4.2	0.569	0.132
HDL-C (mg/dl)	47.8 ± 5.2	46.2 ± 7.0	0.331	0.259
Creatinine (mg/dl)	0.81 ± 0.15	0.85 ± 0.21	0.347	−0.219
HbA_{1c} (%)	5.20 ± 0.71	5.4 ± 0.49	0.166	−0.327
hsCRP (mg/dl)	0.14 (0.03–0.2)	0.15 (0.04–0.18)	0.211	−0.103
Stent diameter (mm)	3.2 ± 0.18	3.17 ± 0.20	0.355	0.157
Total stent length (mm)	24 ± 9	21 ± 12	0.209	0.283
Ejection fraction (%)	63.5 ± 7.5	61.4 ± 6.7	0.209	0.295
Medications				
Aspirin	36 (100%)	38 (100%)	1.000	−2
ACE inhibitors/ARBs	21 (59.5%)	19 (50.5%)	0.494	2
Beta-blockers	20 (54.2%)	21 (55.4%)	0.476	−1
Statins	25 (70.2%)	26 (68.5%)	1.000	−1
Diuretic agents	7 (20.1%)	7 (18.1%)	1.000	0

Values are mean ± SD, n (%), or median (interquartile range). Differences between 2 groups were analyzed using either the Student *t* test (parametric distribution) or the Mann-Whitney *U* test (nonparametric distribution). For categorical data, contingency table analysis and the Fisher exact test were used. Cohen's d was calculated to show the standardized difference between two groups for normally distributed data. Cliff's delta was used to show the standardized difference for non-normal distribution. Among 400 patients enrolled, 36 had developed ISR at 6 months after PCI. Thirty-eight matched subjects without ISR (~1:1 ratio) were selected as a control group for untargeted metabolomics from patients without ISR after PCI to search for potential biomarkers. The remaining patients without ISR (n = 362) were used for targeted metabolomic analysis to confirm the results of untargeted biomarker analysis. The laboratory data listed here are from 1-month post-PCI plasma samples for untargeted metabolomics.

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; BMI = body mass index; HbA_{1c} = glycated hemoglobin; HDL-C = high-density lipoprotein cholesterol; hsCRP = high-sensitivity C-reactive protein; ISR = ISR; LDL-C = low-density lipoprotein cholesterol; PCI = percutaneous coronary intervention.

performed on a Shimadzu LC20AD (Shimadzu, Kyoto, Japan) coupled with a Qtrap 5500 hybrid triple-quadrupole mass spectrometer (SCIEX, Framingham, Massachusetts) operated in MRM mode. The absolute concentrations of the biomarkers were determined using matrix-specific standard curves to account for matrix effects and the peak area ratio of biomarkers to the respective isotope-labeled internal standard. Concentrations are expressed in micromoles per liter. Details are provided in the Online Appendix. Plasma samples collected from both 1 and 6 months post-PCI were analyzed.

STATISTICAL ANALYSIS. For subject characteristics, data are reported as mean ± SD or, in situations in which distributions were skewed, as median and interquartile range. Differences between 2 groups were analyzed using either the Student *t* test (parametric distribution) or the Mann-Whitney *U* test (nonparametric distribution). For untargeted metabolomics data, before statistical analysis, the mass and charge features of metabolites were $\log_2$-transformed and autoscaled to make the empirical distribution of intensities the same across samples. Partial least square analysis was used for multivariate analysis and metabolites with variance in projection (VIP) scores >1.5 were considered significant. Sets of 5 to 17 metabolites were selected manually from the top 58 significant metabolites as candidate diagnostic classifiers using 2 multivariate methods: random forests and linear support vector machine implemented in MetaboAnalyst. The classification performance of the selected metabolites was assessed using the area under the receiver-operating characteristic curve. Classifier robustness was estimated by repeated double cross-validation and permutation testing 1,000 times in MetaboAnalyst. The validation phase was performed in a blinded fashion such that the sample group was not known by the statistical team. Spearman's rank correlation analysis was conducted for the correlation between the levels of the biomarkers and stenosis rates. The paired *t* test was used to check the changes of biomarkers between 1 and 6 months post-PCI. Details are provided in the Online Appendix.

RESULTS

PATIENT CHARACTERISTICS. In the discovery phase, among 2,723 patients assessed, 400 subjects were enrolled in this study. The reasons for the large number of exclusions are discussed in the Online Appendix. The intraobserver variability of QCA was excellent, with a mean difference of 0.0181 ± 0.191 mm (minimal luminal diameter) (p = 0.591) (Online Table 2). Similarly, the interobserver variability between 2 cardiologists showed a mean difference of 0.0261 ± 0.281 mm (p = 0.787) (Online Table 2). QCA measurements of late ISR (>50% stenosis rate at 6-month follow-up) were well correlated with IVUS calculation of in-stent neointimal formation (Spearman correlation r = 0.79; p < 0.001). Thirty-six patients (9%) developed ISR (≥50% stenosis) within 6 months after PCI, and the other 364 patients without or with minor ISR were used as control subjects. At 1-month follow-up, all patients had no clinical manifestations except 1 with recurrent chest pain. The clinical presentation and symptoms varied among the patients with ISR at 6 months after angioplasty (Online Appendix).

Initially, for untargeted metabolomic profiling, to ensure the detection of a true statistical difference between 2 populations, we performed power analysis to determine the minimum number of samples required. In addition, our test run showed that the signal intensity of the metabolites was log normally

distributed, with an SD of 1.5 (Online Figure 2). If the true difference between the control and ISR group mean is 2-fold, we have more than 90% power to detect differential metabolites at an overall significance level of 5% with Bonferroni adjustment using 30 subjects per group. By taking into account the power analysis and the cost and efforts of untargeted metabolomic profiling, we selected 38 age- and sex-matched control subjects for untargeted metabolomics (~1:1 ratio). The other 326 control subjects were analyzed using targeted metabolomics for the validation of selected markers. The characteristics of patients in the discovery phase are listed in **Table 1**. There were no significant differences in the coronary risk factor profiles between the 2 groups.

We validated the selected biomarkers using another independent population of 500 subjects. The validation study included 48 patients with ISR and 452 control subjects (Online Table 1). The enrollment criteria were the same as in the discovery phase.

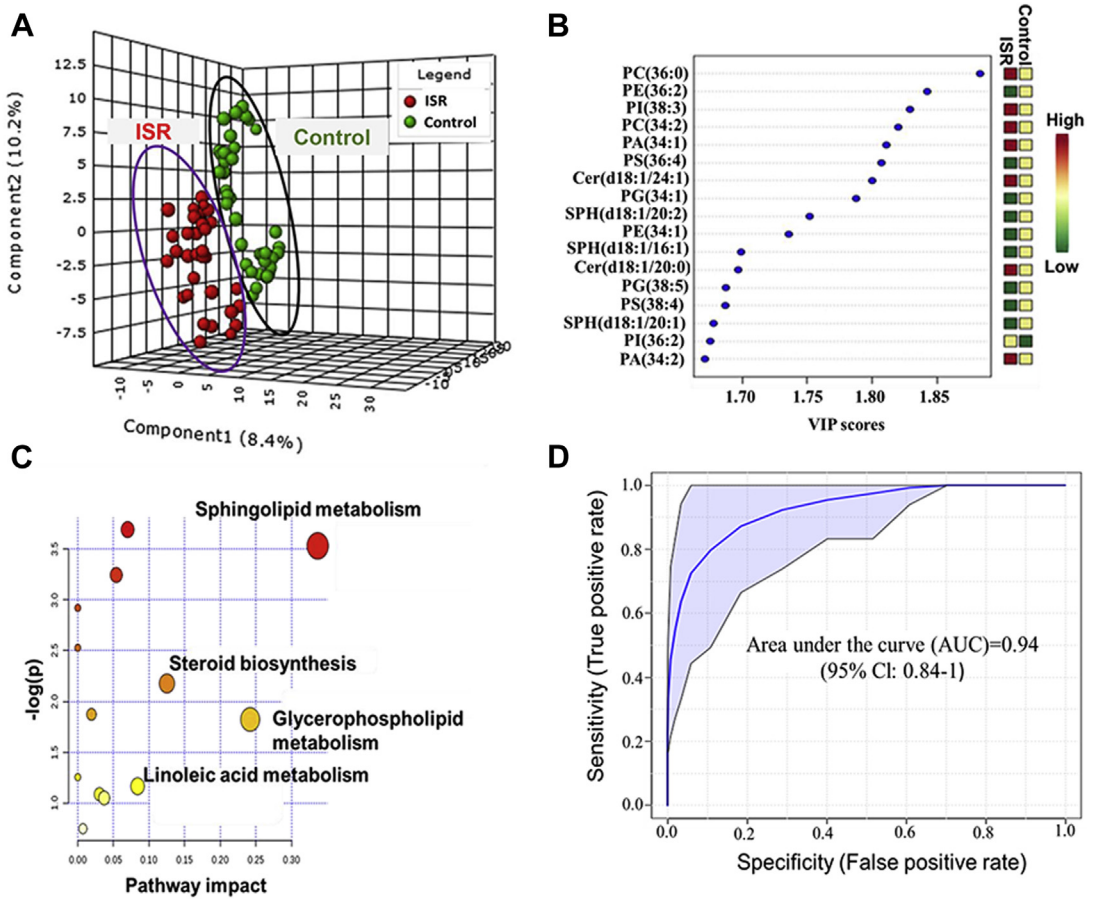
UNTARGETED METABOLOMIC PROFILING. The results of untargeted profiling yielded 2,300 positive features and 1,900 negative features. Multivariate analysis was used to identify metabolomic abnormalities in patients with ISR compared with control subjects. In the 3-dimensional plot of partial least squares analysis, we found complete separation of the 2 groups, which indicated that ISR had a metabolomic signature that was dramatically distinct from control subjects (**Figure 2A**). VIP analysis revealed 58 significantly altered metabolites in plasma that are responsible for discrimination between patients with ISR and control subjects (VIP score >1.5) (**Figure 2B and** Online Table 3). We then performed pathway enrichment analysis for the discriminant metabolites, and the relative pathway impact and statistical significance are visualized in **Figure 2C**. We found that sphingolipids, phospholipids, linoleic acid metabolism, and steroid biosynthesis were the major dysregulated metabolic pathways in patients with ISR. All top 17 discriminators revealed by VIP analysis are sphingolipids or phospholipids, suggesting the important role of these membrane lipids in the pathology and early prediction of ISR (**Figure 2B**).

BIOMARKER ANALYSIS. After identifying more than 60 metabolites that differed between patients with ISR and control subjects, we next sought to find smaller sets of analytes that could be used for the early prediction of ISR. Samples of 5 to 17 metabolites were manually selected using 2 multivariate methods, random forests and linear support vector machine, and the performance of each classifier set of metabolites was then assessed using receiver-operating characteristic curve analysis. We found a set of 6 metabolites—4 phospholipids, 1 sphingomyelin, and 1 ceramide—that yielded a robust area under the curve of 0.94 (**Figure 2D**). The sensitivity and specificity were 91% and 89%, respectively. We also found that even single-analyte classification methods performed surprisingly well (**Table 2**). However, a single biomarker is biologically implausible as a diagnostic test for a complex outcome such as ISR and is likely to perform poorly in larger populations. By using classifiers constructed from 6 metabolites, natural biological variation is more readily accommodated, and diagnostic accuracy is more robust.

CROSS-VALIDATION USING TARGETED ANALYSIS. To further verify the untargeted metabolomic results, we next performed stable isotope dilution–MRM mass spectrometry to unambiguously identify and quantify the selected 6 metabolites. We analyzed all 400 plasma samples, including 364 non-ISR control subjects and 36 patients with ISR taken at 1-month follow-up after stent implantation. This quantitative analysis revealed similar results as observed in untargeted metabolomic profiling. We found dramatic increases in phosphatidylcholine diacyl C36:0, phosphatidylcholine diacyl C34:2, phosphatidylinositol diacyl C36:4, phosphatidic acid C34:1, and ceramide in the ISR group (**Figure 3**). In contrast, the concentration of sphingomyelin diacyl 18:1/20:1 in the plasma of patients with ISR was significantly decreased (**Figure 3**).

INDEPENDENT BLIND VALIDATION USING A SEPARATE GROUP OF PATIENTS. To validate our biomarker-based classification, we recruited a separate group of 500 patients: 48 post-PCI patients with ISR and 452 patients without ISR within 6 months after PCI. Because metabolomic analysis requires special instruments and approaches, to facilitate the use of the selected biomarkers, in the validation experiment, we quantified only the 6 selected biomarkers that could be easily conducted in many clinical laboratories using the same stable isotope dilution–MRM tandem mass spectrometric technique. We applied a separate random-forests classifier model using the discovered 6-metabolite panel from the aforementioned targeted analysis to the independent validation samples. The group information was blinded during analysis. In comparison with IVUS imaging at 6-month follow-up, the model classified patients with ISR and control subjects with sensitivity 91% and specificity 90% using 1-month post-PCI plasma in the validation phase (**Table 2**). The diagnostic accuracy in the validation phase was 90% (95% confidence interval: 87% to 100%) (**Table 2**).

FIGURE 2 Discovery of 6 Plasma Biomarkers for the Early Diagnosis of In-Stent Restenosis Using Untargeted Metabolomics



(A) Partial least squares analysis revealed distinct plasma metabolism between the non-in-stent restenosis (ISR) control group (n = 38) and the ISR group (n = 36). **(B)** Variance in projection (VIP) analysis showed the top 17 most discriminant metabolites between the control and ISR groups. **(C)** Biochemical pathway impact analysis revealed the top 4 disturbed metabolic pathways in patients with ISR. **(D)** Receiver-operating characteristic (ROC) curve analysis using the selected 6 metabolites from the VIP list for classifying ISR and non-ISR control groups in the discovery phase. Training set overfitting was minimized by using random-forest decision tree analysis. To produce a smooth ROC curve, 100 cross-validations were performed, and the results were averaged. Cer = ceramide; CI = confidence interval; PA = phosphatidic acid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; SPH = sphingomyelin.

CHANGES OF PLASMA BIOMARKER LEVELS AND CORRELATION WITH STENOSIS RATES. To further validate the selected biomarkers, we analyzed the levels of 6 plasma biomarkers collected at 6 months in the validation study. Significant correlations were found between the plasma biomarker levels and the stenosis rates at 6 months post-PCI in patients with ISR (Online Figure 3). Compared with their 1-month levels in plasma, phosphatidylcholine diacyl C36:0, phosphatidylcholine diacyl C34:2, phosphatidic acid C34:1, and phosphatidylinositol diacyl C36:4 were increased significantly in the ISR group ($p < 0.01$) (Figure 4). Sphingomyelin diacyl 18:1/20:1 was further decreased at 6 months in the ISR group. In contrast, the changes in these biomarkers after PCI in patients without ISR were not statistically significant. Moreover, the differences were greater at 6 months between patients with ISR and non-ISR control subjects, which supports the potential use of these biomarkers for the prediction of ISR (Figure 4).

DISCUSSION

In the present study, in combination with the next-generation untargeted and targeted metabolomics, we demonstrate the discovery and validation of

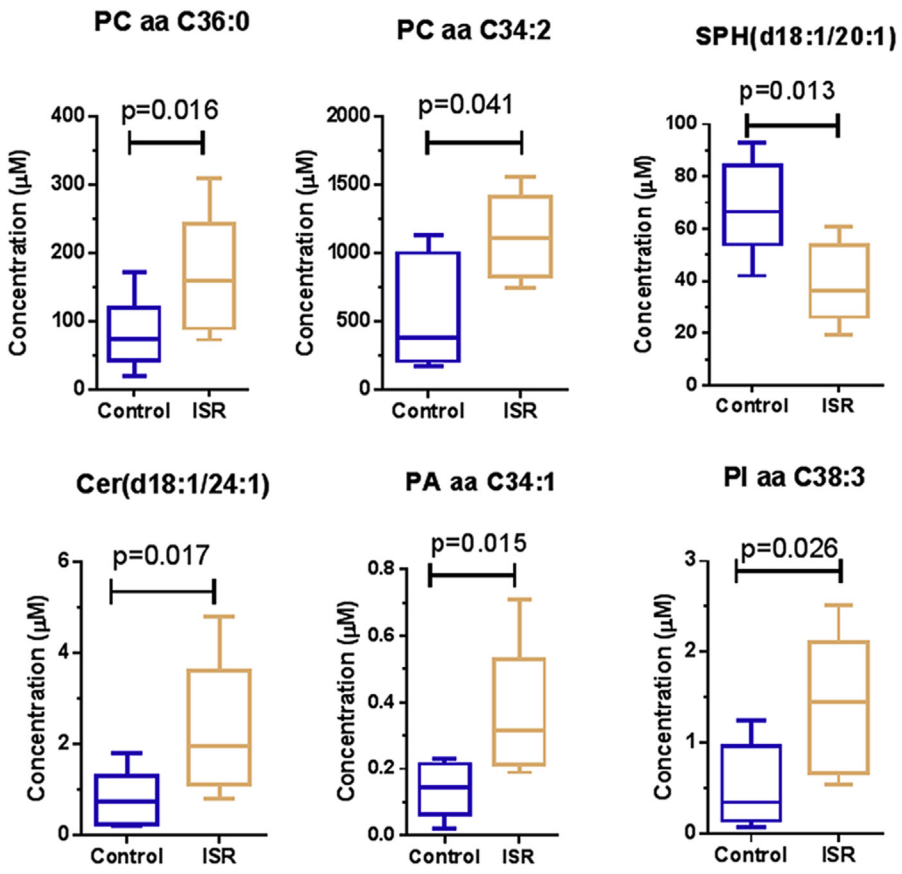
TABLE 2 Diagnostic Accuracy of 6-Metabolite Panel in the Prediction of In-Stent Restenosis in the Discovery and Validation Phases

Phase	Classifiers	AUC	95% CI	rdCV Accuracy	Permutation p Value	Accuracy (%)	Sensitivity (%)	Specificity (%)
Discovery phase	6-metabolite panel	0.94	0.84–1.0	0.84	0.001	90	91	89
	1-metabolite panel	0.71	0.50–0.88	0.62	0.009	72	73	72
Validation phase	6-metabolite panel	0.96	0.87–1.0	0.9	0.001	93	91	95

AUC = area under the receiver-operating characteristic curve; CI = confidence interval; rdCV = repeated double cross-validation.

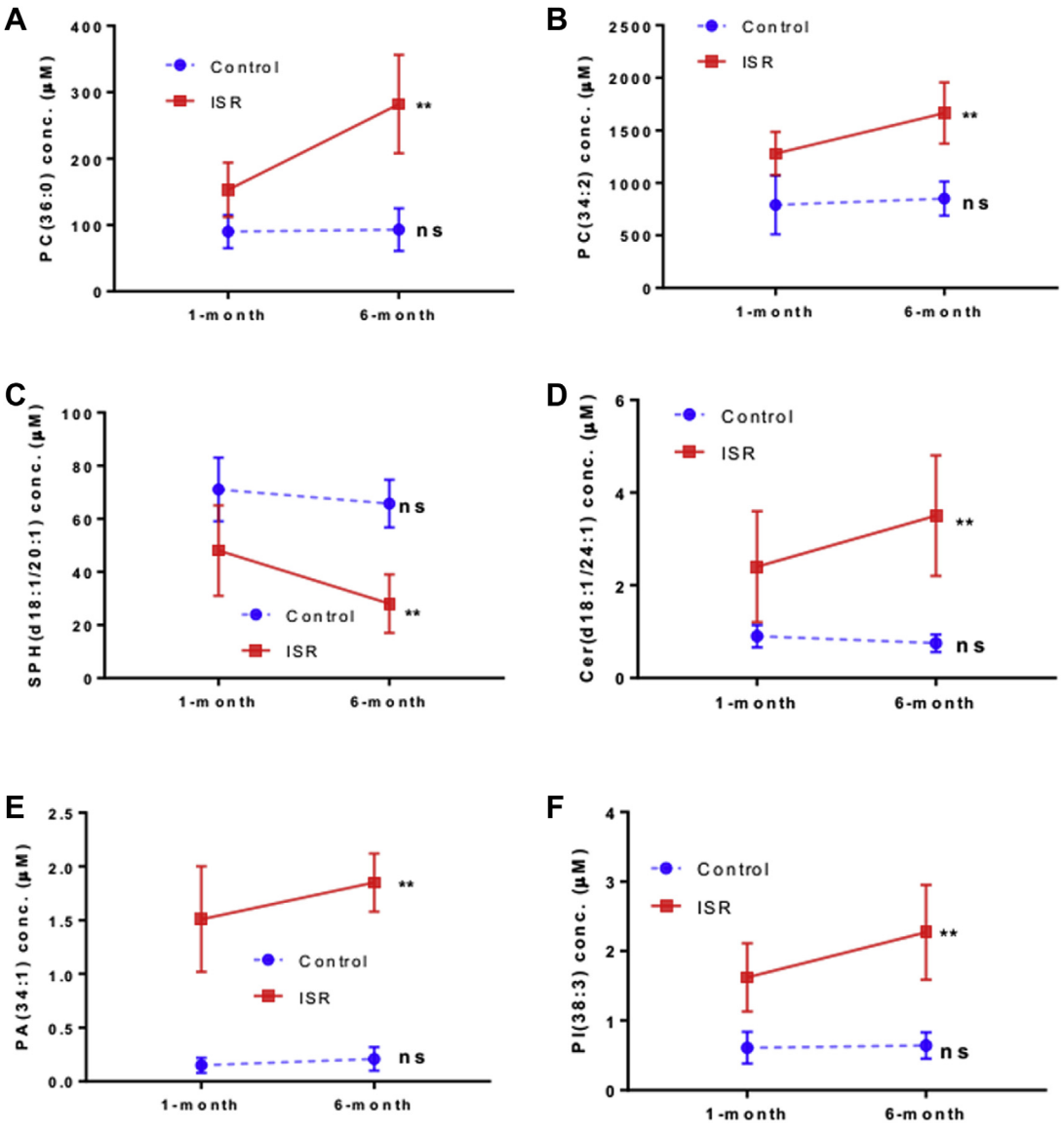
plasma metabolite changes that distinguish patients who will progress to ISR 6 months after PCI. We show that a panel of 6 metabolites in plasma can be used to predict ISR. Blood circulates in the cardiovascular system and maintains homeostasis in all tissue interstitial fluids. Metabolites in blood serve as the essential mediators for the biochemical correspondence between cells and blood (10). Under normal conditions, cells use exosomes, lipoprotein complex, and transporters to exchange metabolites inside and

FIGURE 3 Absolute Quantification of 6 Selected Biomarkers in the Discovery Phase for Cross-Validation Using Liquid Chromatography-Tandem Mass Spectrometry



The abundance of each metabolite is plotted as the absolute concentration in the plasma. The **solid bars** within the **box plot** represent the median abundance. The **whiskers** represent the 10th and 90th percentiles. Data were subjected to the nonparametric Mann-Whitney *U* test. A p value < 0.05 was considered to indicate statistical significance. In-stent restenosis (ISR) group, n = 36; control group, n = 38. Cer = ceramide; PA = phosphatidic acid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; SPH = sphingomyelin.

FIGURE 4 Changes in Plasma Biomarker Levels After Percutaneous Coronary Intervention in the Validation Study



(A) Phosphatidylcholine (PC) diacyl C36:0; **(B)** PC diacyl C34:2; **(C)** sphingomyelin (SPH) (d18:1/20:1); **(D)** ceramide (Cer) (d18:1/24:1); **(E)** phosphatidic acid (PA) C34:1; **(F)** phosphatidylinositol (PI) diacyl C38:3. Control patients without in-stent restenosis (ISR) within 6 months of percutaneous coronary intervention, n = 452; patients with ISR, n = 48. Plasma samples were collected at 1 and 6 months. The 6 selected biomarkers were analyzed using liquid chromatography-tandem mass spectrometry. Nonparametric paired *t* test was used to compare the differences of plasma biomarker levels between 1 and 6 months. **Significant difference ($p < 0.01$) compared with the level at 1 month.

outside of the cells and maintain metabolic homeostasis (Online Figure 4A). This "metabostasis" plays an important role in intracellular communication and cooperation (11). The metabolites in plasma are the direct readout of intracellular metabostasis and the final products of the interactions of genes, proteins, and the environment. Vascular smooth muscle cells (VSMCs) are the major cell type involved in vascular remodeling. At the initiation of restenosis, the disturbance of the metabolic pathways in VSMCs

results in the abnormal pattern of specific metabolites in plasma. These metabolites could serve as biomarkers for restenosis. The metabolic imbalance induces the responses of other cells and tissues and causes the proliferation and migration of the smooth muscle cells (12). Although many factors and mechanisms contribute to the development of restenosis, the migration and proliferation of VSMCs is the hallmark of ISR.

The defined 6-metabolite profile features phospholipids, sphingolipids, and ceramides. The disruption of the sphingomyelin-ceramide-sphingosine metabolic pathway in the early stage of restenosis is intriguing. Sphingomyelins and their metabolic products ceramides are not only the essential structure components of cell membranes but also play important roles in the proliferation and migration of VSMCs. Vascular injury after PCI often induces the overexpression of chemotactic cytokines and a number of inflammatory factors such as tumor necrosis factor-alpha, hypoxia-inducible factors, and platelet-derived growth factor (13-15). These factors subsequently activate the sphingomyelins-ceramide metabolic pathway and enhanced turnover of sphingomyelins (Online Figure 4B). Despite the heterogeneity of factors leading to ISR, the cellular metabolic response in patients with ISR was homogeneous. Ceramides are the direct product of sphingomyelins turnover. It was reported that phosphorylation of ceramides (ceramide-1-phosphate) led to neointimal hyperplasia of VSMCs via the induction of cell proliferation and cell-cycle progression (16). In addition, sphingomyelins are the precursors of sphingosine-1-phosphate (S1P). In the cardiac vascular system, S1P plays an important role in the pathogenesis of many cardiovascular disorders such as coronary artery disease, atherosclerosis, and restenosis (17). Once synthesized in cytosol through sphingolipid metabolism, S1P could be exported to the plasma compartment through ABC transporters on the cell membrane. Plasma S1P communicates with VSMCs via S1P receptors and initiates the proliferation and migration of VSMCs (18). Additionally, fluid shear stress was found to induce S1P release from the platelets into the plasma compartment, enhance S1P responses in cell-cell interactions, and lead to restenosis (19).

Increases in the plasma levels of phosphatidylcholine and phosphatidylinositol lipids in the early stage of restenosis are intriguing. Phospholipids have essential structural and functional roles in the integrity and functionality of cell membranes. Abnormalities in membrane phospholipid homeostasis alter the membrane-associated protein complexes interactions that regulate cell signaling and myocardial metabolism. Phosphatidylcholine biosynthesis has also been reported to be essential for the secretion of cytokines that induce coronary ISR after PCI (20). Phosphatidylinositol lipids are involved in neointima formation, VSMC proliferation, and migration through phosphatidylinositol-3 kinase pathway. It has been reported that disruption of phosphatidylinositol-3 kinase and phospholipase C activity resulted in the neointima formation (21) (Online Figure 4B).

To our knowledge, this is the first published report of a blood-based biomarker panel with very high accuracy for the prediction of stent restenosis as early as 1 month after stent implantation. This metabolic panel robustly identifies (with accuracy better than 90%) patients with PCI who, on average, will develop ISR within 6 months. This has the potential to identify patients at risk for restenosis early after PCI and potentially modify treatment strategy for restenosis and target patients for invasive detection of clinical restenosis especially in cases of multivessel and left main coronary artery stenting.

CONCLUSIONS

In the largest study of the metabolome in relation to incident restenosis to date, we identified phosphatidylcholine diacyl C36:0, phosphatidylcholine diacyl C34:2, phosphatidylinositol diacyl C36:4, phosphatidic acid C34:1, ceramide, and sphingomyelin diacyl 18:1/20:1 as plasma biomarkers for the early prediction of restenosis after PCI. We consider our results a major step toward the American College of Cardiology Foundation, American Heart Association Task Force on Practice Guidelines, and Society for Cardiovascular Angiography and Interventions consensus statement mandate for biomarkers of post-PCI restenosis.

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PERSPECTIVES

WHAT IS KNOWN? ISR remains an important clinical problem for patients after PCI. However, the current tools for evaluation of ISR, including coronary angiography, IVUS, and optical coherence tomography, are invasive and expensive.

WHAT IS NEW? A set of 6 metabolites in plasma belonging to sphingolipids and phospholipids were identified and independently validated by nontargeted and targeted metabolomics for the prediction of restenosis after PCI with high accuracy.

WHAT IS NEXT? The role of sphingolipids and phospholipids in the pathogenesis of ISR should be further investigated. An enzyme-linked immunosorbent assay kit based on the selected 6 biomarkers will be more convenient for clinical use in local hospitals.

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APPENDIX For supplemental methods and results and supplemental figures and tables and their legends, please see the online version of this article.